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THE PREPARATION OF BACTERIAL ANTIGENS

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THE PREPARATION OF BACTERIAL ANTIGENS *

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The power of the so-called "antibodies" to react selectively with the protein antigens employed in their production, occasioned the early use of these "bodies" as reagents in the detection of differences or similarities existing between two or more proteins. In extension, the method was also early applied to define the closeness of the relationship between cells, as displayed by the degree of the similarity of their constituent proteins.

This application was most extensively made in the field of bacteriology and has allowed a biological classification of bacteria more extensive than that afforded by differences in morphology, growth and pathogenicity. The extent of this use of the antibody-antigen reaction has led to a multiplicity of modifications of the method first employed. But although these modifications have in general been directed toward a refinement and possible standardization of the technic, certain phases remain relatively crude and conducive to error both in experiment and in interpretation. Two distinct complications in the methods now practised are; first, the tremendous admixture of non-essential substances complicating the conditions of reaction both physically and chemically; and, secondly, the chemical instability of the essential reacting substances.

It is the purpose of this paper to discuss these complicating factors in the antibody-antigen reaction and to outline methods which I have used successfully in eliminating such complications.

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The experimental work which is the basis of these studies was performed in the differentiation of strains of the colon-typhoid-intermediate group of bacteria by means of the specific antibody-antigen reaction as displayed in the fixation of the complement of a hemolytic complex.

The results to be presented in detail have to do with the production of standard antigens, and fall under three general headings: first, the elimination of admixed culture medium proteins; second, the disintegration of the bacterial cells with a minimal chemical modification and a maximal liberation of their antigenic proteins; third, the conservation of antigens without the addition of chemical preservatives.

I. The elimination of admixed culture medium proteins. — Commonly, the bacteria used in the preparation of antigens have been grown either in protein containing liquid media, such as meat infusion broth, or on the surface of solid media impregnated with protein constituents. For members of the intestinal group of bacteria, beef infusion agar has been most commonly used. Whole blood, blood-serum, ascites fluid, egg, and other substances have been added to the nutrient agar when the organisms required special conditions for maximum growth. In each case, protein substances have been introduced into the medium, including in most instances bacterial proteins derived from the massive number of organisms which develop during the commercial preparation of the so-called peptones.

The removal of bacteria from such a medium is accompanied by a transfer of considerable quantities of the culture medium constituents which cannot be entirely removed from the bacteria even by combined filtration and centrifugalization. The use of such an antigen is untrustworthy. Its injection may well stimulate the production of immune bodies to the admixed culture medium proteins as well as to the proteins of the bacteria themselves. The resulting immune serum therefore, when tested with similarly prepared antigens, may react not only with the bacterial proteins but also with a variety of others derived from the medium itself.

Olitsky and Bernstein,[1] in their studies of such reactions, found that the injection of serum-grown bacteria into animals resulted in the production of a precipitating serum versus the serum present in the medium. The antiserum thus formed reacted with suspensions of other species of bacteria grown on the same serum medium, in regard to precipitation, agglutination, and complement fixation. Their obvious conclusion was that the bacteria themselves were the carriers of the serum proteins of the culture medium in sufficient quantities to function as complicating antigens.

In a later report,[2] the same authors were able to show that when an immune serum was developed by injections of bacteria grown on "serum" medium and was inoculated into guinea pigs, it sensitized the cells of the animal not only to the bacteria but also to the protein present in the medium upon which the bacteria were grown.

These authors are of the opinion that in addition to the traces of culture medium proteins which are carried over as an admixture, certain of the serum proteins of the medium are actually absorbed by the bacterial cells.

While I do not care to discuss this latter conclusion, the results themselves demonstrate the necessity of excluding as far as possible from bacterial antigen preparations all proteins which are not actual constituents of the bacteria themselves. I am convinced that the methods commonly used in preparing bacterial antigens are not satisfactory from this point of view and that fine distinctions based upon their use are not reliable.

For these reasons, in my own studies of the colon-typhoid-intermediate group I sought to cultivate the organisms in media containing no proteins and wish to present here the methods by which this was accomplished. The actual antigenic variations of the organisms so grown as regards complement fixation at periodic intervals are the basis of a future publication.

