THE COMPLEMENT FIXATION REACTION AS APPLIED TO LEPROSY.

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A survey of the more recent literature relative to leprosy shows that many have sought to use the reaction of complement fixation as a means of increasing our knowledge of this disease from one or another of several points of view. Some have hoped to increase our ability to make a certain diagnosis, using the serum of the supposed leper against an extract or suspension of materials derived from the nodular lesions characterizing the disease, employed as an antigen. Others have hoped that by considering that the serum of the leper contained a specific antibody, the use of extracts or suspensions of various bacterial cultures derived from leprous lesions might enable them to assign to certain of these, antigenic qualities, which would help to determine an etiological relationship to the disease. As a development related to the latter purpose, some have immunized animals with certain cultures and sought to determine by agglutination or complement fixation their relation to other bacteria, particularly those of the acid-fast group. In no instance have these efforts to apply complement fixation been conspicuously successful, nor is there so far apparent in the literature any satisfactory generalization covering the work which has been done. The results often seem contradictory and many of them are not even understandable so long as one confines himself to the printed reports.

Our previous work on complement fixation in tuberculosis (55, 57) had led us to a different point of view with regard to this reaction as applied to bacterial antigens, particularly those of the acid-fast group. It appears that the method of Bordet and Gengou, potentially a very general one and susceptible in theory to much modification, has in practise become conventionalized and confined within bounds determined by the marked success of a single application; namely, the Wassermann reaction as related to syphilis. Most who have sought to apply

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the complement fixation reaction to the study of either tuberculosis or leprosy have followed rather precisely one or another of the modifications of the Wassermann reaction.

We found, as a matter of fact, that the fixation reaction with the suspensions of acid-fast bacteria or their extracts ran at a slower rate than the reaction between the active principle of the syphilitic serum and the lipoidal extracts commonly used as Wassermann antigens. Whereas these give perfect fixation in from 20 minutes to 1 hour at 37°C., the reaction with acid-fast bacterial suspensions or extracts from them is scarcely evident in 1 hour and is not complete for 2, or even in certain instances for 4 hours. The intricacies of the technique are such that confusing and apparently positive results may frequently appear within the hour, but when the foregoing point had been developed it was clear that these were accidental. Our observations with tuberculosis are here applied to the study of the serum of a number of cases of leprosy[1] and suitable control sera.

The literature of the subject is extensive and, as has been indicated, not conclusive in its bearings. Since our method is novel we do not devote any considerable space to a formal discussion of the work of our predecessors. A bibliographic index is appended for the use of anyone who may be interested in a review. Cooke in his first paper (8) gives a tabulation of the results obtained up to 1919 from the diagnostic point of view. It is evident from his summary that leprosy cases do give complement fixation in varying degree with a number of antigens, and there is a clear indication that the reaction is in large measure non-specific. So it is now commonly stated that many lepers give positive Wassermann reactions and likewise react with the products of the tubercle bacillus as antigens. This has been held to make the results impossible of interpretation in the diagnostic sense, a point which will be discussed below. It is especially worthy of note that while Cooke was able to tabulate upward of 2,000 cases of leprosy whose blood had been thus examined up to 1919, he does not tabulate the results with non-lepers, and as a matter of fact our study of the literature has shown that few such control

[1] For the serum from leprosy cases we are much indebted to Dr. O. E. Denney, of the United States Public Health Service Hospital No. 66, at Carville, La., and to Dr. G. W. McCoy, Director of the Hygienic Laboratory of that service, who introduced us to Dr. Denney.

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tests have ever been reported. Of the controls reported a number have been syphilitic sera, and these have often given positive fixations with leprosy antigens. This we believe due to inherent defects in the methods employed and we have not, therefore, collected the results or laid stress on them in this discussion.

During the course of our studies with tuberculosis we have developed an immune serum of high potency by treating a goat with living culture of an avirulent bovine type of tubercle bacillus. We sought by experiment to determine the degree of specificity which might pertain to this serum. It was found that as between the bovine and human type of tubercle bacillus no differences could be made out. The avian type gave distinctly less reaction when used as antigen. Other cultures in the acid-fast group commonly carried in stock laboratories as saprophytic acid-fast bacteria gave no reaction whatever with this serum.

