A PRELIMINARY NOTE ON THE USE OF THERMO-STABLE OPSONINS (BACTERIOTROPINS) IN THE ELUCIDATION OF BACTERIAL INFECTIONS.

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(With 5 Charts.)

There are many forms of bacterial disease in which the search for the causal organism is complicated by the fact that it is present, not in pure culture, but associated with the number of other bacteria which may or may not be taking part in the infective process.

This is especially true of diseases of the respiratory and alimentary tracts, in which the search for the infecting germ necessitates the making of cultures from the sputum or the faeces. Under these circumstances, the usual serological tests, such as agglutinin estimations and the "complement fixation" technique are, for the most part, inapplicable, and the Bacteriologist is constrained to arrive at a provisional conclusion on such unsatisfactory grounds as the greater numerical preponderance of certain strains or the fact that some of the germs isolated are known to be more formidable than the others.

It is true that the estimation of the Opsonic Index, by the usual technique of Wright (1921) in which unheated serum is used, was at one time extensively applied for the above purpose, but this test is now seldom employed; nor is it entirely satisfactory, since it takes into account not only the specific but also the non-specific factors of phagocytic activity.

There remain the "thermo-stable" opsonins, the "Bacteriotropins" of Neufeld and Rimpau (1904), the "Incitor Elements," as they were provisionally called by Wright and Reid (1906), which are specific antibodies and which might, therefore, be expected to offer a better prospect of success.

How clear-cut and definite are the results of "thermostable" opsonic tests may be gathered from charts and tables published by the Author and Major C. C. Cumming illustrating investigations on *B. typhosus* and *B. paratyphosus* A (1912 and 1913) and upon the differentiation of *Staphylococci* (1913).

As to the specificity of the thermostable phagocytosis-inducing elements in serum there can be no doubt. This was demonstrated by Savtchenko (1902), by Neufeld and Rimpau (1904), and in this country by G. Dean (1905), as well as by many later observers. For a full bibliography of the subject, the article by F. Neufeld (1913) in Kolle and Wasserman's *Handbuch* may be

consulted. It has always been a source of surprise to the Author that these "bacteriotropins" have been so little used in the elucidation of problems like that now under consideration.

In the course of an extended investigation, still in progress, on the Tuberculo-Opsonic Index in patients suffering from phthisis, it became necessary to envisage the question of secondary infections in this disease; more especially as to the possibility of exploiting these "secondary" organisms in vaccine therapy with such guidance as might be had from a close investigation of the patient's serum.

It was thought necessary, however, to make as sure as possible that the germs used for making the "secondary" vaccines were really infecting the patients and not merely growing as saprophytes in the fluid contents of lung-cavities or upon the respiratory mucous membranes.

To this end, it was decided to compare the phagocytosis induced by both the heated and unheated serum of patients and healthy persons for each of the bacterial strains isolated. It was further decided to apply the same test to non-tubercular patients as occasion might arise.

Before drawing conclusions from this test as applied to human beings, it appeared desirable to ascertain the extent of the alterations in the relation of "bacteriotropins" to "opsonins" in the course of a specific immunization in animals; and the following experiment was accordingly carried out:

EXPERIMENT I.

Two rabbits (Nos. 16 and 17) were subjected to successive inoculations with a recent strain of *Staphylococcus aureus* of human origin, the injections being confined at first to heated emulsions given subcutaneously, and then passing on to the intraperitoneal inoculation of living cultures.