Comparative tests of a number of non-protein synthetic media resulted in favor of a modification of Uschinsky's medium, in the preparation of which especial attention was given to the purity of the reagents and to the final degree of

acidity. The medium as finally standardized is prepared by the following formula:

Redistilled Water	1000.
Sodium Chloride	5.
Asparagin	3.4
Di-Sodium Hydrogen Phosphate	2.
Magnesium Sulphate	.5
Ammonium Lactate	6.3

After dissolving, the reaction of the medium is made +0.2 acid by the addition of sodium carbonate, then autoclaved for ten minutes at ten pounds pressure, tubed and again sterilized as before. Any tubes showing the presence of a precipitate are discarded.

Employing this medium I made daily transfers for from one to two weeks. At the end of this time, all (except a very few) of the one hundred strains with which I worked grew sufficiently in forty-eight to seventy-two hours to be used in the preparation of an antigen. (The few cultures which grew sparingly or not at all when first inoculated in this medium later grew sufficiently by first inoculating them into tubes which contained 1/20 part broth in addition to the protein free medium. These cultures were transferred daily to tubes containing decreasing amounts of the broth and finally after several transfers the cultures grew well in the medium without broth addition.)

With the fact established that an extensive cultivation of the organism could be accomplished in this protein-free medium, a series of experiments was next instituted to determine whether or not the antigenic properties of the bacteria differed when grown in the protein-free and in the protein-containing media.

For this determination twelve rabbits were used, six of which were injected individually with B. typhosus, B. paratyphosus A, B. paratyphosus B, B. enteritidis, B. suipestifer, and B. coli communis, grown on plain agar. The other six were also injected with the same organisms which had been grown in the protein-free medium. For injection, the organisms were killed by heating at 60° C. for one hour, control tests being made as to non-viability. An equal number of organisms from each

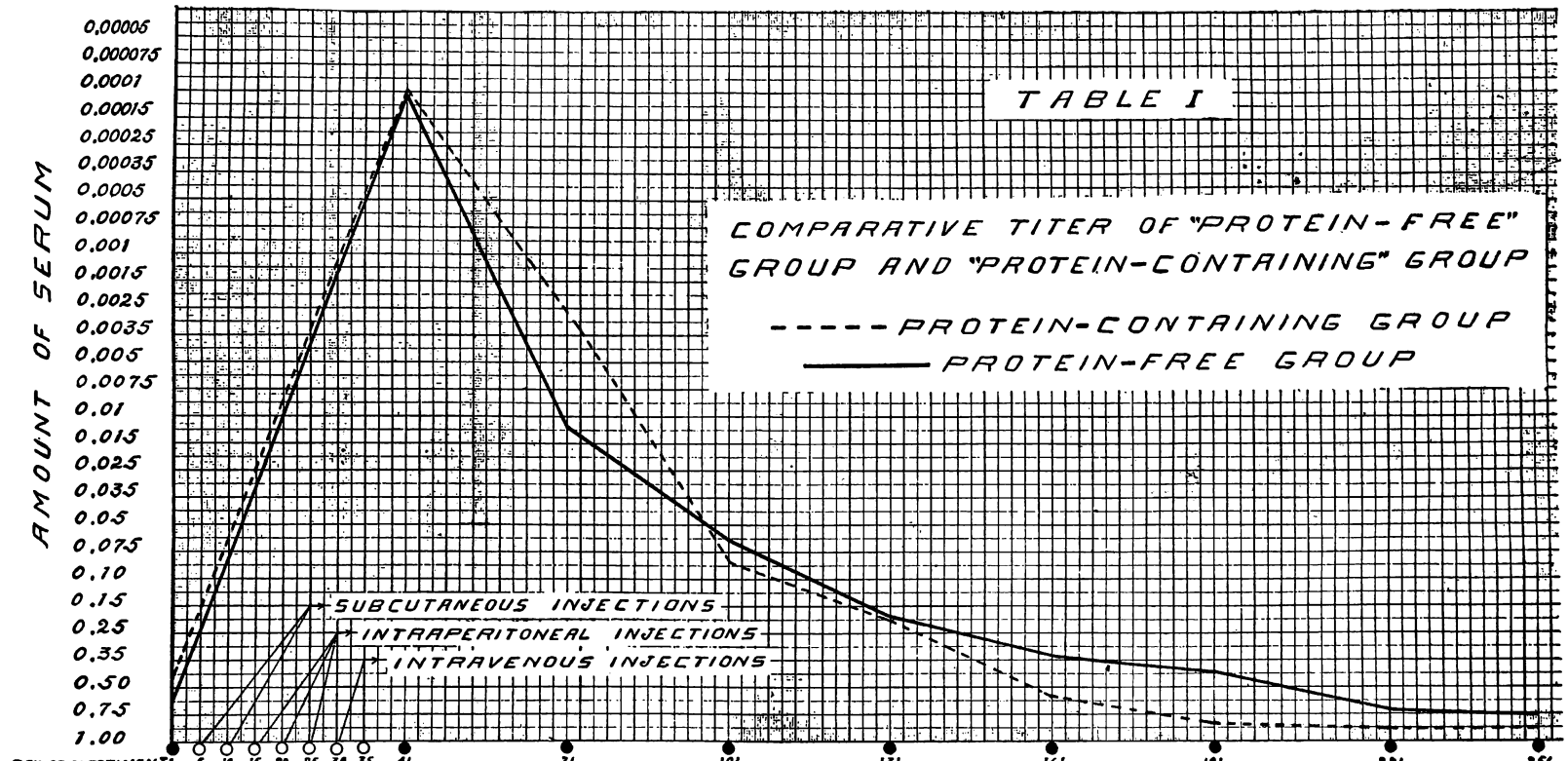
TABLE I
COMPARATIVE TITER OF "PROTEIN-FREE" GROUP AND "PROTEIN-CONTAINING" GROUP
----- PROTEIN-CONTAINING GROUP
——— PROTEIN-FREE GROUP
AMOUNT OF SERUM
0.00005
0.000075
0.0001
0.00015
0.00025
0.00035
0.0005
0.00075
0.001
0.0015
0.0025
0.0035
0.005
0.0075
0.01
0.015
0.025
0.035
0.05
0.075
0.10
0.15
0.25
0.35
0.50
0.75
1.00
SUBCUTANEOUS INJECTIONS
INTRAPERITONEAL INJECTIONS
INTRAVENOUS INJECTIONS
DAY OF EXPERIMENT
1
5
10
15
20
25
30
35
41
71
101
131
161
191
221
251

