ON A SPECIFIC SUBSTANCE OF THE CHOLERA VIBRIO.*

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(Received for publication, April 25, 1927.)

Rather extensive work has been devoted to the question of lipoid antigens in bacilli of the acid-fast group but there are also some reports concerning the solubility in organic solvents of antigens of other microorganisms. Among these are contributions dealing with *V. choleræ.*

Levaditi and Mutermilch (1) were able to prepare solutions of cholera antigen by mixing one volume of an extract in isotonic salt solution with five volumes of absolute alcohol. For the experiments the fluid was centrifuged and the supernatant was evaporated. The residue was found to contain active antigen when tested with anticholera serum in complement fixation tests. After 2 to 3 injections of 40 mg. each of the substance into rabbits the sera of the animals contained specific agglutinins, bactericidal substances, opsonins, and complement-binding antibodies. Guinea pigs treated with the substance acquired active immunity. The substance was insoluble in ether, acetone, or hot absolute alcohol. It resisted boiling in a water solution, and was not easily destroyed by dilute acid or alkali.

Prausnitz (2) in attempting to verify the findings of Levaditi and Mutermilch ascribed the effects observed by these authors to their method of filtration through paper which would not suffice to prevent the passage of bacilli into the filtrate. As a matter of fact after filtration through candles, the author was unable to demonstrate the presence of antigens in the extracts either by immunization or by tests *in vitro.*

In view of the divergent results recorded we undertook a renewed study of the subject.

EXPERIMENTAL.

In order to determine the most suitable method for extraction cholera vibrios were treated successively with ether at room temperature for 24 hours and with various concentrations of alcohol by boiling under reflux for 1 hour. 10 cc. of solvent

* See the preliminary report in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 248.

was employed for the 24 hour growth of each Blake bottle. The alcoholic solutions were filtered by means of a hot water funnel, evaporated on the steam bath to a small volume, the residue taken up in saline, and brought to a volume of 5 cc. per bottle. The solutions were put through common filter paper or kieselguhr paper (Macherey) and precipitin tests were made with the filtrates and cholera immune serum. There was no reaction with the extracts made with ether or strong alcohol but the extracts made with dilute (75 per cent) alcohol were found to be active.

Accordingly for the further work the following technic was adopted. The harvest of a 24 hour growth of cholera vibrio of 150 one quart Blake bottles was washed off with 1.5 liters saline. The microbes were centrifuged, washed twice with about 1–1.5 liters of saline and once with the same quantity of 50 per cent alcohol, centrifuging each time for 30 minutes at high speed.

The sediment was put into 95 per cent alcohol. After 1 or several days the centrifuged bacterial mass was boiled under reflux for 1 hour in 1.5 liters of absolute alcohol and filtered hot. The vibrios were then treated with boiling 75 per cent alcohol. At first two such extractions were made and the solutions joined. Subsequently the first and second extracts were kept separately. The first extraction was made with 500 cc. for 30 minutes; the second with 1500 cc. for 1 to 2 hours. The extracts were filtered through a hot water funnel. Because of the slow rate of filtration the filter paper had to be renewed several times. The hot filtrate ran through clear, but became turbid on cooling and when kept in the ice chest, a flocculent precipitate settled out.[1] This was separated by spinning in a cooled centrifuge and washed with some absolute alcohol and dry ether. From 150 Blake bottles the yield of the first and second extracts approximated 60 mg. and 300 mg. respectively.

It seems, according to preliminary experiments, that a better yield can be obtained by isolating the substance from water extracts.

In the manner described a substance was obtained in the form of a white to grayish white powder. In water the substance swells, and slowly a viscous, more or less turbid fluid is formed; it is more readily soluble on addition of a trace of alkali. The substance was precipitated by cholera immune sera up to dilutions of 1:500,000. In higher concentrations heavy flakes were formed. Both the substances from the first and second extractions in a 1 per cent solution gave positive biuret, xanthoprotein, and Millon reactions and precipitation with trichloracetic acid, tannic acid, phosphotungstic acid, and sulfo-

[1] With another strain of *V. choleræ* and some other vibrios, the alcoholic extracts became faintly turbid on standing in the ice chest and flocculation occurred only after addition of a small quantity of salt solution.

salicylic acid. The reactions were considerably weaker with the second extract. Both preparations gave a strong Molisch test. On heating a 2 per cent solution in N/2 HCl for 90 minutes on the steam bath the liquid became turbid and a rather voluminous precipitate separated. The yield of this precipitate was about 175 mg. per gm. of the hydrolyzed substance. The supernatant fluid gave strong reduction with Fehling's solution and with phenylhydrazine an osazone crystallizing in needles, no pentose reaction with orcinol.

After 10 hours heating the content in reducing sugar was found to be 20.5 per cent, calculated as glucose. After oxidation with nitric acid the solution gave an intense reaction for phosphoric acid.

The precipitate appearing on hydrolysis showed acid character in that it was soluble in alkali, and could be precipitated from the solution by acid. It could be separated by means of methyl alcohol in two parts, one soluble in methyl alcohol and in ether, and another insoluble in these solvents. The former gave the values (calculated for ash-free substance): C, 67.03; H, 10.28; N, 1.35: the latter C, 55.83; H, 8.52; N not determined.