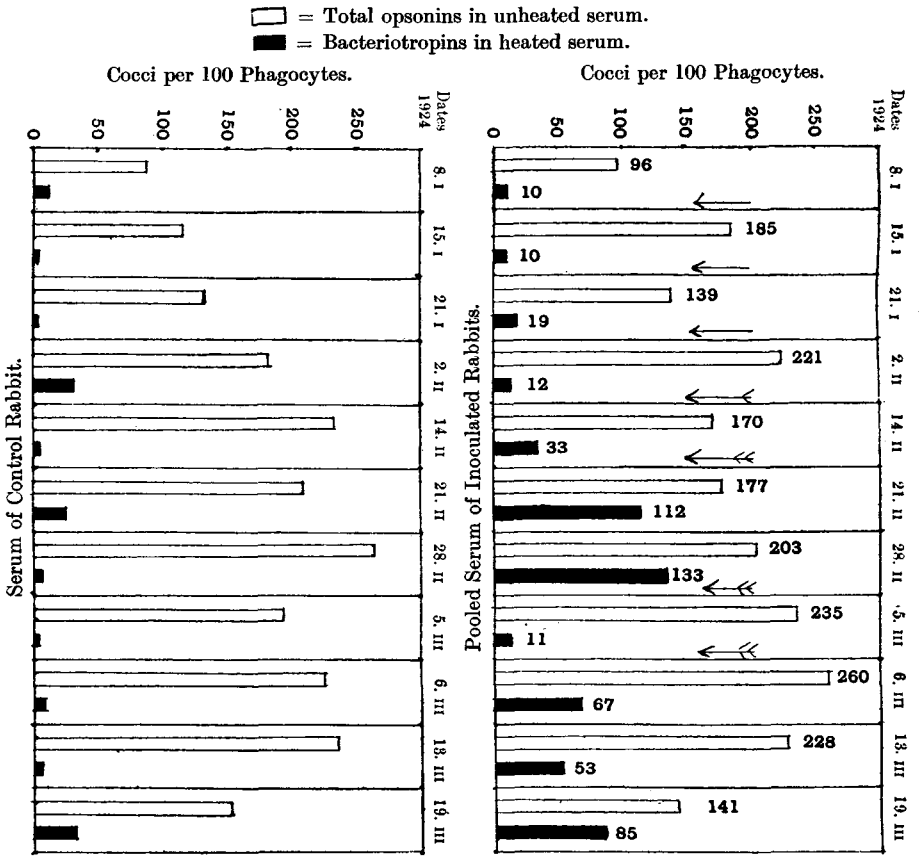
The serum of these two rabbits was collected at intervals and "pooled," the serum of a normal rabbit collected at the same time serving as a "control." Both sera were, for each examination, divided into two portions, one of which was inactivated by heating in a water-bath to 55° C. for half-an-hour, the other being unheated. Opsonic tests were now carried out by Wright's method with both the heated and unheated sera, a standardised living emulsion of staphylococci being used and human leucocytes being employed, always from the same source.

The results of this experiment are set forth in Chart A and serve to demonstrate that while the amount of opsonic power in the control serum is always greatly reduced by heating and remains insignificant throughout the experiment, the "bacteriotropins" in the serum of the inoculated group rise as the immunization proceeds while their quantitative relation to the opsonins steadily increases, attaining to over 65 per cent. when the process is at its height.

The marked fall in bacteriotropin observed on 5. iii. 1924 is to be noted.

On that occasion, the rabbits, through an error, were bled immediately after instead of, as usual, immediately before inoculation.

The apparent rise both in the opsonic power of the "test" and "control" serum may be due to the coccus having been frequently subcultured during the course of the experiment.



This Chart shows the gradual appearance of thermostable opsonins (bacteriotropins) in the course of the "immunization" of a group of two rabbits against *Staphylococcus aureus*. It will be seen that there is *no* formation of thermostable opsonins in the blood of a "control" rabbit of similar age and comparable weight.

→ Subcutaneous inoculation of heated culture of *Staphylococcus*.
→ Intraperitoneal „ „ „ „
→ „ „ living „ „

Chart A (Experiment I.)

The fact that the opsonic power tended to be less in the "inoculated" than in the "control" group, although the bacteriotropins were rising in the former, is a matter of considerable interest. Here it is possible that physical factors were at work. The question of physical factors in the Opsonic Index is, however, too large for consideration here and is still under investigation.

