STUDIES ON COMPLEMENT FIXATION IN TUBERCULOSIS. III.

By PAUL A. LEWIS, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In the course of the work recorded in two previous papers (1, 2) on this subject, increasing interest has developed in a substance extractable from dried tubercle bacilli by hot alcohol, which serves as an antigen in the complement fixation reaction. Much work on the antigenic constituents of the tubercle bacillus and their chemical characterization has appeared recently. Wadsworth, Maltaner and Maltaner (3) have increased our knowledge of the lipoidal constituents and have used them in practical diagnostic work. The experience of these authors is in entire accord with my own in that, in so far as could be determined with the immune serum available to them, bacillary products when subjected to *prolonged* extraction with lipoid solvents, are deprived of residual antigenic activity. Others, particularly Dienes and Schoenheit (4, 5), while recognizing the presence and importance of the constituents extractable by the lipoid solvents, laid stress on the fact that with their sera and antigenic preparations, antigens residing with the protein fractions are also demonstrable and that some sera react with the lipoidal antigens, others with the protein. In no case, however, is it clear that their protein preparations are free from substances extractable with hot alcohol.

The work of Zinsser and Mueller (6) and of Petroff (7) emphasized the importance of "residue" after precipitation of protein-containing extracts with heat and acetic acid. These observations brought the work on the tubercle bacillus into closest conjunction with the work of Avery and Heidelberger (8) on the specifically precipitable substances derived from the pneumococcus, the active substances in the latter case being carbohydrates.

Laidlaw and Dudley (9), working with the tubercle bacillus, have separated a carbohydrate material reacting with a complement-fixing serum in dilution of 1/6,000,000 from residue remaining after prolonged contact of the bacilli with alcohol and chloroform. This substance is said to be insoluble in alcohol, but whether insoluble in hot alcohol is not stated. In many respects the substance is analogous to those obtained from the pneumococcus by Avery and Heidelberger. The possibility remains open that this substance may be found associated with either the proteins or the lipoidal fractions of the tubercle bacillus, according to the circumstances of preparation.

This possibility is emphasized by the observations of Dienes and Schoenheit (10), who have returned to the examination of the lipoidal fraction and have obtained an alcohol-soluble substance, which after a preliminary purification, reacted in the complement fixation reaction in a dilution of 1/2,000,000. The alcoholic extract in the less advanced stages of purification contained carbohydrate to the extent of 26 per cent in some instances. The carbohydrate content of the more highly purified material was not determined.

The determination of the identity or otherwise of the carbohydrates of Laidlaw and Dudley with the alcohol-soluble fraction giving carbohydrate reactions as observed by Dienes and Schoenheit, is likely also to settle finally the much debated question of whether the antigenic relationships of the tubercle bacillus to the sera of animals either naturally diseased or injected one or more times with entire tubercle bacilli, are single or multiple. Till now, it may be repeated, there is no satisfactory evidence that these more natural sera react in complement fixation with any residual material after complete extraction with hot alcohol. It is conceivable, however, that such severe extraction may injure the antigenic qualities of the residue. It is also more than probable that potentially antigenic substances may remain in completely extracted residues and will be made evident by appropriate immunological procedure even though they do not react with sera prepared with the whole bacilli.

I am now able to record a simple method for the extraction, concentration and preliminary purification of the fraction soluble in hot ethyl alcohol. The procedure we have followed should serve as a most convenient starting point for further chemical manipulation of

this portion of the lipoidal fraction. I have also found that normal serum under certain conditions gives a precipitation reaction with the material. This reaction is interesting in itself and its recognition as a possible source of confusion with true immunity reactions may be important.

EXPERIMENTAL.

The work here recorded was all done with a rapidly growing bovine type culture of low virulence (Bovine III). Dried bacilli are extracted repeatedly in a Soxhlet extractor with absolute ethyl alcohol. When the extracts are cooled, a white precipitate forms, which is largely, although not completely, redissolved when the alcohol is again boiled. The extracts from a number of operations may be combined, boiled, filtered through paper while hot, then chilled and filtered in the ice box, giving a considerable mass of whitish paste which when quite dry can easily be ground to a powder. One or two reprecipitations under these conditions result in a powder which is completely soluble in hot alcohol to a practically colorless solution.[1] The experiments to be reported were carried out with material at this stage of purification. Weighed quantities were dissolved in hot alcohol and dilutions made from such a stock solution.