For testing the antigenic properties, the dry substance obtained by alcoholic extraction of the vibrios was dissolved in saline and injected into rabbits. After 2 to 3 injections of 2.0 mg., and also 0.2 mg., precipitins were formed for the extracted substance as well as agglutinins for *V. choleræ*. The antigenic activity of still smaller quantities was not tested.

The substance proved to be toxic and there was loss of animals in the immunization experiments. Guinea pigs died after intravenous injection of 1.0 mg. of the substance and sometimes even 0.1 to 0.2 mg. was lethal.

In order to exclude the presence of bacilli in the injected material immunization experiments were also carried out with 75 per cent alcoholic extracts filtered hot through Berkefeld candles tested for impermeability to a broth culture of *V. choleræ*. The precipitate settling after cooling was employed in quantities of 2 mg. per injection. The results were essentially identical with those recorded in Table III.

The properties of our material bring to mind the specific bacterial substances studied by Avery and Heidelberger on account of the content in carbohydrates, but it differs by virtue of its antigenic

activity and the presence of protein. Consequently efforts were made to determine whether a specifically reacting non-antigenic substance—a haptene according to our nomenclature—could be separated. In this we succeeded in the following manner.

The first alcoholic extract, richer in protein, was discarded. 1 gm. of dry substance obtained in the second extraction was taken up in 10 cc. of water and

TABLE I.

Precipitation Tests.

Antigens: A = crude extract; B = purified product obtained from A in the manner described.

To 0.2 cc. of the antigen dilutions was added 1 capillary drop of immune serum; readings after 5 minutes and 1 hour. The immune serum was obtained by injections of cholera vibrios into a rabbit.

The agglutinin titer of this serum was 1:8000. The intensity of the reactions is indicated as follows: f. tr. = faint trace; tr. = trace; ±, +, +±, etc.

	Antigen	Antigen diluted 1:					Readings after
		5000	50,000	250,000	500,000	1,000,000	
Cholera immune serum No. 82	A	++±	+	tr.	0		5 min.
		++++	+±	±	f. tr.	0	1 hr.
	B	+++	+	tr.	0		5 min.
		++++	++	±	tr.	0	1 hr.
Normal rabbit serum	A	0	0				1 hr.
	B	0	0				1 hr.

In 24 hour readings the precipitate seemed to be partly dissolved especially in the tubes with higher concentrations of antigen.

to it was added 40 cc. of N/10 NaOH. After about 1 hour, the solution was centrifuged from some insoluble material, and 3 volumes of alcohol added. In the supernatant fluid which was kept for several hours, a second precipitate formed on addition of 1 more volume of alcohol. The first precipitate was dissolved in 25 cc. of water, neutralized, and a little Na_2CO_3 added whereupon a slight precipitate formed. After centrifuging the supernatant fluid was acidified (weakly acid to Congo red) with acetic acid so that on addition of 2 volumes of alcohol a flocculating precipitate appeared. The precipitate was washed with 95 per cent alcohol and dried with absolute alcohol and ether. The yield was about 0.4 gm.

TABLE II.

Precipitation Tests.

The antigens used are the crude bacterial extracts prepared in the manner described. Immune serum 32 was obtained by 3 injections each of 2.0 mg. of the crude cholera extract into a rabbit.

Extract prepared from	*V. choleræ* immune serum 82					*V. choleræ* extract immune serum 32					*B. paratyphosus* B immune serum 74					Normal rabbit serum	
	Antigen diluted 1:																
	1000	5000	50,000	250,000	500,000	1000	5000	50,000	250,000	500,000	1000	5000	50,000	250,000	500,000	1000	5000
V. choleræ (Krumwiede)	++++	++++	++	±	f. tr.	++++	++++	+	±	0	0	0	0	0	0	0	0
V. choleræ (Wadsworth)	++++	+++	tr.	f. tr.	0	++++	++±	±	0	0	0	0	0	0	0	0	0
V. metchnikovi	f. tr. ?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. tyrogenus	f. tr. ?	0	0	0	0	f. tr. ?	0	0	0	0	0	0	0	0	0	0	0
V. Finkler-Prior	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyphosus B	0	0	0	0	0	0	0	0	0	0	++	+++	+±	tr.	f. tr.	0	0

This product was a white powder, slowly swelling and dissolving in water, yielding a faintly turbid liquid in a 1 per cent solution. On addition of a trace of alkali it dissolved more readily. The solution

TABLE III, *a*.

Precipitation Tests.

Rabbits injected with A = crude extract: B = purified product obtained from A in the manner described.

