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BARIUM SULPHATE ABSORPTION AND THE SERUM DIAGNOSIS OF SYPHILIS.*

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Negative serum reactions in untreated, well-developed cases of secondary syphilis have been sometimes encountered by those who employ the original method of Wassermann. According to Wechselmann, whose experiments were carried out under Wassermann, this is said to be due to the development of complementoid in the patient's serum during inactivation, and inactivation is essential for Wassermann's method. Wechelsmann states that the treatment of such specimens of syphilitic sera with barium sulphate removes the interfering complementoid and causes the treated sera to react positively. A later report by Lange contains a list of a large number of sera which either became positive or stronger after treatment with barium sulphate. According to Wechselmann and Lange, every specimen of serum sent for diagnosis must be treated with barium sulphate in order to insure a reliable result. Although the absorption of serum with barium sulphate is a simple enough process, yet before its routine adoption in the complement fixation test by the Wassermann method, further examination is demanded. Whatever may be the cause, the phenomenon itself is of interest. In our studies of this subject we employed the antihuman hemolytic system, and have considered the following questions:

1. The antibody content before and after barium sulphate absorption.

2. The effect of barium sulphate upon the complementary activity of guinea pig serum.

3. The effect of barium sulphate upon the antibody content of different syphilitic sera.

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4. The effect of the addition of normal serum upon the barium sulphate absorption of syphilitic antibody.

5. The fate of syphilitic antibodies after barium sulphate absorption.

6. The absorption of hemolytic amboceptors by barium sulphate.

1. *The Antibody Content before and after Barium Sulphate Absorption.*—In this series of experiments we employed unheated and inactivated syphilitic sera. The mixtures of the serum and barium sulphate were made in the proportions originally recommended by Wechselmann. The result shows that the absorption of unheated as well as inactivated serum renders the reaction somewhat stronger, especially with the inactivated sera. This experiment confirms the finding of Wechselmann with inactivated serum, but does not prove that it was the removal of the complementoid that made the reaction stronger. The barium sulphate removed certain serum constituents which interfere with the fixation of the complement. The increase in the intensity of reaction after the

TABLE I.

Absorption with $BaSO_4$ Increases the Number of Antibody Units.

Amounts of the dilution of the centrifugate used for the fixation test.	Unheated syphilitic serum.		The same serum after inactivation.	
	Syphilitic serum 0.1 c.c. + $BaSO_4$ (7%) 0.055 c.c.; incubation; centrifugalization. The centrifugate was made equal to 5 c.c. with salt solution and used for the fixation test.	Control without $BaSO_4$ treatment.	**Syphilitic serum (56° C.) 0.1 c.c. + $BaSO_4$ (7%) 0.055 c.c.; incubation; centrifugalization.** The centrifugate was made equal to 5 c.c. with salt solution and used for the fixation test.	Control without $BaSO_4$ treatment.
1.0	No hemolysis	No hemolysis	No hemolysis	No hemolysis
0.75	No hemolysis	No hemolysis	No hemolysis	No hemolysis
0.625	No hemolysis	No hemolysis	No hemolysis	Slight hemolysis
0.5	No hemolysis	No hemolysis	No hemolysis	Much hemolysis
0.4	No hemolysis	No hemolysis	Much hemolysis	Complete hemolysis
0.3	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.25	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.2	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.15	Trace hemolysis	Slight hemolysis	Complete hemolysis	Complete hemolysis
0.1	Moderate hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.075	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.05	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
	$\frac{5}{0.16}=30$ antibody units	$\frac{5}{0.175}=28.5$ antibody units	$\frac{5}{0.5}=10$ antibody units	$\frac{5}{0.75}=6.6$ antibody units

barium sulphate absorption is almost negligible with the unheated sera.

The results in table I show that a certain quantity of barium sulphate mixed with the syphilitic serum strengthens the reaction. It was of interest, therefore, to see whether barium sulphate in other proportions might not remove the syphilitic antibody. The results of experiments determining this point are recorded in table II.

TABLE II.

Removal of Syphilitic Antibody by Absorption with $BaSO_4$.