If a 0.5 to 1.0 per cent solution of this material be brought to a boil and measured with a warm pipette into 0.85 per cent NaCl solution at the temperature of the room, the 1/10 dilution is opalescent, with a bluish tinge. These solutions, or suspensions, allowed to stand for days at room or refrigerator temperature usually show no precipitation.

Such dilutions in normal saline solution freshly prepared, have been the starting point for the use of the substance in the fixation and precipitation reactions herein presented. When higher dilutions are desired, these are made by using a less concentrated alcoholic solution as a starting point, or a lesser amount of the alcoholic solution. Dilutions in series of the saline suspensions have not been practiced.

The method employed for the fixation test was fully described in our second publication. Essentially, the primary incubation is 2 hours at 37–38°, in the water bath; from 2 to 2½ units of guinea pig complement are used; approximately 10 units of hemolytic amboceptor are employed; the second incubation is ½ hour; and finally, an excess of immune serum, amounting to from 2 to 10 times the fixation unit, has been used.

When, as in this case, the antigenic value of a preparation is under consideration, the experiment may be variously interpreted. The use

[1] I am indebted to Dr. P. A. Levene for his active interest in guiding me to this simple procedure for preliminary purification of the material.

of a definite excess of complement and a large excess of hemolytic amboceptor as outlined above made our conditions very rigid. In the same sense we have considered the end-point to be at the limit of complete fixation. Dilutions of antigen giving partial fixation have been left out of account. Under these conditions the antigenic value of an ordinary sample of the material prepared as above described tested against an immune goat serum was in the neighborhood of 1/1,300,000 dilution calculated on the weight of the powder. Reading partial fixations or otherwise relaxing the conditions of the test would make it possible to recognize the presence of the antigenic substance in two- or possibly fourfold greater dilution than this, but probably a certain degree of accuracy would be lost to comparative experiments.

As an antigen this substance has advantages in the conduct of the fixation reaction. It seems to be very stable when suspended in alcohol and if these suspensions are protected from evaporation, the preparation can be regarded as a standard one, at least for a number of months. Repeated momentary re-solution by boiling, with care to limit evaporation, does not cause deterioration to an appreciable extent. The fixation unit is usually approximately 1/100 of the anti-complementary amount. 2 fixation units give a maximum effect.

Allowing for difference in technique, it would appear that this extract in hot alcohol once or twice reprecipitated by chilling out, approaches in activity the best preparations of Dienes and Schoenheit. It is also of the order of activity of the carbohydrate of Laidlaw and Dudley. This activity together with the previously determined fact that when this substance is completely removed the bacillary bodies no longer react in the complement fixation test, seem to warrant further attention to it.

In connection with fixation studies, Calmette (11) and Caulfeild (12) have repeatedly made reference to another phenomenon spoken of as the inhibition of fixation. For the demonstration as carried out by Caulfeild quantities of the specific antigen in themselves sufficient to be anticomplementary are mixed with the immune serum and incubated with the complement. If the serum is active in the inhibitory sense hemolysis will take place when the hemolytic system is completed. This is paradoxical in that either the antigen alone, or

the antigen + serum would be expected to fix or inactivate the complement.

With the antigen preparation under discussion and sera available during the past year, I made an extra effort to demonstrate the phenomenon of inhibition. The inhibition reaction has sometimes been in evidence but the experiments were marked by many irregularities and I reached no final conclusion as to their significance. The nature of the irregularities were such as to suggest that smaller serum quantities might possibly permit the demonstration where larger quantities would fail. The time factor might also be of decisive importance. I was thus led to set up unusually long dilution series and accidentally to the interesting observation that normal serum gives a precipitation reaction with the antigen under rather unusual conditions.