culture (as determined by Wright's method) were suspended in 1 c.c. of NaCl solution.

After the withdrawal of 10 c.c. of blood from each animal for normal serum controls, the twelve rabbits were injected at five day intervals. The first and second injections were made subcutaneously, the third and fourth intraperitoneally and the fifth, sixth, and seventh intravenously. For test purposes 10 c.c. of blood was drawn six days after the last injection and at successive monthly intervals, and in each instance the serum so obtained was titrated for its agglutinating value on the day following the bleeding.

These determinations showed that the serum from the rabbits which were injected with the organisms grown in the protein-free medium contained as much specific antibody as did the serum from the animals which received organisms grown on the protein-containing medium, even when titrated against organisms grown on the latter medium. The first graph (Table I) displays the curves representing the average titer of each of the two groups of animals at different points.

From this graph it can be seen that at no time was there a distinct contrast in antibody content in the sera of the two groups of animals.

A detailed presentation of the titer of the several sera at the time of maximum observed antibody content (six days following last injection) is given in Table II where the results are shown for each serum as tested with the homologous organism grown in both types of culture medium.

From the results found in Table II it is apparent that with the groups of organisms under consideration, not only can they be cultivated satisfactorily in the absence of admixed proteins, but that when so cultivated they display the same bacterial antigenic values which the organisms possess when grown in protein-containing media.

II. The disintegration of bacterial cells with a minimal chemical modification and a maximal liberation of their antigenic proteins. — While the simple saline suspensions of bacteria as used by Bordet and Gengou [3] in their original work

served the purpose of establishing the principle of complement fixation in a specific antibody-antigen reaction, they were soon found by Moreschi [4] to be unsatisfactory because the bacterial cells so suspended often of themselves so markedly

TABLE II

COMPARISON OF AGGLUTINATION VALUE OF SERA FROM RABBITS INOCULATED WITH
(A) ORGANISMS FROM PROTEIN FREE MEDIUM, AND
(B) SAME ORGANISMS FROM PROTEIN CONTAINING MEDIUM