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The experiment, so far as it went, indicated that there was good ground for applying similar tests to human sera in the attempt to "incriminate" the infecting germs of a symbiosis from the merely saprophytic elements.

EXPERIMENT II.

Ten patients suffering from pulmonary tuberculosis, for whom a "secondary" vaccine was desired, were investigated. From the sputum of each of these cases, two or more bacterial strains were isolated in pure culture and tested with the heated and unheated serum of the patient and a healthy "control" (C. M. A.).

The results are set forth in Table I.

Table I.

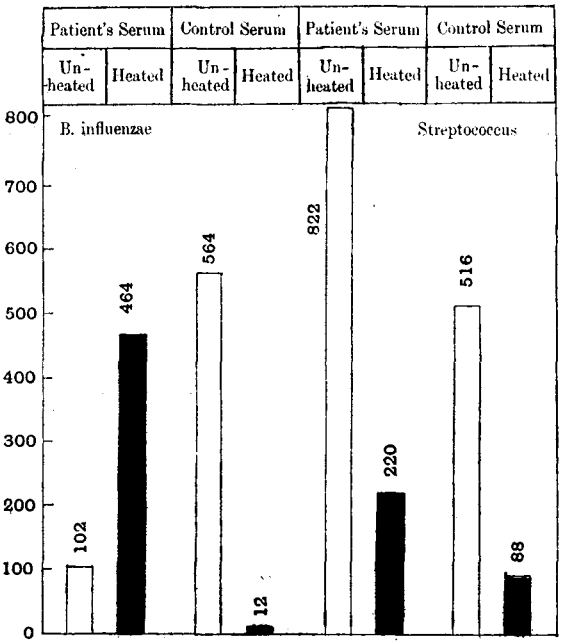
Bacteriotropin Tests in Phthisis Patients.

Case. Name	Germ. Variety	Patient's serum Bacteria in 100 Phagocytes		Control serum Bacteria in 100 Phagocytes	
		Heated	Unheated	Heated	Unheated
Positive Cases:					
1. Miss F. W.	Streptococcus	*108*	122	30	184
	Pneumococcus	2	42	0	40
2. Miss L. D.	B. influenzae	*464*	102	12	564
	Streptococcus	*220*	822	88	516
3. M. J.	Streptococcus longus	*156*	144	44	156
	Streptococcus brevis	8	38	16	74
	? M. catarrhalis	46	268	80	260
4. V. W.	*Streptococcus non-haemolyticus	*226*	366	294	—
	Streptococcus haemolyticus	*798*	570	326	—
Doubtful Cases: (control insufficient)					
5. R. W. J.	? M. catarrhalis	*514*	662	—	520
6. Mrs H.	Streptococcus	*320*	860	—	384
Negative Cases:					
7. Mrs D.	Streptococcus	12	294	4	346
	M. catarrhalis	14	102	34	244
8. Miss C.	Pneumococcus	2	120	78	216
	M. catarrhalis	8	462	82	388
9. J. E.	*Non-haemolyticus Streptococcus	*102*	342	116	370
	M. catarrhalis	0	364	24	302
10. Miss E. E.	Staphylococcus	48	550	—	696
	Streptococcus	98	894	—	838

The words "Positive," "Doubtful," and "Negative" in this table refer to the results of the bacteriotropin test. All the patients were "positive" as regards the tubercle bacillus.