The experiment fully developed after many tentative trials is as follows:

The stock alcoholic solution (¼ per cent) of the antigen as above described, is brought to a boil and an appropriate quantity is added to 20 volumes of 0.85 per cent NaCl solution. Of this 0.9 cc. portions are put in a series of agglutination tubes. A series of serum dilutions is prepared such that when 0.1 cc. or less of each is added to the antigen in the tubes, the serum amounts will range from 0.01 cc. to 0.000002 cc. in a total volume of approximately 1 cc. The tubes are shaken and put in the ice box. The result is the formation of an abundant flocculent precipitate in a few tubes. Tubes adjacent in either direction to those showing precipitation, show an increased turbidity without the formation of flocculi. An essential condition is that the serum used shall have previously been heated to 56°C. for ½ hour or longer. "Fresh" beef serum gives an increased turbidity without flocculation over a much wider zone. The combined results of a number of experiments with beef serum in which the above conditions were fulfilled, although at different times, are shown in Table I.

It will be noted that the precipitation centers at about concentration 0.00007, and that the turbidity change without precipitation is irregularly extended in either direction from the precipitated tubes. Serum 1116 (9-I-f) shown in Column 7 is unheated serum showing the turbidity change only.

Of the serum samples shown in this table, two numbers, 1162 and 1163, are normal, three, 1113, 1116 (13-XI) and 1118 (28-X), were from animals treated previously with avirulent bovine type bacilli,

and showing at this time moderate values in the complement fixation test. The precipitation values all approximate those of the normal animals.

TABLE I.

Serum sample*†	1163 (28-I)	1162 (28-I)	1113 (9-I)	1118 (28-X)	1116 (13-XI)	1116 (9-I-f)	1118 (26-I)
Serum amount *cc.*							
0.01	0‡	0	0	0	0	0	0
0.008	0	0	0	0	0	0	0
0.006	0	0	0	0	0	T	0
0.004	0	0	0	0	0	T	0
0.002	0	0	0	0	0	T	0
0.001	0	0	0	0	0	T	0
0.0008	0	0	0	0	0	T	0
0.0006	0	0	T	T	0	T	0
0.0004	T	0	T	T	0	T	T
0.0002	T	+++	++++	++++	T	T	T
0.0001	++++	++++	++++	++++	++++	T	++++
0.00008	++++	++++	++++	++++	++++	T	++++
0.00006	++++	++++	++++	++++	++++	T	++++
0.00004	++++	++++	++++	++++	+++	T	++++
0.00002	++++	++++	T	T	T	T	++++
0.00001	0	0	T	T	T	0	0
0.000008	0	0	T	T	0	0	0
0.000006	0	0	0	T	0	0	0
0.000004	0	0	0	0	0	0	0
0.000002	0	0	0	0	0	0	0
0.000001	0	0	0	0	0	0	0

Alcoholic extract of tubercle bacilli L. 1. 0.5 per cent hot alcoholic solution diluted 1/19 with normal salt solution. Of this 0.9 cc. used in test. Serum dilutions added to total volume 1 cc. Readings after 24 hours in ice box.

* 1162 and 1163, normal calves. 1113, 1118 (28-X), 1116, calves treated with living avirulent tubercle bacilli. 1116 (9-I-f) serum not heated. 1118 (2601) calf 4 weeks after infection with virulent culture of tubercle bacillus; died 2 weeks later.

† Except 1116 (9-I-f) all sera heated to 57–58°C. for ½ hour.

‡ ++++ = complete precipitation. T = increase in turbidity without precipitation. 0 = no change in fluid.

The reaction may be developed at either 37° or at 56°C., but it is not materially hastened or more delicate at these temperatures. If the completed reaction series is placed at 56°, the flocculi contract and

the precipitate becomes heavy almost at once, but precipitation does not occur in tubes where it was not previously present. Sometimes the reaction is not fully developed in less than 48 hours.

Following Calmette's method for the separation of his inhibitory substance from the fixing substances in immune serum, serum diluted

TABLE II.