The cultures which have been associated with leprosy by reason of their origin reacted variously.[2] *Bacillus lepræ* (Duval) and *Bacillus lepræ* (Kedrowsky) have given, with this goat serum, reactions of about the same quantitative value as the avian tubercle bacillus. *Bacillus lepræ* (Clegg) gives no reaction whatever. In extension of this it was found that when human serum from cases of tuberculosis was used they might react somewhat with Duval, Kedrowsky, or avian tubercle cultures but never with Clegg. It did seem possible then, in view of the clear-cut results which the complement fixation reaction as used by us was capable of giving, that an examination of lepra serum might develop points of interest.

Methods.

Antigens.—A number of antigens were employed.

(*a*) *Bacillary Emulsions.*—These emulsions were prepared by removing the organisms from the slant of glycerol agar, weighing the mass, and adding sufficient salt solution to bring about a concentration of 10 mg. per cc. In order to make

[2] These cultures have come to us from Professor C. W. Duval. They are: (1) A culture of the type isolated by Clegg, which tends to grow in a heavy moist mass and produces much light yellow pigment. The culture which we have used was isolated by Duval but is spoken of throughout as *B. lepræ* (Clegg). (2) A culture isolated by Duval which originally was propagated with great difficulty and which produces no pigment. This is spoken of as *B. lepræ* (Duval). (3) A culture of similar characters isolated by Kedrowsky and spoken of by his name.

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this emulsion as uniform as possible, the organisms were first ground in an agate mortar and the salt solution added slowly. The emulsion was then filtered through cotton in order to remove the larger clumps which might remain. Emulsions were prepared from the following organisms: *B. leprœ* (Clegg), *B. leprœ* (Duval), *B. leprœ* (Kedrowsky), *B. smegmatis*, Timothy hay bacillus, Mist bacillus, Marpman's urine bacillus, Rabinowitch's bacillus, butter bacillus, an acid-fast bacillus which has been designated as paratuberculosis, and a saprophytic chromogenic acid-fast organism which we have isolated and designated "Jacob."[3]

(*b*) *Alcoholic Extracts.*—An alcoholic extract of the bovine *B. tuberculosis* and of *B. leprœ* (Clegg) was prepared by first growing the organisms on glycerol bouillon until the surface of the liquid was covered. The culture was then filtered through filter paper and the mass of bacteria washed several times with salt solution until the filtrate was clear. The bacterial mass was collected and placed in a vacuum desiccator over sulfuric acid until dry. The dried organisms were placed in a Soxhlet extractor, and extraction with boiling absolute ethyl alcohol was continued for a week or longer, the alcohol being renewed several times during the period of extraction. The extracts, before being used, were brought to the boiling point, and a measured amount of the hot extract was added to cold salt solution to make the desired concentration.

(*c*) *Petroff's Antigen.*—A glycerol extract of B. tuberculosis, prepared according to the method of Petroff (54) was employed as a control for the alcoholic extract of *B. tuberculosis*.

(*d*) *Cholesterol and Acetone Antigens.*—A cholesterolized alcoholic extract of human heart and an acetone-insoluble fraction prepared from the same organ were used as in the Wassermann reaction.

With the exception of the alcoholic extracts and Petroff's antigen, the recently prepared antigens were used, since we have found that the bacillary emulsions, even when kept at a low temperature, are not stable and in a short time become highly anticomplementary. The emulsions were diluted further with salt solution so that each cubic centimeter contained 1 mg. of the bacteria. One-sixth of the anticomplementary dose was employed in the reaction. This amount, varying from 0.1 to 0.2 mg., was found to be fairly constant with the organisms which give a homogeneous emulsion, while, with those cultures which did not give a homogeneous emulsion, the amount fluctuated more widely. The antigenic dose of the alcoholic extract of *B. tuberculosis* and of Petroff's antigen was determined by adding varying amounts of the diluted extracts to 10 units of the homologous immune goat serum, and the minimal fixing dose was ascertained. The alcoholic extract of *B. leprœ* (Clegg) was employed in one-sixth of the anticomplementary dose.

