EXPERIMENTAL VIBRIO INFECTIONS OF DEVELOPING CHICK EMBRYOS*

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There is no laboratory animal in which cholera can be produced with manifestations characteristic of the disease in man. Illness and sometimes death can be produced in rabbits, guinea pigs, and mice, but the disease which occurs in them is quite different from human cholera, and is so erratic in its appearance and characteristics as to be an unreliable laboratory procedure. A virulence test has been devised in which white mice are given small intraperitoneal inocula consisting of mixtures of vibrios and mucin (1). By means of the latter test, it has been possible to measure the protection afforded mice by previous injections of anticholera vaccines or by the injection of immune sera (2). Since none of the animals customarily used are satisfactory in all respects, an investigation was made of the susceptibility of the developing chick embryo to vibrios and of the features of infections thus produced, in relation to the antigenic structure of the organisms and to protection induced by means of specific immune sera.

Methods and Materials

Preparation of Antisera.—Vaccines of living organisms were prepared freshly for each inoculation by growing the vibrios on rabbit blood agar slants overnight and then washing them from the slants with sufficient 0.85 per cent sodium chloride solution to make a moderately heavy suspension.

Vaccines of heat-killed organisms were prepared in a manner similar to that used for vaccines of living vibrios with the exception that the former were immersed in a water bath at 56°C. for 20 minutes.

Boiled vaccines were prepared by growing vibrios on veal infusion agar in Kolle flasks. After overnight incubation at 37°C., the organisms were washed from the agar and then suspended in sufficient 0.85 per cent sodium chloride solution to make a moderately dense suspension. About 100 cc. of saline solution was needed for the yield from a single Kolle flask. The suspension was placed in a flask, to which a reflux condenser was attached, and boiled for 2 hours.

Adult rabbits were inoculated intravenously with increasing doses of the desired vaccine at 3 or 4 day intervals; the initial dose was 0.25 cc.; then 0.5 cc. was used; and finally two or three doses of 1.0 cc. were given. Five days after the last inoculation, the rabbits were bled from the ear or the heart, and the blood was allowed to clot in vaseline-lined centrifuge tubes. The

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clear, supernatant serum was separated after centrifugation and was stored in suitable vials without the addition of preservatives.

Preparation of Absorbed Sera.—Organisms for absorption of sera were grown overnight on veal infusion agar in Kolle flasks. They were washed from the agar with cold 0.85 per cent sodium chloride solution, were killed by immersion in a water bath at 56°C. for 30 minutes, and then were centrifuged at high speed (13,000 R.P.M.) for 20 minutes. The supernatant fluid was discarded and the serum to be absorbed was added in the proportion of approximately 6 parts of serum to one part sedimented vibrios. The serum and vibrios were thoroughly mixed with a sterile pipette and the mixture was placed in the incubator at 37°C. for 2 hours, being shaken occasionally. The mixture was then centrifuged at high speed and the resulting clear supernate constituted the absorbed serum. The entire procedure was done with strict precautions for asepsis and the final serum, to which no preservative was added, was tested for sterility. If contaminated, it was sterilized by filtration through a Berkefeld filter.

Bactericidal (Bacteriolytic) Test.—Although the terms "bactericidal" and "bacteriolytic" are used interchangeably, the test described here actually measures the bactericidal action of serum, which presumably is a reflection of the lytic phenomenon.

Serial dilutions, usually in multiples of 5, of inactivated immune serum were made in neopeptone broth to which had been added enough fresh guinea pig complement to make a concentration of 1 part of complement in 20 parts of final mixture. To each tube containing serum dilution and complement was added 0.1 cc. of diluted vibrionic culture representing 10^{-5} cc. of original culture. The final volume in each tube was 1.0 cc. The tubes were placed in the incubator at 37°C. and after 24 hours were inspected for the presence or absence of visible growth. The endpoint of the test was usually quite sharp, and was recorded in terms of the dilution of serum in the tube of greatest dilution in which visible growth failed to occur.

In earlier tests, subcultures on rabbit blood agar plates were made from each of the tubes of the test after a period of incubation of 2 hours. The agreement in results of the two methods was so close that the method depending on visible growth at 24 hours, because of its greater ease in performance and reading, was adopted for regular use.

