tion products, the increase in yield of phenol when hydrolyzed in the presence of a reducing agent and the precipitation of triphenyl-methyl peroxide when triphenyl-methyl-magnesium bromide is oxidized, suggests the formation of phenyl peroxide as an intermediate product.

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[CONTRIBUTION OF THE MULFORD BIOLOGICAL LABORATORIES.]

ANTIBODY STUDIES—PART 3. A PRELIMINARY REPORT ON THE CHEMICAL NATURE OF BACTERIAL ANTIBODIES.[1]

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Received September 23, 1920.

I. Introductory.

This paper deals merely with certain chemical facts concerning bacterial antibodies. A complete discussion of the antigen-antibody reaction will be published at a later date.

Immediately following the signing of the Armistice, a large supply of anti-pneumococcic serum became available. Experiments were undertaken to devise methods for extracting or isolating the protective antibodies from such serums as free from other serum constituents as possible.

The application of the principle of bacterial sensitization was utilized. Pneumococcus antigen variously treated was immersed in its specific serum and after allowing the antibodies to combine with the antigen or better, perhaps, after the antibodies were adsorbed by the antigen, the mixture was centrifuged. The sediment consisting of the highly sensitized bacteria was then treated with various reagents in order to split off the protective antibodies from the antigen. The resultant solution was candle filtered and its antibody content determined by protection tests on mice according to the U. S. Hygienic Lab. method for testing anti-pneumococcic serum.[2]

The filtered solution was almost as clear as water, very rarely showing any opal-escense. A typical proximate chemical analysis of such extracts as well as their content of antibodies shown by protection tests is given in Tables I and II, respectively.

[1] Presented by P. Masucci before the Division of Biological Chemistry at the Chicago Meeting of the American Chemical Society, September 6–10, 1920.

[2] The protection test is carried out as follows. For each serum or extract tested, 3 white mice, approximately 18 g. in weight are injected with 0.2 cc. of serum, followed within less than 5 minutes by 0.1 cc. or less of culture. Both injections are made intraperitoneally. Similar injections are made at the same time with the Hygienic Laboratory Control Serum on 6 mice, 3 of which are injected at the beginning of the test, and 3 at the end.

The mice are observed for 96 hours, and mice living over 96 hours are taken as survivals (S). Those dying before 96 hours are marked with the number of hours they lived. (See Table II.)

TABLE I.—THE CHEMICAL COMPOSITION OF ANTI-PNEUMOCOCCIC ANTIBODY SOLUTION.

Protein Color Reactions.	
Biuret	Negative
Xanthoproteic	Negative
Adamkiewicz	Negative
Ninhydrin	Negative
Ehrlich's	Negative
Millon's	Negative
Other Tests.	
Sulfur	Negative
Phosphorus	Negative
Total nitrogen	0.035 mg. per cc.
Mono-amino acids	None

Nitrogen in phosphotungstic acid precipitate, 0.028 mg. per cc.

TABLE II.—PROTECTION TESTS ON ANTI-PNEUMOCOCCIC ANTIBODY SOLUTION.

Comparative protection tests are given on the original immune serum, the immune antibody solution, normal serum, and normal serum extract.

Protective substance.	Amt. of culture used. Cc.	Mice.	
None	0.000,000,001	48	48
	0.000,000,01	48	48
Immune serum 0.2 cc	0.01	S	S
	0.1	S	18
	0.3	S	18
Normal serum 0.2 cc	0.01	48	48
Immune antibody sol. 0.2 cc	0.0001	S	S
	0.001	S	S
	0.01	S	S
	0.1	S	S
Normal antibody sol. 0.2 cc	0.0001	48	48

II. Nature of Antibodies.

The chemical nature of antibodies can only be definitely determined by direct tests on the pure product. Unfortunately, these substances occur in such small proportions that it is impossible to isolate them in pure form.

Whether the substances involved in immunity reactions are colloids or not, we know that antibodies are closely associated with proteins, and that both are taken up by antigen in the process of sensitization. Thus other serum constituents may be given off during the final extraction.

Again, the question of the delicacy of the chemical tests at our disposal, and the competence of any chemical reaction to detect the presence of antibodies as a chemical entity must be considered. The fundamental fact to be borne in mind is that these substances are comparatively unstable and are easily altered by strong chemical reagents.