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[3] The cultures of *B. smegmatis*, Timothy hay bacillus, Marpman's bacillus, Rabinowitch's bacillus, butter bacillus, Mist bacillus, and paratuberculosis bacillus were obtained from Dr. D. H. Bergey, of the Department of Bacteriology of the University of Pennsylvania.

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Serum.—The leprosy blood was collected by Dr. Denney in Keidel tubes and mailed to us by parcel post. It was always in apparently good condition on arrival and was used as soon as possible. The serum separated clear and there is no definite reason to assume that it was seriously altered in any way. It should, however, be noted that the number of anticomplementary sera encountered was greater than with the control sera collected from non-lepers in Philadelphia, and this may be accounted for by the necessary delay in transport. The serum was inactivated at 56°C. for 30 minutes before use. 0.1 cc. of serum was used in the tests and 0.2 cc. as the control for anticomplementary action.

Complement.—The complement was obtained by bleeding guinea pigs from the heart. The blood was defibrinated and permitted to remain in contact with the clot over night. Blood was always obtained from two or more animals which had been previously tested to be sure that their complement was capable of giving fixation with tuberculosis antigens. The complement was separated by centrifuging and the unit of complement determined by mixing varying amounts of complement with 2 units of amboceptor and 0.1 cc. of 5 per cent sheep cell suspension. After the addition of sufficient salt solution to bring the total to 1 cc., the tubes were placed in the incubator, and the minimum amount of complement which had completely hemolyzed the cells was taken as the unit.

Tests.—0.1 cc. of the serum was apportioned out in a number of tubes, to which was then added the antigen. 2 units of complement were added and then sufficient salt solution to bring the total volume to 1 cc. The tubes were incubated for 2 hours at 37°C., at the expiration of which time 4 units of anti-sheep amboceptor and 0.1 cc. of a 5 per cent suspension of sheep cells were added. After 30 minutes incubation in the water bath, the results were noted. The tubes were placed in the ice box and again read the next morning. The increase of the period of primary incubation to 2 hours is, we believe, one of the fundamental features of our method. The other feature of importance is the use of 4 or more units of anti-sheep amboceptor. The effect of this is to mask any variations due to the presence of natural anti-sheep amboceptor which occurs in variable amounts in human serum and possibly also to detach any loosely bound complement. In practise this procedure somewhat reduces the delicacy of the test but greatly increases the certainty of result. For a more complete discussion of this the reader is referred to previous papers on tuberculosis (55, 57).

EXPERIMENTAL.

Of the forty-five specimens of sera obtained from thirty-nine cases of leprosy, four were anticomplementary in a dose of 0.1 cc. but smaller quantities, except with one specimen, were not anticomplementary. A comparison of the anticomplementary property of non-leprous sera with that of leprous sera shows that, while the non-leprous were anti-

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complementary in 2.3 per cent of 604 sera examined, sera from leprosy cases were anticomplementary in 8.8 per cent of forty-five cases studied, when the same dose of serum was employed.

An attempt was made to determine, by means of the method outlined above, in what way non-leprous sera differed in their complement-fixing property from leprous sera.[4] The results of the complement fixation reaction with leprous sera and non-leprous sera and with various antigens are shown in comparison in Table I.

It will be noted that the leprosy sera give reactions quite generally with all of the antigens employed. While the differences are not great, the number of reactions with the tubercle bacillus or its products is ac-

TABLE I.