	Syphilitic serum No. 1.		Syphilitic serum No. 2.	
	0.002 c.c.	0.02 c.c.	0.003 c.c.	0.03 c.c.
$BaSO_4$ (7 per cent.)				
1.0	Complete H.	Complete H.	Complete H.	Complete H.
0.7	Complete H.	Complete H.	Complete H.	Complete H.
0.5	Complete H.	Complete H.	Complete H.	Complete H.
0.4	Complete H.	Complete H.	Complete H.	Complete H.
0.3	Complete H.	Much H.	Complete H.	Complete H.
0.25	Complete H.	No H.	Complete H.	Much H.
0.2	Complete H.	No H.	Complete H.	No H.
0.15	Complete H.	No H.	Complete H.	No H.
0.1	Complete H.	No H.	Complete H.	No H.
0.07	Complete H.	No H.	Complete H.	No H.
0.05	Complete H.	No H.	Complete H.	No H.
0.04	Complete H.	No H.	Complete H.	No H.
0.03	Much H.	No H.	Complete H.	No H.
0.025	No H.	No H.	Complete H.	No H.
0.02	No H.	No H.	Much H.	No H.
0.015	No H.	No H.	No H.	No H.
0.01	No H.	No H.	No H.	No H.
0.007	No H.	No H.	No H.	No H.
0.005	No H.	No H.	No H.	No H.
control	No H.	No H.	No H.	No H.
Amount of $BaSO_4$ (7 per cent.) required for complete absorption.	0.04 c.c.	0.4 c.c.	0.025 c.c.	0.3 c.c.

H = hemolysis.

Table II shows that the syphilitic antibody can be completely removed by absorption with a sufficiently large amount of barium sulphate. With a given serum the amounts of the antibodies removed by barium sulphate are approximately proportional to the amounts of barium sulphate employed. Thus, to remove ten times the number of antibody units, ten times the quantity of barium sulphate is required. On the other hand, we have noticed that the

absolute amount of the sera containing the antibodies bears no quantitative relationship to the amount of barium sulphate required for different sera. In the experiment (table II), a somewhat larger amount of barium sulphate is seen to be necessary to remove the antibodies from a somewhat smaller amount of the serum. The number of antibody units contained in 0.002 cubic centimeter of the first specimen was the same as that in 0.003 cubic centimeter of the second. From this fact it is clear that the absorption by barium sulphate is not directed in a selective manner to the antibodies.

2. *The Effect of Barium Sulphate upon the Complementary Activity of Guinea Pig Serum.*—Incidentally, it may be mentioned that barium sulphate has almost no anticomplementary action upon guinea pig serum. A slight antihemolytic effect has been noticed when one cubic centimeter of a 7 per cent. suspension of this salt was mixed with 0.05 cubic centimeter of the complement.

3. *The Effect of Barium Sulphate upon the Antibody Content of Different Syphilitic Sera.*—In this series of experiments we selected six samples of syphilitic sera, the antibody contents of which were very variable. The titers of these sera are recorded in table III, where other details of the experiments are also given.

The results were rather unexpected and reveal wide variations in the ease or difficulty with which the removal of the antibodies from different sera could be effected with barium sulphate absorption. At the foot of the table we have recorded figures for the amounts of barium sulphate necessary for removing one antibody unit from different sera and the relative quantities of the barium salt necessary to absorb a given unit volume of these six sera. This numerical expression aids us in seeing how each specimen behaved towards the barium sulphate absorption. The most difficult serum to fix, No. 1, is found to be nearly forty times less sensitive to the barium sulphate absorption than the most easily fixable specimen of serum, No. 2. Here again we obtain the evidence that the absorption by barium sulphate is not proportional to the amount of the serum. Nor is it parallel to the antibody unit, because the amount of each serum employed in this experiment represented about one unit. If the absorption were selectively directed toward the antibodies, the amount of barium sulphate necessary to remove

TABLE III.

Antibody Content of Different Sera Removed by $BaSO_4$.

	Determination of the quantities of $BaSO_4$ necessary to remove 1 unit of syphilitic antibody from different samples of positive sera.					
	Serum No. 1. Titer, 0.00125 c.c.	Serum No. 2. Titer, 0.00175 c.c.	Serum No. 3. Titer, 0.0025 c.c.	Serum No. 4. Titer, 0.0125 c.c.	Serum No. 5. Titer, 0.0125 c.c.	Serum No. 6. Titer, 0.025 c.c.
$BaSO_4$(7 %)						
0.5	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.4	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.3	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.2	Complete H.	Complete H.	Complete H.	No H.	Complete H.	Complete H.
0.1	No H.	Complete H.	Complete H.		Moderate H.	No H.
0.07		Complete H.	No H.		No H.	
0.05		Complete H.				
0.04		Complete H.				
0.03		Complete H.				
0.02		Complete H.				
0.015		Complete H.				
0.01		Complete H.				
0.007		Complete H.				
0.005		No H.				
control	No H.	No H.	No H.	No H.	No H.	No H.
$BaSO_4$ for removal of unit antibody	0.2 c.c.	0.007 c.c.	0.1 c.c.	0.3 c.c.	0.2 c.c.	0.2 c.c.
$BaSO_4$ for removal of 1 volume unit of serum	$\frac{0.2}{0.00125} = 160$	$\frac{0.007}{0.00175} = 4$	$\frac{0.1}{0.0025} = 40$	$\frac{0.3}{0.0125} = 24$	$\frac{0.2}{0.0125} = 16$	$\frac{0.2}{0.025} = 8$