Certain fresh guinea pig sera are, in themselves, lytic for some strains of vibrio. For this reason, it is always necessary to control the experiment by testing the organism with the complement which is to be used. To allow a margin of error, it is desirable to test the complement for undesirable lytic acrivity in a dilution of 1:10; that is, in twice the concentration in which it will be used in the test. Certain guinea pig sera are not active as complement for some vibrios or for some sera, and it is necessary to select, in a preliminary experiment, complement which will be active. Here again, to allow a margin of safety, it is advantageous to use the complement in a dilution of 1:40, which is half the concentration in which it will be present when the final test is run.

Infections of Chick Embryos.—The methods of Goodpasture (3) and Burnet (4), with minor modifications, have been used. The details of these methods are so familiar that they will not be described here. All embryos which died were cultured on rabbit blood agar plates, material being taken from any desired part of the egg. In many cases, surviving embryos were similarly cultured.

Strain 35. V. cholerae, group I, Type Inaba. Received in the dried state from the National Institute of Health. Original source: Dr. Punja, Calcutta, India; October, 1941.

Strain 41-B. V. cholerae, group I, Type Ogawa. Received in the dried state from the National Institute of Health. Original source: Royal Army College, London, England; March, 1942.

Strain VCH 5. V. cholerae, group I, Type Ogawa. Received in the dried state from Col. J. T. Tripp, U. S. Army, through the courtest of the National Institute ot Health. From the 1945 epidemic in Chungking.

Strain VC 11. Group III. Received from Dr. William Burrows, University of Chicago. British National Collection of Type Cultures No. 4711.

Strain VC 12. Group IV. Received from Dr. Burrows. British National Collection of Type Cultures No. 4712.

All the above strains were in the smooth state, as judged by the usual criteria. Strain VCH 5 showed a marked tendency to develop rugose variants on serial passage through neopeptone broth. The rugosity of the strain had no influence on its susceptibility to bacteriolysis.

RESULTS

When groups of 7 day old chick embryos were inoculated *via* the allantoic sac with serially decreasing numbers of vibrios, all the embryos died within a day or two, except those which received doses so small that probably no vibrios were contained in the inoculum. Cultures of the allantoic fluid of embryos which had received the small inocula and survived to the 5th day, were sterile. It is apparent, therefore, that 7 day embryos are highly susceptible to vibrionic infection. This was found to be true for all vibrios tested, including representatives of the six groups of Gardner and Venkatraman (5), as well as the 3 types or variants of group I (6).

Effect of Age of Embryos.—The younger the chick embryo at the time of infection, the more susceptible it is. Embryos which were inoculated when they were 7 days old died within 48 hours of the inoculation. On the contrary, when 15 day old embryos were given similar inocula, several of them survived the arbitrary 5 day period of observation, and there was a definite tendency for death to occur later in the period of observation than in the case of younger embryos. In some instances, embryos which were inoculated intra-allantoically when they were 15 days old, and survived 5 days, were free from vibrionic infection as measured by subculture of their allantoic fluids, but other similarly surviving embryos contained living vibrios in their allantoic fluids. Embryos of ages between 7 and 15 days showed intermediate degrees of susceptibility (Table I).

Effect of Dosage.—It is strikingly demonstrated in Table I that the size of the initial inoculum had little influence on the survival rates of chick embryos, except in the case of doses which were so small that it was difficult to be sure that any vibrios had been injected. Survival times were influenced to a greater degree than were survival rates, and this was particularly true of the older embryos. A dose of 10^{-5} cc. containing in the neighborhood of 10,000 to 50,000 vibrios was chosen for use in most of the single-dose experiments reported here. The failure of various doses to influence survival rates is in all likelihood due to the remarkable lethal effect of very small numbers of vibrios. A possible reason for the effectiveness of small inocula is that the allantoic fluid itself is an excellent medium for the multiplication of vibrios, as is easily shown *in vitro* with sterile allantoic fluid withdrawn from the egg.