Antigen.	Leprous serum.				Non-leprous serum.			
	No.		Per cent.		No.		Per cent.	
	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.
B. lepræ (Clegg) alcoholic extract	41	3	93.2	6.8	0	152	0.0	100.0
" " "	31	2	93.9	6.1	0	120	0.0	100.0
" " (Duval)	36	8	81.8	18.2	51	99	34.0	66.0
" " (Kedrowsky)	37	6	86.1	13.9	35	56	38.5	61.5
" *tuberculosis*, alcoholic extract	43	1	97.7	2.3	48	104	31.6	68.4
Petroff's antigen (T.B.)	30	1	96.8	7.2	11	28	28.2	71.8
Cholesterol (Wassermann reagents)	29	15	65.9	34.1	37	115	24.3	75.7
Acetone-insoluble (Wassermann reagents)	28	16	63.6	36.4	25	109	18.7	81.3

tually higher than with any of the cultures having leprosy as a source. The reaction is, then, as has generally been assumed, non-specific. The result can have little value in terms of the etiological significance of any of the cultures derived from lepers. On the other hand, certain of the leprosy cultures do give a high percentage of reaction with the control sera, which may possibly indicate a close relationship of the cultures Duval and Kedrowsky with the tubercle bacillus.

[4] For this purpose sera were employed which were obtained from patients who attended the prenatal clinic, the clinic for the diseases of the chest, and the clinic for syphilitics at the Henry Phipps Institute, Philadelphia. Many of these cases are known to have tuberculosis or syphilis or both diseases.

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Among the control sera none were found to give a reaction with *Bacillus lepræ* (Clegg). This observation has been amplified in two directions. First, an attempt was made to determine whether the reaction with *Bacillus lepræ* (Clegg) might be specific if examined quantita-

TABLE II.

Case No.	Type.	Least quantity of serum reacting with antigens given.				
		B. lepræ, chromogenic.	*B. lepræ*, non-chromogenic.	*B. lepræ* (Kedrowsky).	*B. tuberculosis.*	Cholesterol.
		cc.	*cc.*	*cc.*	*cc.*	*cc.*
108	Anesthetic.	Negative.	Negative.	Negative.	0.05	Negative.
112	Nodular.	0.01	0.02	0.02	0.02	0.02
151	Anesthetic.	Negative.	Negative.	Negative.	0.05	Negative.
203	Nodular.	0.02	0.02	0.05	0.02	"
210	Mixed.	0.01	0.01	0.02	0.02	0.01
211	"	0.01	0.02	0.02	0.02	0.05
187	Anesthetic.	0.01	0.05	0.02	0.01	0.01
172	Nodular.	0.02	0.1	0.05	0.01	0.1
197	"	0.01	0.05	0.05	0.02	0.05
209	"	0.01	0.05	0.05	0.02	0.05
15	Mixed.	0.01	0.01	0.01	0.01	0.02
25	Nodular.	0.01	0.01	0.01	0.01	0.1
95	"	0.01	0.01	0.01	0.01	0.1
134	Mixed.	0.01	0.01	0.01	0.01	0.02
151	Anesthetic.	0.10	0.01	0.01	0.02	Negative.
83	Mixed.	0.01	0.01	0.01	0.02	"
174	"	0.01	0.01	0.01	0.01	0.01
217	Anesthetic.	0.02	0.01	0.01	0.02	Negative.
5	Nodular.	0.01	0.01	0.01	0.01	0.02
157	Mixed.	0.01	0.01	0.01	0.01	0.01
186	Anesthetic.	0.02	0.01	0.01	0.02	Negative.
173	Mixed.	0.01	0.01	0.01	0.01	0.01
144	Nodular.	0.01	0.01	0.01	0.01	0.05
203	"	0.01	0.01	0.01	0.01	0.1
150	Mixed.	0.02	0.01	0.02	0.05	0.1
177	"	0.01	0.01	0.01	0.01	0.01

tively. The differences in this sense as shown in Table II are not material.

Secondly, the study was extended to include a considerable number of antigens prepared with other acid-fast bacteria. Again it is seen in Table III that the reaction of leper serum with this group of antigens is

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more or less universal. It is true that with none of the saprophytic acid-fast bacteria is the percentage of reacting serum so high as with *Bacillus lepræ* (Clegg), and this applies also to *Bacillus lepræ* (Kedrowsky) and *Bacillus lepræ* (Duval). On the other hand, among the control sera there is an almost universal failure to react with the cultures to which no suspicion of pathogenicity attaches. Two sera reacted with "Jacob," one of these also with syphilitic reagents, the other also with *Bacillus lepræ* (Duval) and *Bacillus lepræ* (Kedrowsky).