Serum	1122 (26-I)	1122 (26-I)	1122 (26-I)	1122 (9-I)	1121 (26-I)	1121 (9-I)
Preparation	Whole serum	Supernatant fluid	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*
Serum amount *cc.*						
0.01	0†	0	T	T	T	T
0.008	0	0	T	T	T	T
0.006	0	0	T	T	T	T
0.004	0	0	T	T	T	T
0.002	0	0	++++	++++	++++	+++
0.001	0	0	++++	++++	++++	++++
0.0008	0	0	++++	++++	++++	++++
0.0006	T	0	++++	++++	++++	++++
0.0004	T	0	++++	++++	++++	++++
0.0002	T	T	++++	++++	++++	++++
0.0001	++++	++++	T	T	++++	++++
0.00008	++++	++++	0	T	++++	T
0.00006	++++	++++	0	0	+++	T
0.00004	++++	++++	0	0	T	T
0.00002	+++	+++	0	0	T	0
0.00001	T	T	0	0	0	0
0.000008	0	0	0	0	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Serum heated to 56–57° for ½ hour before precipitation with CO_2.

† Terms as in Table I.

to 1/20 with distilled water has been precipitated with CO_2. If the CO_2 precipitate is redissolved in salt solution, and the salt content of the supernatant fluid restored, comparison shows that the CO_2 precipitate gives a similar reaction but the zone of precipitation is moved toward the stronger concentrations centering about 0.0006 instead of the tenth dilution of this amount. The fluid remaining after the

removal of the CO_2 precipitate may show no reaction whatever. In order to achieve this complete separation it is necessary that the serum be heated to 58°C. for 2 hours before the CO_2 precipitation. If CO_2 precipitation is done on fresh serum or on serum heated for a shorter time, or to 56°C., the separation is at least imperfect.

TABLE III.

Serum	1162	1162	1116	1116
Preparation	Supernatant fluid	CO_2 precipitate*	Supernatant fluid	CO_2 precipitate*
Serum amount *cc.*				
0.01	0†	T	0	T
0.008	0	T	0	T
0.006	0	T	0	T
0.004	0	T	0	T
0.002	0	++++	0	++++
0.001	0	++++	0	++++
0.0008	0	++++	0	++++
0.0006	0	++++	0	++++
0.0004	0	++++	0	++++
0.0002	0	++++	0	++++
0.0001	0	++++	d.T	T
0.00008	0	T	d.T	T
0.00006	0	T	d.T	T
0.00004	0	T	d.T	0
0.00002	d.T	0	d.T	0
0.00001	0	0	d.T	0
0.000008	0	0	0	0
0.000006	0	0	0	0
0.000004	0	0	0	0
0.000002	0	0	0	0
0.000001	0	0	0	0

* Serum heated to 58–60° for 2 hours before CO_2 precipitation.

† Terms as in Table I. d.T = questionable increase in turbidity.

Table II gives the results of serum of Calf 1122, bleeding of January 26, 1925, and compares the whole serum, the supernatant fluid after CO_2 precipitation and the redissolved CO_2 precipitate for this sample. The CO_2 precipitates of one other sample from Calf 1122 and two samples from Calf 1121 are also shown. In this experiment the whole serum, as used in the test and also as preliminary to the CO_2 pre-

cipitation, was heated to 56–57° for $\frac{1}{2}$ hour. It will be noted that the precipitations with the CO_2 precipitates are much alike. Those from Calf 1121 show a somewhat wider range. It is especially interesting that in the case of Serum 1122, the whole serum and the supernatant fluid react alike and yet the precipitate is likewise fully active.

Table III gives the results with the two samples of serum which were heated to 58–60° for 2 hours preliminary to the CO_2 precipitation. In this case the supernatant fluid is without activity while the precipitates are comparable to those shown in Table II.

The animals whose serum was used in the experiments of Table II were both infected with a virulent culture about 1 month before the samples were drawn. They had fever at this time. They had not been immunized.

The samples used in the experiments of Table III were of animals also used in those of Table I, 1162 being normal, 1116 immunized and infected.