It is of interest to note that of nineteen "secondary" organisms isolated from the sputum of ten phthisical patients, there was definite evidence to incriminate seven organisms as actually infecting the tissues; while it is probable that two other strains, making a total of nine, were etiologically connected with the patient's condition. In the case of the two doubtful

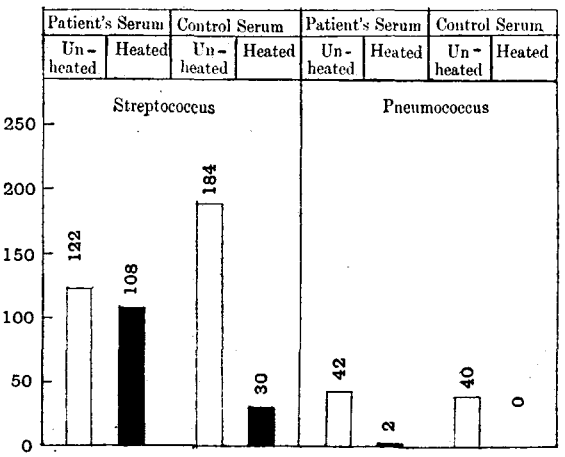
From the sputum were isolated



The high thermostable opsonin for *B. influenzae*, which contrasts sharply with the control, indicates specific infection with the germ. There is reason to think that the streptococcus, too, is infecting.

Chart B. (Experiment II. Case 2. Table I.)

Streptococcus and Pneumococcus isolated from sputum.



This test indicates that there is specific infection by the streptococcus, for which the thermostable opsonins represent 88 % of the total; while it is clear that the Pneumococcus is merely a saprophytic element.

Chart C. (Experiment II. Case 1. Table I.)

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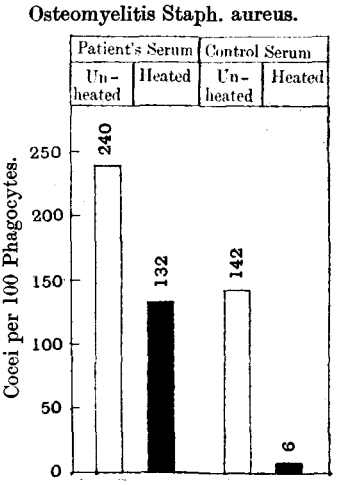
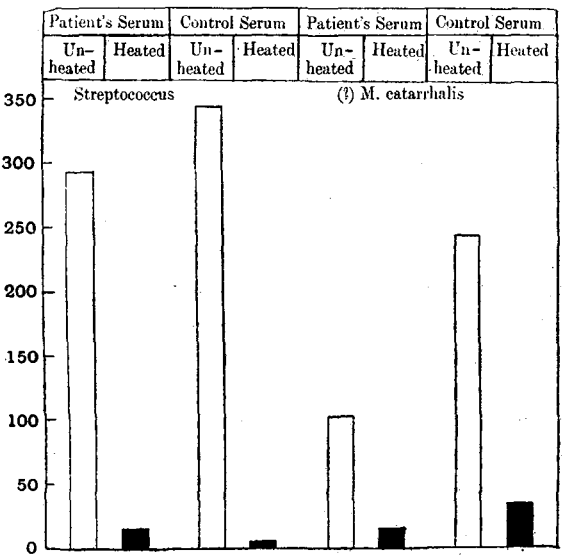
organisms, marked with a "star" in the table, it will be noticed that both were non-haemolytic streptococci and that, in both instances, the doubt arose



Here there is definite evidence of specific reaction to infection, the patient's thermostable opsonins being 55 % of the total; the control being just over 4 % only.

Chart D. (Experiment III. Case 1. Table II.)

The organisms cultivated from the sputum were a streptococcus and (?) *M. catarrhalis.*



In this patient, there is no evidence to suggest that either of the germs isolated has infected the tissues. Both organisms would appear to be purely saprophytic.

Chart E. (Experiment II. Case 7. Table I.)

owing to the presence of equally high or higher bacteriotropins in the heated serum of the "control."

It is probable that this finding should be interpreted, not as exempting

the germ as an agent of infection of the patient but rather as incriminating it as infecting the "control" also.

The sharp differences elicited in this test are more easily appreciated graphically; and three "positive" tests are illustrated in Charts B, C and D, a "negative" result in Chart E.