A SERUM FROM RABBITS INOCULATED WITH ORGANISMS FROM PROTEIN FREE MEDIUM, AND TESTED AGAINST (a) ORGANISMS GROWN IN PROTEIN FREE MEDIUM (P −), AND (b) ORGANISMS GROWN IN PROTEIN CONTAINING MEDIUM (P +)

RABBIT NO	INJECTED WITH PROTEIN FREE CULTURES OF	TESTED AGAINST	AMOUNT OF SERUM																	CONTROLS	
			.01	.0075	.005	.0035	.0025	.0015	.001	.00075	.0005	.00035	.00025	.00015	.0001	.000075	.00005	.000035	.000025	.000015	SERUM CONT	PATHOL CONT
1	B. TYPHOSUS	B. TYPHOSUS (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
		B. TYPHOSUS (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
2	B. PARA. A	B. PARA. A (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
		B. PARA. A (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
3	B. PARA. B	B. PARA. B (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
		B. PARA. B (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
4	B. COLI COM.	B. COLI (P−)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		B. COLI (P+)	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
5	B. SUIPESTIFER	B. SUIPESTIFER (P−)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		B. SUIPESTIFER (P+)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
6	B. ENTERITIDIS	B. ENTERITIDIS (P−)	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
		B. ENTERITIDIS (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−

B SERUM FROM RABBITS INOCULATED WITH ORGANISMS FROM PROTEIN CONTAINING MEDIUM, AND TESTED AGAINST (a) ORGANISMS GROWN IN PROTEIN FREE MEDIUM (P −) AND (b) ORGANISMS GROWN ON PROTEIN CONTAINING MEDIUM (P +)

RABBIT NO	INJECTED WITH AGAR GROWN CULTURES OF	TESTED AGAINST	AMOUNT OF SERUM																	CONTROLS	
			.01	.0075	.005	.0035	.0025	.0015	.001	.00075	.0005	.00035	.00025	.00015	.0001	.000075	.00005	.000035	.000025	.000015	SERUM CONT	PATHOL CONT
7	B. TYPHOSUS	B. TYPHOSUS (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
		B. TYPHOSUS (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
8	B. PARA. A	B. PARA. A (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
		B. PARA. A (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
9	B. PARA B	B. PARA B (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
		B. PARA B (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
10	B. COLI COM.	B. COLI COM. (P−)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		B. COLI COM. (P+)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
11	B. SUIPESTIFER	B. SUIPESTIFER (P−)	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		B. SUIPESTIFER (P+)	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
12	B. ENTERITIDIS	B. ENTERITIDIS (P−)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
		B. ENTERITIDIS (P+)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−

inhibited the action of the complement as to disallow a conclusion concerning a true fixation of the complement in a specific antibody-antigen reaction.

That the complication could be minimized by the substitution of extracts of the bacteria for a suspension of the cells was demonstrated in a considerable series of experiments by

Wassermann and Bruck,[5] Citron,[6] Wassermann,[7] and by Leucles.[8]

Thus it early became recognized that the products of bacterial disintegration were to be preferred to intact bacterial cells in the preparation of antigens for test tube determinations by the complement fixation method. Of the many means used in the attempt to obtain a suitable disintegration, autolysis is perhaps the oldest. Mechanical procedures such as grinding the dried organisms, either alone or admixed with a cutting material such as sand have also been extensively employed. In general, these processes are of slight efficiency. Direct treatment by chemical reagents has also been resorted to, the organism often being subjected to the action of alcohol, ether, chloroform, antiformin or the proteolytic ferments. In the more recent attempts to obtain thorough disintegration, the several procedures have been combined as in Besredka's method by Gay, where alcoholic precipitation, desiccation and grinding with sodium chloride crystals are resorted to, in order.

None of these methods is more than partially satisfactory in obtaining favorable antigen products from bacterial cells. In the case of the mechanical procedures, the yield of cell-free antigen is slight. In the case of the chemical methods a modification of the antigenic bacterial proteins by the reagents employed is sure to occur and may in any given instance be sufficient to change the antigenic properties of the native proteins. This latter point, I cannot emphasize too strongly; for it has been disregarded with great regularity. If there is one generalization which can be made concerning antigenic substances, it is that they are protein in structure; and equally valid is the observation that as such, they are highly susceptible to modification by chemical reagents. The marked specificity of the antibody-antigen reaction demonstrates in itself the delicacy of the chemical differences which determine characteristic antigenic properties. That these differences are readily modified by chemical reagents is shown by the work of Pick,[9] Kahn and McNeil,[10] Perry and Kolmer[11] and others. There is no guarantee that any cell protein subjected to reagents such as

referred to, may not be profoundly changed in its structure and methods which involve such possibilities are to be avoided.