It is also observed from Table I that occasionally a chick embryo will survive

TABLE I

Variation in Susceptibility of Chick Embryos of Different Ages to Vibrionic Infections

Colony count	Dose	Fate of individual embryos					
		1	2	3	4	5	6
		7 day embryos					
	cc.						
	10^{-3}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-4}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-5}	D1+	D1+	D1+	D1+	D1+	D1+
254	10^{-6}	D1+	D2+	D1+	D1+	D1+	D1+
36	10^{-7}	D1+	D1+	D1+	D1+	D1+	D1+
2	10^{-8}	D1+	D2+	D1+	D2+	D1+	D1+
		9 day embryos					
	10^{-3}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-4}	D1+	S+	D1+	D1+	D1+	D1+
	10^{-5}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-6}	D1+	D1+	D1+	D1+	D1+	D1+
78	10^{-7}	D1+	D1+	D2+	D2+	D1+	D1+
6	10^{-8}	D2+	D1+	D2+	D2+	D2+	D2+
		11 day embryos					
	10^{-3}	D1+	D4+	D1+	D1+	D2+	D1+
	10^{-4}	D1+	D1+	D1+	D1+	D4+	D1+
	10^{-5}	D5+	D2+	D2+	D4+	D2+	D1+
254	10^{-6}	D1+	D2+	D4+	D1+	D1+	D5+
36	10^{-7}	D1+	D2+	D2+	D2+	S−	D5+
2	10^{-8}	S−	S+	S+	D2+	D2+	D1+
		13 day embryos					
	10^{-3}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-4}	D1+	D1+	D1+	D1+	D1+	D1+
	10^{-5}	D1+	D2+	D2+	D2+	D1+	D2+
	10^{-6}	D2+	D1+	D2+	S+	D2+	S−
78	10^{-7}	D2+	S−	D2+	D3+	D2+	S+
6	10^{-8}	D2+	D5+	D1+	S−	D2+	S−
		15 day embryos					
	10^{-1}	D1+	D4+	D1+	D2+	D1+	D1+
	10^{-2}	D2+	D1+	D3+	D2+	D5+	D2+
	10^{-3}	D5+	D2+	D5+	D2+	D2+	D2+
	10^{-4}	D4+	D5+	S+	D5+	D2+	D5+
	10^{-5}	S−	D4+	D2+	S+	D5+	D2+
108	10^{-6}	D3+	D5+	D4+	D5+	D5+	S−
16	10^{-7}	D4+	D5+	D2+	D5+	D2+	D5+
1	10^{-8}	D5+	S−	D5+	D5+	S−	D5+

All embryos inoculated intraallantoically with strain VCH 5.

D = died, followed by number to indicate day following inoculation on which death occurred.

S = surviving on the 5th day after inoculation, followed by + or − sign to indicate whether or not vibrios were recovered on culture of allantoic fluid on the 5th day.

a dose of vibrios which is greatly in excess of the smallest dose necessary to kill most of the embryos of the same age. This presumably is attributable to an individual resistance of the particular embryo involved. It becomes necessary, therefore, to employ fairly large numbers of embryos for experiments designed to demonstrate induced protection, if the results are not to be unduly disturbed by occasional resistant members of the group.

Nature of the Infection.—Vibrios dropped on the exposed chorio-allantoic membrane proliferate rapidly and produce edema and clouding of the membrane. If the embryo is not killed too quickly, considerable thickening of the membrane also occurs. Invasion of other parts of the egg occurs, and shortly before the embryo dies, vibrios can be cultured from the allantoic fluid, the amniotic fluid, and the yolk. No specific gross lesions occur on the membranes or the embryo. Extensive thromboses of the chorio-allantoic vessels are commonly seen. Microscopic sections show intense congestion of the membranes and of the viscera of the embryo; and hemorrhages are seen in the tissues.

Passive Protection by Specific Immune Sera.—Two types of specific antisera are customarily prepared for antigenic studies of cholera vibrios: (*a*) the so called H-O antisera, prepared by inoculating rabbits with gently heat-killed organisms, followed by living organisms, and (*b*) the so called O antisera, prepared by inoculating rabbits with bacterial suspensions which have been boiled for 2 hours to inactivate the H antigen. At the same time, of course, any other heat-labile antigens that may be present are inactivated.

Antisera of these two types were prepared from strains representative of the several subgroups and types of *V. cholerae*, and were tested for their ability to protect chick embryos of various ages against fatal infections with homologous vibrios. The antisera were mixed with homologous strains and were injected into allantoic sacs. The protective effect observed was very slight, and amounted to a "partial" protection, which consisted in a certain propotion of cases in the prolongation of life, but which almost never resulted in the sterilization of the infection. The embryos which survived to the 5th day after inoculation contained living vibrios which had multiplied to some extent, but which had not killed the embryos (Table II).