TABLE III.

Antigen.	Leprous serum.				Non-leprous serum.			
	No.		Per cent.		No.		Per cent.	
	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.	Positive.	Negative.
B. lepræ, chromogenic, alcoholic extract	14	1	93.3	6.7	0	33	0.0	100.0
" " " (Clegg)	13	2	86.7	13.3	0	33	0.0	100.0
" " non-chromogenic (Duval)	9	6	60.0	40.0	6	26	18.7	81.3
" " (Kedrowsky)	11	4	73.3	26.7	7	26	21.2	78.8
" *tuberculosis*, alcoholic extract	14	1	93.3	6.7	8	27	22.9	77.1
Cholesterol	9	6	60.0	40.0	8	27	22.9	77.1
Acetone	8	7	53.3	46.7	5	30	14.3	85.7
Timothy hay bacillus	12	3	80.0	20.0	0	30	0.0	100.0
Marpman's urine bacillus	4	11	26.7	73.3	0	31	0.0	100.0
Rabinowitch's bacillus	6	9	60.0	90.0	0	29	0.0	100.0
Butter bacillus	10	5	66.7	33.3	0	28	0.0	100.0
Mist bacillus	9	6	60.0	40.0	0	27	0.0	100.0
Paratuberculosis bacillus	9	6	60.0	40.0	0	25	0.0	100.0
"Jacob"	11	4	73.3	26.7	2	23	8.0	92.0
B. smegmatis	7	8	46.7	53.3	0	22	0.0	100.0

It is not altogether clear why, considering for the moment that *Bacillus lepræ* (Clegg) may be saprophytic and incidental in its relation to the lesions of leprosy, it should still be a better antigen than other acid-fast bacteria. It makes a better emulsion than many of them, which may have an influence, but this is for the present only a suggestion. If it were possible for us to continue this work we should proceed to the examination of the alcoholic extracts of a number of other cultures, particularly *Bacillus lepræ* (Duval) and *Bacillus smegmatis*.

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A classification of the results with the leper sera based on the type of disease is also presented (Table IV). That there exists a relationship between the type of the disease and the activity of the serum in the reaction of complement fixation has been recognized by a number of observers. From this table it is apparent that the mixed type of the disease yields the highest percentage of positively reacting sera while

TABLE IV.

Reactions According to Type of Leprosy.

Antigen.	Anesthetic.				Nodular.				Mixed.			
	Positive.		Negative.		Positive.		Negative.		Positive.		Negative.	
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
B. leprœ, chromogenic	6	13.6	2	4.5	15	34.1	1	2.3	20	45.5	0	0.0
" " non-chromogenic	5	11.3	3	6.8	14	31.8	2	4.6	17	38.7	3	6.8
" " (Kedrowsky)	5	11.6	3	7.0	14	32.6	1	2.3	18	41.9	2	4.6
" *tuberculosis*, alcoholic extract	8	18.2	0	0.0	16	36.3	0	0.0	19	43.2	1	2.3
Petroff's antigen	5	16.7	0	0.0	8	26.6	0	0.0	16	53.4	1	3.3
Cholesterol	3	6.9	5	11.3	11	25.0	5	11.3	15	34.2	5	11.3
Acetone	3	6.9	5	11.3	11	25.0	5	11.3	14	31.9	6	13.6
Timothy hay bacillus	2	13.3	0	0.0	3	20.0	1	6.7	7	46.7	2	13.3
Marpman's bacillus	0	0.0	2	13.3	1	6.7	3	20.0	3	20.0	6	40.0
Rabinowitch's bacillus	2	13.3	0	0.0	1	6.7	3	20.0	3	20.0	6	40.0
Butter bacillus	2	13.3	0	0 0	2	13.3	2	13.3	6	40.0	3	20.0
Mist bacillus	2	13.3	0	0.0	2	13.3	2	13.3	5	33.4	4	26.7
Paratuberculosis bacillus	2	13.3	0	0.0	2	13.3	2	13.3	5	33.4	4	26.7
"Jacob"	2	13.3	0	0.0	3	20.0	1	6.7	6	40.0	3	20.0
B. smegmatis	1	6.7	1	6.7	2	13.3	2	13.3	4	26.6	5	33.4

the anesthetic type yields the smallest number of such sera. There does not appear to exist any relationship between the type of disease and any given antigen.