The method of choice for the preparation of a bacterial antigenic product is one which liberates the characteristic proteins from the cell bodies in maximal amounts and with minimal chemical modification. I have sought to accomplish this by the use of physical means, and to this end have developed a method in which the essential feature is the rapid freezing and thawing of the bacteria in aqueous suspension. By this method large amounts of the native antigenic proteins are obtained in solution and the anticomplementary constituents of the cells are removed. The method is simple, and as I have used it with the colon-typhoid-intermediate group, is as follows:

The organisms are grown for from forty-eight to seventy-two hours in culture tubes containing the synthetic medium previously described, at the end of which time the entire contents are transferred to silver plated metal tubes. (The tubes are made in the following manner: A hole $\frac{3}{4}$ inch is drilled $4\frac{7}{8}$ inches deep in the center of a 1 inch diam. solid steel rod. The rod is cut off at a length of 5 inches thus leaving a $\frac{1}{8}$ inch wall. The ends are rounded off and both the inside and outside are polished. The tubes are then heavily silver plated. In order to keep the tubes from bursting during the freezing process, a tapering silver plated pin is placed in them. This pin is 4 inches long and $\frac{3}{16}$ inch in diameter at the top, through which 2 smaller pins are inserted at right angles in order to hold it in the center of the tube. After the material is added to the tubes, a cork is placed in the end and it is sealed tightly by applying several layers of adhesive tape. A small metal handle is inserted in the cork. The tubes are immersed in a flask of liquid air to within 1 inch of their tops for from three to four minutes. They are then removed and the contents thawed quickly by dipping the tubes for a few seconds at a time in boiling water. By shaking the tubes vigorously, the material in the tubes, if carefully watched, may be very rapidly thawed without the temperature of the contents rising above 40° C. In this way, a change of temperature of more than 200° C. is obtained in a period of from four to five minutes.

This process is repeated in rapid succession from twenty to twenty-five times. The contents of the tubes are then centrifugalized, and a clear supernatant fluid is obtained with a compact sediment consisting of fragments of the disintegrated bacterial cells. The supernatant fluid is drawn off and re-centrifugalized. Without further treatment the resulting clear solution constitutes the antigen preparation. It is tested for antigenic and anticomplementary properties. The sediment contains the great bulk of anticomplementary substances with