Chart B (from Case 2) is especially interesting in that the opsonins for *B. influenzae* in the unheated serum *appear* as in the case of the immune rabbits in Exp. I to be less in amount than those in the heated serum. It will be noticed that a similar phenomenon occurs with a *Streptococcus longus* in Case 3 and, more markedly, with a *Streptococcus haemolyticus* in Case 4.

The authors (Cummins and Acland, 1923) have sometimes found this to happen with tubercle bacilli, especially in advanced cases, and have also reproduced the same finding in rabbits infected with Koch's bacillus. The phenomenon may perhaps depend on an increase of "antibody" above the optimum concentration for the strength of antigen used, the heating of the serum reducing the total opsonin to a more favourable concentration and thus leading to increased phagocytosis. H. R. Dean (1911) has shown, in Complement Deviation tests with bacteria, that such a quantitative relationship does actually exist between antigen and antibody, excess of either constituent being unfavourable to the fixation of complement.

Experiment III.

The bacteriotropin test was also applied in a group of ten non-phthisical patients, details of these cases being given in Table II.

It will be noticed that, in this group of cases, the evidence to be gained from the test is less sharp, as a rule, than in the phthisical group where existing lesions may be supposed to have given a readier access of the secondary germs to the tissues.

Case 11, a patient suffering from osteomyelitis, is perhaps the most striking. Here the answer was definitely positive.

Cases 12 and 15 are of interest, both being instances of acute streptococcus empyema following influenzal attacks. In both, the organism was isolated in pure and profuse culture from the pus aspirated by means of a syringe. It may be assumed, therefore, that the streptococci were from the respiratory tract and not from the skin. In Case 15, the test may be taken as indicating that there had not, as yet, been much general infection by the streptococcus isolated and which was pullulating in the liquid contents of a closed serous sac. That some infection was taking place was, however, strongly suggested by a "dilution opsonic," carried out at the same time but not here illustrated; and the result of the bacteriotropin test must therefore be noted as a mark *against* its value in this instance. In Case 12, the emulsion used was very much too dilute, as the result of difficulty with "clumps" which had to be got rid of by centrifugalisation. This explains the negative findings in both

heated and unheated "control" serum. The amount of bacteriotropin in the patient's heated serum, though higher than in the "control," was very low in proportion to the opsonic power of the same serum unheated; but the observation clearly indicates that the germ was "infecting," the most striking result being that obtained with the patient's unheated serum, more especially in consideration of the very dilute bacterial emulsion used.

Table II.

Bacteriotropin Tests in Non-phthisical Patients.

Case. Name and disease	Germ. Variety	Patient's serum Bacteria in 100 Phagocytes		Control serum Bacteria in 100 Phagocytes	
		Heated	Unheated	Heated	Unheated
11. Mrs S. Osteomyelitis (pus)	Staphylococcus aureus	*132*	240	6	142
12. MacA. Empyema (pus)	Streptococcus	*14*	322	0	2
13. Miss M. G.	Streptococcus	30	530	24	104
Lupus facialis (culture from lesion)	Staphylococcus aureus	102	822	160	198
14. J. S. C.	B. coli	24	12	14	40
Colitis (faeces)	Streptococcus	10	118	22	52
15. A. E. F. Empyema (pus)	*Streptococcus	76	506	124	508
16. C. Robinson	*Streptococcus	*256*	632	260	—
Chronic bronchitis (sputum)	? M. catarrhalis	*392*	402	62	—
17. S. L. C.	M. tetragenus	4	40	14	72
Chronic cold (sputum)	*Streptococcus viridans	*204*	398	336	554
18. S. L. C.	Streptococcus faecalis	20	434	4	146
Rheumatism	Gram + bacillus	4	46	—	72
(faeces culture)	*B. coli	14	190	40	62
19. Mrs S.	Staphylococcus	8	150	—	474
Colitis	*B. coli	*216*	700	156	164
(faeces culture)	Gram + bacillus	—	230	2	232
20. K. M. H.	*Streptococcus	*234*	674	258	284
Chronic cold	*? M. catarrhalis	146	678	*672*	564
(sputum)	? M. pharyngeus	100	400	52	208