We have reason to believe that the concentration of antibodies in the extracts is, at the least, in a dilution of 1- 100,000. This concentration could hardly be detected chemically. It is, therefore, evident that common chemical methods are of very limited value.

Much information, however, may be gained by indirect chemical and biological methods and by analogous reactions to known chemical substances.

The first question to determine in such a complex problem, is whether antibodies are similar chemically to any of the known serum proteins or whether they are of a non-protein nature. It has long been known that antibodies are apparently associated with certain serum fractions obtained by salting-out methods, and the impression prevails that antibodies are probably of a globulin nature. The salting-out methods, however useful from a practical standpoint, are of little value in determining the true nature of antibodies owing to the adsorption of the latter by serum proteins.

The most that can be stated is that antibodies are carried down by certain precipitates, which when dissolved show some of the characteristic properties of such bodies.

Landsteiner and Jagic,[1] working with extracts of hemagglutinins, concentrated such material until the agglutinin content equaled the original serum, and found the albumin content to be $^1/_{33}$ that of serum.

Liebermann and Fenyvessy[2] produced concentrated and purified extracts of immune hemolysin. They obtained no tests for protein by the biuret, sulfosalicylic acid, the potassium ferrocyanide and the acetic acid methods, and only traces with the xanthoproteic or Millon's reagent. They also state that only a trace of sulfur could be obtained with alkaline lead solutions and heat.

Kosaki[3] states that with his solutions of hemolysin the sulfosalicylic acid and the test with salt and acetic acid did not give the same reactions as were obtained with true proteins.

Presumably these investigators did not work with pure solutions of antibodies as there is always present a certain amount of impurities derived from the antigen employed. As we have already indicated, it is extremely doubtful that even a pure solution of antibodies could be analyzed by the usual protein reagents.

Some of our chemical tests on antibody extracts are given for the purpose of comparison, and also to show that these tests are not as delicate as the biological methods for determining serum proteins. The extracts tested have a comparatively small amount of serum proteins.

The results shown above indicate that the serum protein content of the extracts tested approaches the minimum amount which can be detected with the chemical reagents at our disposal.

The immune antibody solution employed in Table II was also used to de-

[1] Landsteiner and Jagic, *Münch. Klin. Wochenschr*, **18**, 764 (1903).

[2] Liebermann and Fenyvessy, *Cent. Bakt. Parasitenk.*, **47**, 274 (1908).

[3] Kosaki, *J. Immunol.*, **3**, 109 (1918).

termine its ability to produce serum sensitiveness. Five cc. was injected intraperitoneally into each of 2 guinea pigs. After an incubation period of 15 days, one guinea pig received an intravenous injection of one cc. of normal horse serum. This animal showed a severe anaphylactic reaction but did not die. The second guinea pig received an intravenous injection of one cc. of antipneumococcic immune horse serum and showed some symptoms but not as severe as the other animal.

This experiment led to the routine testing of our extracts by this method. A summary and discussion of the results will be introduced at a later point.

Our results showed that a considerable number of our extracts would produce serum sensitiveness to subsequent injections of horse serum, and it became necessary to consider the source of the proteins producing such sensitiveness. Were the antibodies responsible or were other serum proteins adsorbed in the process of sensitization?

Except in rare cases, the calculated possible dilutions of serum in most of our extracts were too high to expect sensitization from this source. Tests made on the fluids employed for wash purposes failed to show any reactions, so that it must be assumed that such results as we obtained were due to proteins present with the antigen until the final dissociation.

An experiment was made to determine whether the antigen would take up such substances from normal serum. The nitrogen content of this extract was practically the same as that treated with immune serum.

An attempt was also made to determine the nature of the antibody content by employing in the sensitizing tests both normal and immune serum for the final dosage. This was a total failure, for no consistent differences appeared between the response to normal and immune serum.

It must be acknowledged that these tests have failed to demonstrate in one way or another the question of the protein nature of antibodies, but have shown that with the technique employed for sensitization, washing and dissociation , that a certain amount of serum protein remains in solution, in sufficient amount to sensitize a certain proportion of guinea pigs to horse serum. By continued purifications and final concentration of such antibody extracts, we hope to determine definitely whether highly potent solutions may be obtained free from serum proteins which will sensitize guinea pigs to subsequent injection of serum.