TABLE III

UNIT DETERMINATIONS OF ANTIGEN NO 12. P.F. 3 DAY GROWTH 37°C. FROZEN-UNHEATED

ANTICOMPLEMENTARY TITRATION							ANTIGENIC TITRATION							Ac-Ag INDEX
	ANTIGEN	NORMAL SERUM	COMP.	AMBO.	R.B.C. 2.5%	DEGREE OF FIXATION		ANTIGEN	IMMUNE SERUM	COMP.	AMBO.	R.B.C. 2.5%	DEGREE OF FIXATION	ANTICOMP ANTIGENIC
ORIGINAL SUSPENSION AFTER FREEZING AND THAWING 20 TIMES	0.05	0.05	1.5 UNITS	2 UNITS	1 C.C.	−	ORIGINAL SUSPENSION AFTER FREEZING + THAWING 20 TIMES	0.0001	0.05	1.5 UNITS	2 UNITS	1 C.C.	−	
	0.10	" "	" "	" "	" "	−		0.005	" "	" "	" "	" "	−	
	0.15	" "	" "	" "	" "	−		0.010	" "	" "	" "	" "	+	
	0.20	" "	" "	" "	" "	−		0.015	" "	" "	" "	" "	+ + +	
	0.25	" "	" "	" "	" "	−		0.020	" "	" "	" "	" "	+ + + +	15
	0.30	" "	" "	" "	" "	+		0.025	" "	" "	" "	" "	+ + + +	
	0.35	" "	" "	" "	" "	+		0.030	" "	" "	" "	" "	+ + + +	
	0.40	" "	" "	" "	" "	+		0.035	" "	" "	" "	" "	+ + + +	
	0.45	" "	" "	" "	" "	+		0.040	" "	" "	" "	" "	+ + + +	
	0.00	" "	" "	" "	" "	−		0.000	" "	" "	" "	" "	−	
SUPERNATANT FLUID	0.50	0.05	" "	2 UNITS	" "	−	SUPERNATANT FLUID	0.005	0.05	" "	" "	" "	−	
	0.75	" "	" "	" "	" "	−		0.010	"	" "	" "	"	+ + + +	
	1.00	" "	" "	" "	" "	−		0.015	" "	" "	" "	" "	+ + + +	
	1.50	" "	" "	" "	" "	−		0.020	" "	" "	" "	" "	+ + + +	
	2.00	" "	" "	" "	" "	−		0.025	" "	" "	" "	" "	+ + + +	>350
	2.50	" "	" "	" "	" "	−		0.030	" "	" "	" "	" "	+ + + +	
	3.00	" "	" "	" "	" "	−		0.035	" "	" "	" "	" "	+ + + +	
	3.50	" "	" "	" "	" "	−		0.040	" "	" "	"	" "	+ + + +	
	0.00	" "	" "	" "	" "	−		0.045	" "	" "	" "	" "	+ + + +	
	3.50	" "	" "	N.O.	" "	+ + + +		0.000	" "	" "	" "	" "	−	
RESUSPENDED CELLS IN NaCl TO ORIGINAL VOLUME	0.05	0.05	" "	2 UNITS	" "	−	RESUSPENDED CELLS IN NaCl TO ORIGINAL VOLUME	0.020	0.05	"	" "	" "	−	
	0.10	" "	" "	" "	" "	−		0.025	" "	" "	" "	" "	−	
	0.15	" "	" "	" "	" "	−		0.030	"	" "	" "	" "	−	
	0.20	" "	" "	" "	" "	−		0.035	" "	" "	" "	" "	+	
	0.25	" "	" "	"	" "	−		0.040	" "	" "	" "	" "	+	3
	0.30	" "	" "	" "	" "	−		0.045	" "	" "	" "	" "	+	
	0.35	" "	" "	" "	" "	+		0.050	" "	" "	" "	" "	+	
	0.40	" "	" "	" "	" "	+		0.075	" "	" "	" "	" "	+ +	
	0.45	" "	" "	" "	" "	+		0.100	" "	" "	" "	" "	+ + + +	
	0.00	" "	" "	" "	" "	−		0.000	" "	" "	" "	" "	−	

but little antigen and is discarded. For completeness, a protocol of the antigenic and anticomplementary titrations obtained in preparing a given antigen from B. paratyphosus B by the above method are given in Table III. The organism "Paratyphosus B no. 12" was grown seventy-two hours in synthetic medium at 37° C.

This table shows that the antigen preparation when titrated by the complement fixation method displayed a very great antigen content, in fact distinctly greater than did the total suspension; on the other hand, it completely lacked an anti-

complementary action of its own. The sediment, on the contrary, possessed almost the full anticomplementary power of the original suspension, but showed only a slight antigenic value.

The method is constant in providing a satisfactory antigen preparation without anticomplementary action when applied to the group of organisms in question. The only point in the method at which special care must need be exercised is at the time of centrifugalization. The antigen solution must be centrifugalized until it is perfectly clear, otherwise it may still possess a degree of anticomplementary action.

How far the method is applicable to other types of organisms than those here considered has not been extensively determined. But that it has a general application seems probable from the fact that in a slightly modified form I have by its use succeeded in preparing an antigen from tubercle bacilli with so low an anticomplementary action as to allow its use in a reliable serum diagnosis of tuberculosis in man.

III. The conservation of antigens without the addition of chemical preservatives. — In the preceding section I have given methods by which bacteria may be cultivated for use as antigens, without protein admixture and by which the essential antigenic substances may be released from the bacterial cell without modification by chemical reagents.

A third necessity in the comprehensive use of antigens for quantitative test-tube determinations is the preservation of a given antigen over a relatively long period without great loss in antigenic value or marked increase in its anticomplementary action.

The usual method of adding to antigen solutions such chemical preservatives as phenol and trikresol has the distinct disadvantage of inviting progressive chemical changes by these reagents often resulting in precipitates with considerable anticomplementary power.