Case 13, too, is of interest as showing that there are occasions when the comparison of the unheated sera of patient and control gives a clear answer when the bacteriotropin test is negative. It will be remarked that the strains tested in this case were from the surface of an extensive and long-standing lupus patch, under which, no doubt, the lymphatic drainage was almost completely obliterated by the tuberculous process. Under these circumstances, the auto-inoculations of staphylococcus and streptococcus must have been considerably reduced.

Cases 12, 13 and 15 suggest that where the infective agent has only very recently come into operation, as in acute cases, or where, for some mechanical reason, an old-standing infection is almost completely shut off from the circulation, the bacteriotropin test is liable to fail. In such cases the standard

"opsonic index" readings, according to Wright's method, would appear more likely to be of service.

In Case 14, the observation as regards the streptococcus proved that this was "non-infecting" and, in the case of the *B. coli*, though suggesting the presence of bacteriotropins, the test was unsatisfactory through a technical error in using too thin an emulsion. A vaccine of the colon bacillus and streptococcus gave excellent results.

In Case 16, the test was satisfactory and there seems definite evidence to incriminate the *M. catarrhalis*.

Case 17 requires further comment.

In the course of an acute exacerbation of a chronic cold met with in one of us (S.L.C.) it was found that while there were no specific antibodies for a strain of *M. tetragenus* from the throat, there were high thermostable opsonins for a *Streptococcus viridans* also present in large numbers on the plates. It was found, however, that the thermostable opsonins for the latter organisms were even higher in the control serum (C. M. A.).

It happened that the donor of the control serum in this instance had been acting for months as Assistant to the "Patient" and had also, during this period, suffered from several "colds." A swab taken from the throat of the "control" the day after the test produced numerous colonies of *Streptococcus viridans* on plating. In Case 20, the bacteriotropins and opsonins for each of the three organisms tested were present in about the same degree, except in the case of *M. catarrhalis* in which the "control" serum was very rich and no *definite* conclusions could be drawn. Here, too, the "patient" and "control" had for long been exposed to the same source of catarrhal infection.

These findings raise once more the question of the occasional presence of a considerable measure of bacteriotropins in the serum of healthy persons.

I have marked with a star the seven organisms out of the twenty bacterial strains dealt with in Table II for which the "control" (C. M. A.) possessed bacteriotropins, and it will be noticed that, as in the case of the organisms similarly "starred" in Table I, four of these germs are streptococci.

The isolation of *Streptococcus viridans* in large numbers in throat swabs from this "control" gives point to the above observations. It is now too late to be sure whether the other streptococci for which bacteriotropins were demonstrated in the control (C. M. A.) serum were of the "viridans" type, as the time available did not permit of satisfactory classification of all the strains isolated; but it is at least known that none of them were haemolytic.

It has long been recognised that "normal" sera contain a variable amount of thermostable opsonin, especially for the more widely distributed organisms such as tubercle bacilli, colon bacilli and staphylococci. It seems probable that this depends on occasional infection or sub-infection by the organisms concerned or their near antigenic relatives. In the case of such a widely distributed and persistently infective organism as the tubercle bacillus, this fact amounts to a serious interference with the value of the bacteriotropin test;

but for less resistant or less common bacteria it is not difficult to find suitable "controls."

From the point of view of technique, the method used was that devised by Sir A. E. Wright for the estimation of the Opsonic Index. Films were made with the type of "spreader" introduced by him; the staining was carried out with Leishman's stain; and no account was taken of the content of any cellular type except the polymorphonuclear phagocyte. Except in two unavoidable instances, all the enumerations were carried out by the same observer (S. L. C.). Control serum and washed blood cells were always from the same source (C. M. A.).