Injected with	Rabbit No.	Substance A diluted 1: 500	5000	50,000	125,000	250,000	500,000	1,000,000	2,000,000	Read after
A	32	−	++++	+	±	tr.	0			1 hr.
		−	++++	+++	+±	±	tr.	0		24 hrs.
	33	−	+++	+	tr.	0				1 hr.
			++++	+++	+±	±	f. tr.	0		24 hrs.
	41	−	++++	+	±	f. tr.	0			1 hr.
			++++	++	+	±	f. tr.	0		24 hrs.
B	90	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	91	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	92	0	0	0						1 hr.
		f. tr.	0	0						24 hrs.
V. choleræ	82	−	++++	+±	±	±	f. tr.	0		1 hr.
		−	++++	+++	++	+	±	tr.	0	24 hrs.
Normal rabbit serum	1	0	0	0						1 hr.
		0	0	0						24 hrs.
	2	0	0	0						1 hr.
		0	0	0						24 hrs.

gave a negative biuret and Millon's reaction, a very slight xanthoprotein reaction, faint traces of turbidity with trichloracetic, tannic acid, and sulfosalicylic acid, and no turbidity with phosphotungstic acid.

With Molisch's reagent it reacted intensely. A 2.5 per cent solution in N/50 sodium hydroxide gave a rotation of +.16 in a 5 cm. tube. An

TABLE III, *b*.

Agglutination Tests.

To 0.5 cc. of the stated dilutions of inactivated serum was added 0.5 cc. saline suspension of heat-killed *V. choleræ* grown for 18 hours on agar slants. The tubes were kept for 2 hours at 37° and overnight in the ice box. A = crude extract; B = purified product obtained from A in the manner described.

Injected with	Rabbit No.	Serum diluted 1: 50	500	1000	2000	4000	8000	16,000	Read after
A	32	−	++++	+++	++±	+	0		2 hrs.
			++++	++++	+++	+±	±	0	24 hrs.
	33	−	+++	+++	+	f. tr.	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
	34	−	++++	+++	+±	±	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
B	90	0	0	0					2 hrs.
		0	0	0					24 hrs.
	91	0	0	0					2 hrs.
		0	0	0					24 hrs.
	92	0	0	0					2 hrs.
		0	0	0					24 hrs.
V. choleræ			++++	++++	+++	+±	tr.	0	2 hrs.
			++++	++++	++++	+++	+	0	24 hrs.
Normal rabbit serum	1	0	0	0					2 hrs.
		0	0	0					24 hrs.
	2	0	0	0					2 hrs.
		0	0	0					24 hrs.

analysis gave the following values calculated for ash-free material: C, 49.05; H, 7.17; N, 4.34; P, 1.67; no S; ash, 4.98. The second precipitate mentioned above, after reprecipitation with alcohol in acid

solution, analyzed as follows: C, 47.04; H, 7.09; N, 4.31; P, 1.66; ash, 2.84.

When hydrolyzed with N/2 HCl, sugar could be demonstrated by Fehling's solution and by the osazone test, and a precipitate appeared as in the experiments mentioned above.

With cholera immune serum it was precipitated to the same titer as the original product but rather more intensely (Table I). The precipitin reaction was not diminished by heating a 2 per cent solution for 1 hour in the steam bath; neither was it appreciably affected by digestion with pepsin or trypsin. It resisted the action of nitrous acid.

The specificity of the substance was investigated with the results shown in Table II.

The tests demonstrate the specificity of the reaction with the precipitable substance. A third strain of cholera gave a product which reacted only weakly with cholera immune serum. This strain was atypical in that it showed spontaneous agglutination and was considerably less agglutinated by immune serum than the other strains.

The antigenic activity of the crude and purified extract was tested as follows:

Two batches of rabbits were injected—one with the crude material used for the purification; another with the purified substance. Three injections each of 2 mg. of the substances dissolved in 1 cc. saline were given intravenously at intervals of 5 days. 7 days after the last injection the sera were tested. The results are presented in Tables III, *a* and III, *b*. Before the injections the sera of the animals did not agglutinate in dilutions of 1:10 and 1:50 and gave no precipitin reactions.

It appears from the experiment that substance B while reacting strongly *in vitro* (Table I) had little if any antigenic activity.

SUMMARY.

The investigations described show that it is possible to extract specific substances from *V. choleræ* by means of hot dilute alcohol. This result cannot be attributed to the presence of bacilli in the extracts as would follow from the statements made by Prausnitz in his criticism of the work of Levaditi.

The original extract contains protein and exhibits antigenic properties. From this preparation an almost protein-free product was obtained. It was fully active in the precipitin test but had lost almost completely the antigenic activity. Accordingly the latter preparation belongs to the class of substances described by Zinsser as residue antigens and studied chemically by Avery and Heidelberger. The fact that the immune sera resulting from the injections of the crude extracts acted upon the non-immunizing precipitable substance indicates that in the crude extract there is present an antigenic complex consisting of protein and the specific substance.

Regarding its chemical nature it follows from the foregoing that the precipitable but non-immunizing substance is not a protein. On hydrolysis it yielded a considerable quantity of sugar although less than that given for the specific carbohydrates of pneumococci and *B. friedlænderi*. The product contains nitrogen and phosphorus and on hydrolysis a substance of acid character separates from the solution. Accordingly the substance prepared would appear to have either a rather intricate structure or to be a complex carbohydrate, similar to those described by Avery and Heidelberger, but still containing impurities. This issue can probably be decided by further studies.

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