H = hemolysis.

the antibodies would have been nearly the same for all specimens. Just what determines this variation in the fixability of human serum by barium sulphate was not investigated further.

In the next series of experiments we determined whether barium sulphate absorbs the anticomplementary substances of human serum as in the case of the syphilitic antibodies. For this purpose we selected some syphilitic sera which became anticomplementary upon standing for many days. The results are recorded in table IV.

The experiments in table IV demonstrate that barium sulphate removes both the anticomplementary substances and the syphilitic antibodies.

4. *The Effect of the Addition of Normal Serum Upon the Barium Sulphate Absorption of Syphilitic Antibody.*—We have already shown that barium sulphate removes the antibodies from a syphilitic

TABLE IV.

Removal of the Syphilitic Antibodies and Anticomplementary Substances with $BaSO_4$.

0.02 c.c. of each serum was mixed with 1 c.c. of $BaSO_4$ (7 per cent.) and incubated at 37°C. for 1 hour. The mixture was centrifugalized and the centrifugate was tested for the antibody in the usual way. The fixation test was made with 0.05 c.c. and 0.1 c.c. of guinea pig complement. Controls were also made with the untreated sera.

		Serum No. 1.		Serum No. 2.		Serum No. 3.		Serum No. 4.	
		No antigen.	Plus antigen.	No antigen.	Plus antigen.	No antigen.	Plus antigen	No antigen.	Plus antigen.
Serum treated with $BaSO_4$	Complement 0.05 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
	Complement 0.1 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
Untreated serum (control)	Complement 0.05 c.c.	No H.	No H.	No H.	No H.	Slight H.	No H.	No H.	No H.
	Complement 0.1 c.c.	Complete H.	Much H.	Complete H.	No H.	Complete H.	No H.	Complete H.	No H.
		Serum No. 5.		Serum No. 6.		Serum No. 7.			
		No antigen.	Plus antigen.	No antigen.	Plus antigen.	No antigen.	Plus antigen.		
Serum treated with $BaSO_4$	Complement 0.05 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.		
	Complement 0.1 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.		
Untreated serum (control)	Complement 0.05 c.c.	Slight H.	No H.	Slight H.	No H.	Slight H.	No H.		
	Complement 0.1 c.c.	Complete H.	No H.	Complete H.	No H.	Complete H.	No H.		

serum in almost direct proportion to the amount of this salt used for absorption. We have also stated that the treatment of some syphilitic sera with a *comparatively small amount* of barium sulphate removes certain serum constituents and renders the reaction somewhat stronger than in the untreated serum. We have also shown elsewhere that when the syphilitic antibody is associated with comparatively large amounts of normal serum constituents, the fixation reaction is proportionately less sensitive. Reversing this consideration, we derive the conclusion that by removing the normal serum constituents as much as possible we can make the reaction more distinct; in other words, we might be able to purify the syphilitic antibodies. Such a purification is possible, especially when the other interfering serum constituents possess a greater affinity than the antibodies for barium sulphate. It is also possible to purify the syphilitic antibodies to a certain extent, even when the affinities of the antibodies and other serum constituents for barium sulphate absorption are the same, provided the amount of the serum constituents is greater than that of the antibodies. This is not possible, however, when the amounts of the indifferent serum constituents and the antibodies are in the reverse proportion, because here we would remove more antibodies than interfering serum constituents. It is, therefore, necessary to determine experimentally whether the non-syphilitic serum possesses the same affinity for barium sulphate as the antibodies of syphilitic serum do. We have accordingly estimated the number of the antibody units after barium sulphate absorption in syphilitic serum, syphilitic serum plus an equal amount of normal serum, and syphilitic serum plus four times the quantity of normal serum. If the normal serum possesses the same affinity as the syphilitic antibodies, its presence must interfere with the removal of the latter by a given amount of barium sulphate.

The protocol given in table V shows that the addition of one part of normal serum to one part of syphilitic serum reduced the removal of the antibodies to one-half of that obtained when no normal serum was added, and the addition of four parts of the normal serum to one part of syphilitic serum reduced the removal to about one-fifth. This indicated undoubtedly that the normal serum em-

TABLE V.