In Exp. I, the opacity of the bacterial emulsion was always adjusted to correspond to No. 1 on Brown's scale (1914). In Exps. II and III, the emulsions were "guessed" to about a suitable opacity but were not standardised by comparison with the scale. It was an invariable rule that the Enumerator remained in ignorance of what film he was counting, the slides being marked by an Assistant, "shuffled" and dealt to him in any order that chance happened to dictate. It was only when the day's counting was completed that the "results" were "assembled" and sorted into their proper order. There was, therefore, no question of unconscious bias.

As was to be expected in a group of advanced phthisis cases, the "secondary vaccine" treatment based on the above tests gave but inconclusive results. One point of interest, however, emerged; as it was found that there was definite "sensitivity" to the vaccines used in Cases 2, 3 and 5, the patients exhibiting a tendency to marked reactions after even minimal doses.

There were no reactions of any kind amongst the "negative" cases to any of the mixed vaccines made for them from their own germs. This bacterial sensitivity of infected persons, noticed, too, in several of the non-phthisical cases, requires to be taken into account in determining the doses of vaccines, especially in the case of debilitated patients.

Amongst the non-phthisical patients, marked benefit followed vaccine treatment in Cases 11, 12, 14, 17 and 20. There was no benefit in Case 13. In Case 15, no vaccine was used, and in Case 16, the patient, a West Indian negro, left the hospital too soon to allow evaluation of the treatment. In Case 18, there was marked sensitivity to the vaccine of streptococcus and *B. coli*. In Case 19, the results are not yet to hand as the patient is travelling abroad.

It is hardly to be expected that a laboratory test involving a considerable amount of time should come into general use in the guidance of vaccine therapy; but, in intricate cases where there appears to be special reason for caution in dosage, the bacteriotropin test would appear to be well worth the trouble.

Conclusions.

1. The comparison of the bacteriotropins and opsonins of the patient' serum is often of definite value in distinguishing between the infective agents and the harmless saprophytes in the bacterial flora of sputum, faeces, etc.

2. The fact that "healthy" control serum may contain well-marked bacteriotropins for certain bacteria must be taken into account in interpreting the findings and may necessitate the use of several "controls" as suggested by Wright.

3. The presence of bacteriotropins in the serum of a patient should be taken as suggesting that there may be marked sensitivity to the germ in question. In such cases, the greatest caution in dosage is necessary.

4. From the point of view of clinical medicine, it is of interest to note that there was evidence of secondary infection by nine out of nineteen bacterial strains, other than tubercle bacilli, isolated from the sputum.

REFERENCES.

Brown, H. C. (1914). *Indian Journ. Med. Research*, vii.
Cummins, S. L. and Acland, C. M. (1923). *Journ. Pathol. and Bacteriol.* xxvi.
—— and Cumming, C. C. (x. 1912, ix. 1913). *Journ. Roy. Army Med. Corps.*
—— —— (v. 1913). *Journ. Roy. Army Med. Corps.*
Dean, G. (xi. 1905, xi. 1907). *Proc. Roy. Soc.* B. lxxvi and lxxix.
Dean, H. R. (iv. 1911). *Proc. Roy. Soc. Med.* (Path. Section).
Neufeld, F. (1913). *Handbuch der pathogenen Microorganismen*, Jena, xi. (1), pp. 401 *et seq.*
—— and Rimpau (1904). *Deutsche med. Wochenschr.* No. 40.
Savtchenko, J. G. (i. 1902). *Ann. Inst. Pasteur*, xvi. No. 1.
Wright, A. E. and Colebrook, L. (1921). *Technique of the Teat and the Capillary Glass Tube*, London, p. 207.
—— and Reid, S. T. (1906). *Proc. Roy. Soc.* B. lxxvii.

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