The complement fixation reaction must, of course, be carried out with much smaller quantities of the antigen than those which show the precipitation reaction, these being anticomplementary. The fixation value of the sera in question has ranged from a maximum of 0.004 in the case of the immune sera to an immeasurable minimum of less than 0.01 in the case of the normal samples. In certain cases we have added the hemolytic system to the series after precipitation was complete, incubating the complement with the mixtures for varying lengths of time. The results have been so irregular as to be incapable of interpretation.

In one experiment, the tubes showing precipitation were pooled and filtered through paper, the filtrate being tested by complement fixation for the presence of antigen. The test was negative, showing that the specific antigenic substance with which we are dealing is included in the precipitate. The amount of serum involved in the tubes showing precipitation is much less than that required to demonstrate the fixation antibody in any of the serum samples in question and the relation of the precipitate to the immune principles of an active serum remains undetermined.

Finally the sera of a number of other species were tested; horse, sheep, goat, rabbit, guinea pig and fowl were included and all gave

precipitation. The sera were heated to 56°C. for ½ hour before the test. The reaction zones as expressed in serum concentration vary in extent and location but no distinctive character was developed by any. These results are summarized in Table IV.

TABLE IV.

Serum* of.....	Horse	Sheep	Goat	Rabbit	Guinea pig	Fowl
Serum amount cc.						
0.01	0	0	0	0	0	T
0.008	0	0	0	0	0	T
0.006	0	0	0	0	0	T
0.004	0	0	0	0	0	T
0.002	0	0	T	0	T	++++
0.001	T	T	++++	0	T	++++
0.0008	T	++++	++++	0	T	++++
0.0006	++++	++++	++++	0	T	++++
0.0004	++++	++++	++++	T	++++	++++
0.0002	++++	++++	++++	++++	++++	++++
0.0001	++++	++++	++++	++++	++++	T
0.00008	++++	++++	++++	++++	++++	T
0.00006	T	T	++++	++++	++++	T
0.00004	T	++++	++++	++++	T	T
0.00002	T	T	T	T	T	0
0.00001	T	T	0	T	0	0
0.000008	T	0	0	T	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Sera heated 56–57° for ½ hour.

DISCUSSION.

The experiments present a precipitation reaction between normal serum and an extractive preparation of the tubercle bacillus. The reaction is evidently a very delicate one considering the quantities of material involved, comparing in this respect with rather highly developed immune reactions. The quantities being adjusted on the other hand the phenomenon has the greatest constancy.

The reaction considered as an analog of immune precipitin reactions, which it certainly resembles in its main features, is of

interest in that it presents so wide a pro-zone. The serum dilutions in which activity is evident are quite beyond the range of those it would usually be thought necessary to examine in cases where the more concentrated numbers of the series failed to react, and the discovery of the precipitation was quite accidental.

The results with the fractions of serum after CO_2 precipitation, suggest some direct connection of the serum globulins with the precipitation. Conventional reasoning in consideration of the observation that in the case of serum heated to 56°C. the precipitate and the residual fluid are both active while after heating to 58°C. for a longer time, only the globulin fraction is active, would suggest that a multiplicity of substances might be involved.

The reaction may possibly be another aspect of a result obtained by Wadsworth and Maltaner (13), who found that if crude antigenic preparation from the tubercle bacillus were mixed with 5 volumes per cent of normal horse serum, and precipitated by CO_2, the antigen active in the complement fixation reaction was carried down on the globulin and could be extracted from the precipitate with alcohol.

SUMMARY.

The prolonged extraction of the tubercle bacillus with boiling ethyl alcohol, followed by one or more reprecipitations by chilling the hot alcoholic solution, easily yields a preparation very active as antigen in the complement fixation reaction.

This preparation gives a precipitation reaction with high dilution of the normal blood serum of a number of species.

The precipitation reaction presents as a peculiar feature a very long pro-zone and is further dependent on a preceding heat treatment of the serum for its demonstration.

Occurring as a reaction of normal serum, the reaction is apparently not influenced by immunization sufficient to develop moderate specific complement fixation reactions.

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