Interference by Normal Serum with the $BaSO_4$ Absorption of Syphilitic Antibody.

Amounts of diluted centrifugate used in the fixation test with antigen.	Series 1. Syphilitic serum and $BaSO_4$. **Syphilitic serum (1:10) 1.2 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation at 37° C., 1 hour; centrifugalization.** The centrifugate is made equal to 25 c.c. with salt solution.	Series 2. Syphilitic serum and normal serum, equal parts, and $BaSO_4$. **Syphilitic serum (1:10) 1.2 c.c. + normal serum (1:10) 1.2 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation; centrifugalization.** The centrifugate is made equal to 25 c.c. with salt solution.	Series 3. Syphilitic serum with 4 parts of normal serum, and $BaSO_4$. **Syphilitic serum (1:10) 1.2 c.c. + normal serum (undiluted) 0.5 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation; centrifugalization.** The centrifugate is made equal to 25 c.c. with salt solution.	Series 4. Control: syphilitic serum alone without $BaSO_4$. **Syphilitic serum (1:10) 1.2 c.c. + salt solution 0.6 c.c.; incubation; centrifugalization.** The fluid is made equal to 25 c.c. with salt solution.
1.0	No hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.7	Much hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.5	Complete hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.4	Complete hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.3	Complete hemolysis.	Almost complete hemolysis.	No hemolysis.	No hemolysis.
0.25	Complete hemolysis.	Complete hemolysis.	Trace hemolysis.	No hemolysis.
0.2		Complete hemolysis.	Complete hemolysis.	Much hemolysis.
0.15		Complete hemolysis.	Complete hemolysis.	Complete hemolysis.
0.1		Complete hemolysis.	Complete hemolysis.	Complete hemolysis.
0.07			Complete hemolysis.	Complete hemolysis.
0.05				Complete hemolysis.
0.04				Complete hemolysis.
0.03				Complete hemolysis.
	$\frac{25}{1}$ = 25 units; removed 75 units;	$\frac{25}{0.4}$ = 62.5 units; removed 37.5 units;	$\frac{25}{0.3}$ = 83.3 units; removed 16.6 units;	$\frac{25}{0.25}$ = 100 units; original content.

ployed, possessed the same affinity as the syphilitic antibodies for barium sulphate. In another experiment we found that the number of demonstrable antibodies in a mixture of normal and syphilitic sera, in the proportions of 1 to 1 and 1 to 4, was considerably smaller when barium sulphate was not used than when it was used. As we have shown in another communication, when normal serum[1] is added to a mixture containing syphilitic antigen and antibody, it interferes with the fixation of complement subsequently to be added, by uniting with the molecules which under the usual conditions are capable of fixing complement.

It would manifestly be unsafe to conclude from these experiments that all normal sera behave in the same way. On the con-

[1] In fixation experiments, white of egg has a strong interfering property which can be removed to a large extent by absorption with barium sulphate.

trary, we already know that there are many human sera which fix with great difficulty.

5. *The Fate of Syphilitic Antibodies after Barium Sulphate Absorption.*—We have endeavored to trace the antibodies which disappear during the process of barium sulphate absorption. The results are given in table VI.

TABLE VI.

Fate of the Syphilitic Antibody after $BaSO_4$ Absorption.

	Syphilitic serum (1:20) 0.6 c.c. + $BaSO_4$(7%) incubation; 0.6 c.c.; centrifugalization.		Control: no $BaSO_4$ absorption; syphilitic serum (1:20) 0.6 c.c. in 4 c.c. salt solution.
	Sediment resuspended in 4 c.c. salt solution, and used for fixation test.	Centrifugate was made equal to 4 c.c. with salt solution and used for fixation test.	
	1	2	3
Quantities of: (1) sediment suspension, (2) $BaSO_4$ treated serum, (3) untreated serum (control) for fixation test. Complement, 0.05 c.c. The rest of the procedure was as usual.			
1.0		No H.	
0.8		Moderate H.	
0.6	No H.	Complete H.	No H.
0.5	No H.	Complete H.	No H.
0.4	No H.	Complete H.	No H.
0.3	Complete H.		No H.
0.25	Complete H.		No H.
0.2	Complete H.		Much H.
0.1	Complete H.		Complete H.
Number of antibody units contained in the total 4 c.c. of each series.	10 units.	4 units.	16 units.