Reasoning from analogy it is conceivable that absorption experiments with the acid-fast bacterial antigens might lead to some differentiation in instances in which the direct analysis has given none. Such experiments have failed in the past because when serum is incubated with suspensions of acid-fast bacteria and the latter are removed by centrifugalization the resulting fluid is anticomplementary. It was found by one of us that this difficulty could be avoided by removing the bacterial

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bodies by filtration through Berkefeld or Mandler candles. The complete removal of the complement-fixing bodies from a strong antituberculosis serum may be satisfactorily demonstrated in this way. We have sought to apply this observation to the present problem. The complement-fixing body of leper serum is not removed by absorption either with *Bacillus tuberculosis* or with *Bacillus leprœ* (Clegg). Neither is it removed by treatment with charcoal or kaolin, as are many antibodies.

DISCUSSION.

The wide range of antigenic substances fixed by serum from cases of leprosy precludes the use of the complement fixation reaction for the determination of the specific organism in the causative sense. Absorption with the bacterial suspensions does not remove the complement-fixing bodies from the serum.

The fact that leprosy sera give so high a percentage of positive reactions with antigens ordinarily used for the Wassermann reaction and with the tuberculosis antigens cannot be interpreted as being indicative of a superimposed or underlying infection with either syphilis or tuberculosis. When one realizes that only 8 to 10 per cent of the general population give a positive Wassermann reaction and that extremely unmoral groups give about 30 per cent, it does not seem reasonable to suppose that 66 per cent of lepers are syphilitic. As further suggestion that a positive Wassermann reaction in leprosy is not necessarily indicative of an accompanying syphilitic infection, is the fact that the Wassermann reaction in leprosy is not affected by the administration of arsenicals. Again, in earlier studies in complement fixation (55, 57) with the same antigens that were employed in this study, it was found that but 71 per cent of cases of frank tuberculosis gave a positive complement fixation reaction with tuberculosis antigen, whereas with the sera from leprosy cases a positive reaction was obtained with over 93 per cent of the cases, a higher percentage than with any of the other antigens employed.

We know of no other disease in which the blood serum develops this property of multiple fixation. If our controls are adequate this property might be of diagnostic value in either the positive or negative sense. However, opinion as to this point must for the present be reserved. The control sera which have been employed come from a different commun-

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ity, and unforeseen complications to any interpretation of this order may conceivably arise. Granted that the results obtained are truly applicable, the fixation reaction done by our method with the alcoholic extract of *Bacillus leprœ* (Clegg), or with the emulsion of this organism, would seem likely to be of use practically in spite of the fact that it would at present have no clear foundation in any etiological relationship between this bacterium and leprosy as a disease.

SUMMARY.

By means of a method differing in important details from those of previous investigators it has been determined that the blood serum of cases of leprosy exhibits the ability to fix complement with a wide variety of antigens including to a greater or less extent those derived from any culture of the acid-fast group of bacteria available to us.

This property of multiple fixation may sufficiently characterize the disease to be of diagnostic significance, although our experience is hardly sufficient to enable us to speak with complete assurance on this point. Certainly, control sera from normal individuals, from cases of tuberculosis, or from cases of syphilis as obtained in our locality have entirely failed to react with certain antigens, whereas serum from cases of leprosy have so reacted to the extent of over 93 per cent.

The most characteristic fixation given by the leprosy sera is that with *Bacillus leprœ* (Clegg) used as antigen, either in the form of a bacterial emulsion or of an alcoholic extract of the dried culture.

Antibody absorption may be demonstrated in the acid-fast group if the absorbing bacteria are removed by filtration. Otherwise the resulting fluid is strongly anticomplementary. Leper serum is not deprived of the complement-fixing body when so treated with either *Bacillus tuberculosis* or *Bacillus leprœ* (Clegg).

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