III. Experimental.

The fact that direct chemical tests are yet not available to determine the chemical nature of antibodies, and that it is doubtful whether solutions can be prepared totally free from all other serum constituents and extractive substances derived from the antigen used, necessitates the application of indirect physical, chemical and biological methods in order to throw some light on the problem.

In order to determine whether a solution of antibodies is colloidal it may be tested by dialysis, diffusion or ultra-filtration. Of the 3, the simplest one, at our disposal is dialysis. Although this method gives purely qualitative results, it gives, however, an indication of the size of the particle involved, in other words, it tells us whether or not the substance is colloidal in nature.

Kosaki[1] had not been able to dialyze solutions of hemolysin. Some previous work by one of the authors indicated that agglutinins were not dialyzable. Using a parchment bag as a dialyzing medium, we were able to dialyze a protective antibody solution over a considerable period of time without much loss.

In another experiment in which larger quantities were employed there was apparently some loss but when the precipitate which formed during the process was removed and dissolved, it was found that a portion of the antibody content had gone with the precipitate in sufficient amount to account for the loss.

The formation of this precipitate during dialysis is a constant factor being more marked when salt solution and sodium bicarbonate are used for dissociation instead of distilled water.

It can be stated, however, that protective antibodies will remain in solution when the sodium chloride content is $^1/_{1000}$ that of physiological salt solution and that little or none is lost through dialysis.

Assuming that these antibodies have not been adsorbed by protein impurities present, an assumption which is probable, these experiments point to the conclusion that antibodies have a large molecule, ranging from 0.1 micron to 1 micron. In other words, we are dealing with colloidal solutions. Evidence that this colloidal behavior is not due to the protein impurities present but to the antibodies themselves is very strong. During dialysis, as already noted, a great deal of the proteins present is precipitated out of solution, leaving behind the bulk of the antibodies. Were the antibodies combined with these proteins, we should expect to find them largely in the precipitate, which is not the case. We are, therefore, forced to admit that antibodies do not dialyze on account of the large size of their molecule.

Dialysis experiments indicate that antibodies are of a complex nature composed of large molecules. Assuming that they are protein in nature and approaching in chemical composition globulins, we should expect, on digesting with trypsin, the breaking down of the complex molecule into its individual amino acids, with consequent destruction of the antibodies as shown by protection tests.

Experiments were carried out to demonstrate this. As shown in Table III, an excess of trypsin failed to show any reduction in antibody con-

[1] Kosaki, *J. Immunol.*, 3, 109 (1918).

tent. We made sure that the trypsin was active by running suitable controls on casein. When the acid-alcohol precipitating agent was added to tubes, there was hardly any precipitate in any of the tubes containing trypsin as compared with the control. The enzyme had apparently digested the serum proteins present, but had not attacked the antibodies.

TABLE III.—SHOWING EFFECT OF TRYPSIN ON PROTECTIVE ANTIBODY EXTRACT.

A. Protective antibody extract plus 0.2% of 1/1000 solution trypsin.
B. Protective antibody extract (control).
C. 0.2% of 1/1000 solution trypsin (trypsin control).

Substance. Cc.	Length of digestion. Hours.	Culture used. Cc.	Mice.	
A 0.2	24	0.001	S	S
		0.01	S	40
		0.04	S	70
	48	0.001	S	41
		0.01	S	88
		0.04	S	27
	168	0.001	S	..
		0.01	52	..
		0.04	18	..
B 0.2	24	0.001	S	S
		0.01	S	S
		0.04	S	70
	48	0.001	S	S
		0.01	S	S
		0.04	S	88
	168	0.001	S	..
		0.01	S	..
		0.04	90	..
C 0.2	24	0.000, 000, 01	28	..
		0.000, 000, 1	27	..
Virulence	...	0.000, 000, 01	S	42
		0.000, 000, 1	40	28

Besides shedding light on the nature of antibodies, these experiments reveal the rather remarkable resistance that such an extract offers to the deteriorating influence of a temperature of 37.5° and such an active enzyme as trypsin.

The fact that trypsin fails to digest antibodies is very significant. We are either dealing with a non-protein substance which cannot be broken into tri- di- and mono-peptides and ultimately amino acids, or if we are dealing with a protein substance its composition is such that trypsin does not attack it. The ability to be digested by the enzyme trypsin depends of course, on the several properties of the molecule. As is well known, peptides having the carboxyl-amino linkages (NH — CO) are not split by trypsin. Again, Dakin found that casein and other proteins which have been racemized by dil. alkali are no longer digested by trypsin.