The experiments given in table VI show (1) that the syphilitic antibodies are removed by barium sulphate (to which they seem to adhere), and (2) that the antibodies thus absorbed retain their activity.

6. *The Absorption of Hemolytic Amboceptors by Barium Sulphate.*—Several experiments were performed with antihuman amboceptor (rabbit), and the results show that the amboceptpor is quite readily removed from the solution by barium sulphate absorption. The barium sulphate treated with the amboceptor solution retains the amboceptor and causes hemolysis when mixed with human corpuscles and guinea pig complement. Although we have not made similar absorption tests with antisheep amboceptor, it is probable that this acts in the same way.

CONCLUSIONS.

The so-called syphilitic antibodies can be removed from a serum by means of absorption with barium sulphate. The removal is due either to an adsorption or a mechanical absorption. The activity of the syphilitic antibodies is thereby unimpaired. The readiness with which the absorption is accomplished with barium sulphate varies considerably with different syphilitic sera. That barium sulphate exerts the same absorbing effect upon non-syphilitic serum components is made evident by the interfering property which the latter manifest in the absorption experiment of the syphilitic antibodies. The selective removal of the serum components, other than the syphilitic antibodies, by means of barium sulphate absorption is, therefore, impossible.

On the other hand, a partial removal of these components, with but little removal of the syphilitic antibodies, may be effected when the content of a given serum is poor in syphilitic antibodies and comparatively rich in the indifferent serum components. But this is impossible if the conditions are reversed. The main reasons why some negative syphilitic sera may be so modified by the barium sulphate treatment as to give positive reactions, are explained below, but these apply only to those methods in which inactivated serum is employed. The inactivation reduces the antibody content to about one-fourth to one-fifth of the original. When the serum is very rich in antibodies, this does not affect the result of the fixation test. But when the amount of the antibodies is small, the process of inactivation creates conditions quite unexpected. It may produce such a condition that a given amount of the serum contains, after inactivation, only one or two antibody units, while the other serum components remain undiminished. Here one must not lose sight of the vital fact that these apparently indifferent serum constituents are not at all indifferent in the fixation processes. They may possess affinities which are similar to those of complement for the fixing combination of syphilitic serum and antigen. Speaking quantitatively, one unit of the syphilitic antibodies plus antigen will fix 0.1 cubic centimeter of guinea pig complement, but this unit can also be saturated and blocked by nearly the same amount of the seemingly indifferent

serum component of the serum to be tested. Moreover, the regular amount of inactivated serum used in the Wassermann system is 0.2 cubic centimeter, a quantity sufficient to saturate two units of the fixing combination. Fortunately, this *self-saturation* of the syphilitic antibody-antigen combination by the other serum components is not constant in occurrence, owing to the wide variations of the fixability of the serum components of man. Here the benefit of Wechselmann's procedure becomes obvious. By removing a surplus of the fixable indifferent serum components by means of barium sulphate, the serum is made to react positively, or more strongly than before the treatment with barium sulphate. This masking of the positive reaction through the self-saturation is liable to occur in any system in which inactivated serum is recommended.

Another equally important factor in masking the positive reaction in a serum in which the antibodies are poor, is the presence in considerable amount of natural antisheep amboceptor in human serum. It is a plain and simple fact that an excess of hemolytic amboceptor renders a positive reaction feeble or completely negative. As we have shown in our present investigation, a hemolytic amboceptor can be removed from the serum by means of absorption with barium sulphate. Thus it is easy to understand why Wechselmann found that barium sulphate absorption improves the reaction in the original Wassermann system.

The treatment of syphilitic serum with this salt can have a twofold benefit in the case of the original method of Wassermann; namely, the removal of certain interfering serum components and the removal or diminishing of the natural antisheep amboceptor present in the syphilitic serum.

In the method of Noguchi, there is no necessity for applying the barium sulphate absorption. Noguchi recommends the use of unheated serum, hence the absolute amount of the serum employed is only one-half of the absolute amount of complement. Eventually an old serum may be anticomplementary and need inactivation, but if the result is doubtful in this instance, a fresh serum from the same patient may be secured and subjected to reëxamination. In this method there is no danger of introducing an amount of hemolytic amboceptor which is both unknown and uncontrollable, for the

reason that human serum is usually devoid of isolysin (antihuman hemolytic amboceptor), and, if the latter is present, it never reaches the strength which shows any effect upon the hemolytic system employed.

We conclude, therefore, that the barium sulphate absorption is to be recommended for the original method of Wassermann under certain conditions, but that it is unnecessary for the antihuman hemolytic system of Noguchi.