A far more dependable method I have found to be the preservation of stock antigens in a frozen state. In this method, the perfectly clear antigen solution obtained by centrifugalization

as described above, is subdivided into small glass tubes. Each tube is filled to about two-thirds its capacity and sealed. The tubes are then placed in a refrigerating chamber maintained at — 18° C. and remain there until required for use.

Antigens so preserved are serviceable for a period of at least three years. Slight decrease in antigenic value does occur in that time, but it is not of such degree as to impair seriously the antigen as a reagent.

TABLE IV

ANTIGENIC TITRATION							ANTICOMPLEMENTARY TITRATION						
DATE OF TEST	ANTIGEN	IMMUNE SERUM	COMP.	AMBO.	R.B.C. 2.5%	DEGREE OF FIXATION	ANTIGEN	NORMAL SERUM	COMP.	AMBO.	R.B.C. 2.5%	DEGREE OF FIXATION	AC-AS INDEX (ANTICOMPLEMENTARY ANTIGENIC
FEB. 11 1917	0.005	0.05	.5 UNITS	2 UNITS	1.0 C.C.	—	0.5	0.05	.5 UNITS	2 UNITS	1.0 C.C.	—	
	0.010	" "	" "	" "	" "	+ +	0.6	" "	" "	" "	" "	—	
	0.015	" "	" "	" "	" "	+ + + +	0.7	" "	" "	" "	" "	—	
	0.020	" "	" "	" "	" "	+ + + +	0.8	" "	" "	" "	" "	—	
	0.025	" "	" "	" "	" "	+ + + +	0.9	" "	" "	" "	" "	—	
	0.030	" "	" "	" "	" "	+ + + +	1.0	" "	" "	" "	" "	—	>166
	0.035	" "	" "	" "	" "	+ + + +	1.5	" "	" "	" "	" "	—	
	0.040	" "	" "	" "	" "	+ + + +	2.0	" "	" "	" "	" "	—	
	0.045	" "	" "	" "	" "	+ + + +	2.5	" "	" "	" "	" "	—	
	0.000	" "	" "	" "	" "	—	0.0	" "	" "	" "	" "	—	
	0.045	0.00	" "	" "	" "	—	2.5	NO	" "	" "	" "	—	
	0.045	0.05	" "	NO	" "	+ + + +	2.5	0.05	" "	NO	" "	+ + + +	
AUG. 17 1917	0.005	0.05	" "	2 UNITS	" "	—	0.5	0.05	" "	2 UNITS	" "	—	
	0.010	" "	" "	" "	" "	—	0.6	" "	" "	" "	" "	—	
	0.015	" "	" "	" "	" "	+ +	0.7	" "	" "	" "	" "	—	
	0.020	" "	" "	" "	" "	+ + + +	0.8	" "	" "	" "	" "	—	
	0.025	" "	" "	" "	" "	+ + + +	0.9	" "	" "	" "	" "	—	
	0.030	" "	" "	" "	" "	+ + + +	1.0	" "	" "	" "	" "	—	>126
	0.035	" "	" "	" "	" "	+ + + +	1.5	" "	" "	" "	" "	—	
	0.040	" "	" "	" "	" "	+ + + +	2.0	" "	" "	" "	" "	—	
	0.045	" "	" "	" "	" "	+ + + +	2.5	" "	" "	" "	" "	—	
	0.000	" "	" "	" "	" "	—	0.0	" "	" "	" "	" "	—	
	0.045	NO	" "	" "	" "	—	2.5	NO	" "	" "	" "	—	
	0.045	0.05	" "	NO	" "	+ + + +	2.5	0.05	" "	NO	" "	+ + + +	
FEB 15 1918	0.005	0.05	" "	2 UNITS	" "	—	0.5	0.05	" "	2 UNITS	" "	—	
	0.010	" "	" "	" "	" "	—	0.6	" "	" "	" "	" "	—	
	0.015	" "	" "	" "	" "	+ +	0.7	" "	" "	" "	" "	—	
	0.020	" "	" "	" "	" "	+ + + +	0.8	" "	" "	" "	" "	—	
	0.025	" "	" "	" "	" "	+ + + +	0.9	" "	" "	" "	" "	—	
	0.030	" "	" "	" "	" "	+ + + +	1.0	" "	" "	" "	" "	—	100
	0.035	" "	" "	" "	" "	+ + + +	1.5	" "	" "	" "	" "	—	
	0.040	" "	" "	" "	" "	+ + + +	2.0	" "	" "	" "	" "	—	
	0.045	" "	" "	" "	" "	+ + + +	2.5	" "	" "	" "	" "	+	
	0.000	" "	" "	" "	" "	—	0.0	" "	" "	" "	" "	—	
	0.045	NO	" "	" "	" "	—	2.5	NO	" "	" "	" "	—	