We have, therefore, these 2 facts to work upon. As this is merely a preliminary report on the chemical nature of antibodies, further work is being done to determine more definitely whether we are dealing with a complex peptide containing the carboxyl-amino group or whether the antibodies have been racemized by dil. alkali, which in either case resists the action of trypsin.

Experiments were undertaken to determine the effect of weak bases and acids on antibodies. It was found that 0.5% sodium hydrogen carbonate solution did not injure them, but facilitated filtration due, perhaps, to the reversal of the electrical charge.

The addition of acetic acid produced a slight precipitate, but as shown in Table IV, no great injury took place, although a certain amount of the antibodies was adsorbed by the precipitate formed.

TABLE IV.—SHOWING THE EFFECT OF ACETIC ACID ON PROTECTIVE ANTIBODY SOLUTION.

The precipitate obtained by adding 1% glacial acetic to the solution was dissolved in the proper amount of physiological salt solution and designated A.

The supernatant liquid after neutralization with NaOH was designated B.

Substance. Cc.	Culture used. Cc.	Protective Tests. Mice.
Original ext. 0.2	0.001	S ..
	0.01	S ..
	0.04	23 ..
A. (Ppt. dissolved in solution) 0.2	0.001	S ..
	0.01	S ..
	0.04	16 ..
B. (Supernatant) 0.2	0.001	S ..
	0.01	S ..
	0.04	42 ..
Virulence	0.000, 000, 001	S ..
	0.000, 000, 01	42 ..
	0.000, 000, 1	28 ..

By means of protection tests it was likewise found that a 30% solution of sodium chloride did not destroy antibodies, and that ether does not dissolve them.

IV. Summary.

1. The antibody molecules are of large size, not being dialyzable indicating the colloidal nature of the substance.

2. Antibodies are not affected by trypsin over considerable periods, indicating either that they are not protein in nature, or have been racemized by the dil. alkali used, or belong to the peptide group having a carboxyl-amino linkage.

3. Antibodies are not precipitated by solutions containing little or no electrolyte content, indicating that they are not of a globulin nature.

4. Antibodies are not soluble in ether.

5. Antibodies are not precipitated nor affected by a short exposure to 30% sodium chloride solution.

6. Antibodies are not injured by certain dil. alkalies or acids.

7. Antibodies are not affected by temperature up to 60°. Higher temperatures progressively destroy or alter their nature.

We may state, therefore, that antibodies do not belong to that group of proteins usually considered under the head of serum proteins.

It is felt that in the present state of knowledge of protein chemistry the negative information such as we have obtained by indirect methods is of value in narrowing the possible field of investigation.

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[FORTY-SEVENTH CONTRIBUTION FROM THE COLOR LABORATORY OF THE BUREAU OF CHEMISTRY.]

KRYPTOCYANINES. A NEW SERIES OF PHOTOSENSITIZING DYES.

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Received September 23, 1920.

The alkyl halides (or other quaternary addition compounds) of lepidine of sufficient purity give, when treated with alcoholic alkalies in hot, concentrated solution, dyes of the isocyanine type.[1] When the reaction is carried on in very dilute solution, if the radical attached to the nitrogen is *iso*-propyl, or if the reaction is carried on with exclusion of air and the addition of chloroform or formaldehyde, there result dyes of a type not hitherto described, having an absorption maximum near 7000 Å, and a maximum of photosensitizing action near 7400 Å. For these dyes, prepared with the aid of chloroform or formaldehyde[2] we have adopted the name of "kryptocyanines."

The structure of these dyes has not been established, but a comparison of the 6 types of dyes of the cyanine series (see Table I and Fig. 1) suggests that the kryptocyanines and hypocyanines[2] are related to the dicyanines as the pinacyanols and cyanines, respectively, are to the isocyanines. Since the structure of both pinacyanols and dicyanines is still uncertain this does not determine the structure of the kryptocyanines, but the suggestion is made that they result from abimolecular 4,5′-condensation.

[1] E. Q. Adams and H. L. Haller, THIS JOURNAL, 42 2389-91(1920).

[2] Since we have not yet established their identity with the dyes of similar properties formed without formaldehyde, etc., we shall provisionally refer to these latter as "hypocyanines."