	0.045	0.05	" "	NO	" "	+ + + +	2.5	0.05	" "	NO	" "	+ + + +	
FEB. 13 1919	0.005	0.05	" "	2 UNITS	" "	—	0.5	0.05	" "	2 UNITS	" "	—	
	0.010	" "	" "	" "	" "	—	0.6	" "	" "	" "	" "	—	
	0.015	" "	" "	" "	" "	—	0.7	" "	" "	" "	" "	—	
	0.020	" "	" "	" "	" "	+ +	0.8	" "	" "	" "	" "	—	
	0.025	" "	" "	" "	" "	+ + + +	0.9	" "	" "	" "	" "	—	
	0.030	" "	" "	" "	" "	+ + + +	1.0	" "	" "	" "	" "	—	80
	0.035	" "	" "	" "	" "	+ + + +	1.5	" "	" "	" "	" "	—	
	0.040	" "	" "	" "	" "	+ + + +	2.0	" "	" "	" "	" "	+	
	0.045	" "	" "	" "	" "	+ + + +	2.5	" "	" "	" "	" "	+ +	
	0.000	" "	" "	" "	" "	—	0.0	" "	" "	" "	" "	—	
	0.045	NO	" "	" "	" "	—	2.5	NO	" "	" "	" "	—	
	0.045	0.05	" "	NO	" "	+ + + +	2.5	0.05	" "	NO	" "	+ + + +	
FEB. 20 1920	0.005	0.05	" "	2 UNITS	" "	—	0.5	0.05	" "	2 UNITS	" "	—	
	0.010	" "	" "	" "	" "	—	0.6	" "	" "	" "	" "	—	
	0.015	" "	" "	" "	" "	—	0.7	" "	" "	" "	" "	—	
	0.020	" "	" "	" "	" "	+ +	0.8	" "	" "	" "	" "	—	
	0.025	" "	" "	" "	" "	+ + + +	0.9	" "	" "	" "	" "	—	
	0.030	" "	" "	" "	" "	+ + + +	1.0	" "	" "	" "	" "	—	60
	0.035	" "	" "	" "	" "	+ + + +	1.5	" "	" "	" "	" "	+	
	0.040	" "	" "	" "	" "	+ + + +	2.0	" "	" "	" "	" "	+	
	0.045	" "	" "	" "	" "	+ + + +	2.5	" "	" "	" "	" "	+ + +	
	0.000	" "	" "	" "	" "	+	0.0	" "	" "	" "	" "	—	
	0.045	NO	" "	" "	" "	+	2.5	NO	" "	" "	" "	—	
	0.045	0.05	" "	NO	" "	+ + + +	2.5	0.05	" "	NO	" "	+ + + +	

The periodic titration of an antigen so preserved is displayed in Table IV.

As shown above, the antigen unit when first tested was .015 c.c. while 2.5 c.c. displayed no anticomplementary effect. After six months, the antigenic unit was .02 c.c. whereas 2.5 c.c. still was not anticomplementary. After one year, the values were the same as at six months. At two years, the antigenic unit was found to be .025 c.c. and 2.5 c.c. displayed a slight anticomplementary action. At three years, the findings were the same as at two, except that the anticomplementary action was slightly increased. Throughout the whole period however, the antigenic value decreased less than one half and the final anticomplementary-antigenic [Ac.-Ag.] index was 60, thus affording an entirely satisfactory antigen for complement fixation reactions.

Conclusions

As stated at the beginning of this paper, the work has been directed to the elimination of certain factors tending to lessen the accuracy of bacterial identification by the antigen-antibody reaction.

I conclude that the methods as given above are efficient:

1. In eliminating complicating non-essential proteins derived from the culture medium.

2. In obtaining from the bacterial cell an increased amount of its essential antigenic proteins with a minimal modification of their chemical structure.

3. In preserving antigens without desiccation and without the addition of chemical reagents.

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