The failure to achieve high degrees of protection with antisera suggested that for some reason the specific immune mechanisms were not functioning properly. In 1938, Polk, Buddingh, and Goodpasture (7), observed that complement was not present in developing chick embryos until shortly before hatching. Inasmuch as bacteriolysis, which involves the mediation of complement, is considered to play an important rôle in immunity to Gram-negative organisms, fresh guinea pig complement was added to the mixture of serum and vibrios with the result that the protective activity of the antiserum was enormously enhanced. In many instances, the added complement enabled the serum to protect completely against as many as 100,000 organisms. The

allantoic fluids of the embryos which survived to the 5th day after inoculation were found to be sterile. In Table III, an experiment is presented in which it is shown that exceedingly small quantities of antiserum were effective in protecting embryos against large infecting doses of homologous organisms. The embryos had been prepared by the injection of guinea pig complement into the allantoic sac 6 hours prior to the injection of antiserum.

The protection of the embryos which has just been described is certainly the result of bacteriolysis, and a similar destruction of the vibrios can be demonstrated and studied more economically and more conveniently in the test tube

TABLE II

Difference in Protection of Chick Embryos from Vibrionic Infections in the Presence and in the Absence of Complement

Dose	Controls						Homologous immune serum No complement			Homologous immune serum Complement added		
	Organisms alone			Complement alone								
	1	2	3	1	2	3	1	2	3	1	2	3
10^{-1}	X	X	X	X	X	X	D1+	D1+	D1+	D1+	D3+	D4+
10^{-2}	X	X	X	X	X	X	D1+	D1+	D1+	D3+	S−	S−
10^{-3}	X	X	X	X	X	X	D1+	D3+	D1+	D3+	S−	S−
10^{-4}	X	X	X	X	X	X	D2+	S+	D3+	S−	S−	S−
10^{-5}	D1+	D1+	D1+	D1+	D1+	D1+	S+	S+	D2+	S−	S−	S−
10^{-6}	D1+	D1+	D1+	D1+	D2+	D1+	S+	D4+	D3+	S−	S−	S−
10^{-7}	D2+	D1+	D1+	D1+	D1+	D1+	S+	S+	S+	S−	S−	S−
10^{-8}	D1+	D1+	D1+	D2+	D1+	D1+	D3+	D4+	S+	S−	S−	S−

9 day embryos. Window technique. Serum, complement, and diluted culture mixed before implantation on the chorio-allantoic membrane. Strain VCH 5 used for inoculations. Homologous serum prepared by inoculating living organisms. Complement used was fresh, pooled guinea pig serum, tested *in vitro* for activity as complement and lack of lytic activity in absence of immune serum. Symbols as in Table I. X = not done.

than in the embryo. The above findings are presented to show that the bacteriolytic or bactericidal process is active in the chick embryo when the necessary reagents of the reaction (immume serum, complement, and organism) are present, and that no interfering substances are contributed by the egg.

Passive protection of the two types just described, *i.e.* the complete protection in which complement is involved, and the partial protection in which no complement is involved, can be conferred on the embryos by the use of either the H-O antiserum or the O antiserum. There is possibly a small quantitative reduction in the antigenicity of the boiled suspensions, but the differences which were observed in antibody content of sera prepared with living and boiled antigens were not greater than differences which were present in sera taken from several rabbits immunized in the same way with a single vaccine. It is ap-

parent that the antigen or antigens involved in the protective phenomena are highly heat-stable.

Relation of Passive Protection to Serological Group and Type.—It is of considerable importance to know whether immune protection in cholera is strain-specific, type-specific, group-specific, or genus-specific. The bearing of this

TABLE III

Effectiveness of Protection by Specific Immune Serum in the Presence of Complement

Serum, complement, and organisms. Serum dilutions	Fate of individual embryos									
	1	2	3	4	5	6	7	8	9	10
1:1	S−	S−	S−	S−	S−	S−	S−	D1−	S−	S−
1:5	S−	S−	S−	S−	S−	S−	S−	S−	S−	S−
1:25	S−	S−	S−	S−	D1+	S−	S−	D2−	S−	D1+
1:125	S−	S−	S−	S−	S−	S−	S−	S−	S−	S−
1:625	S−	S−	D3+	S−	S−	S−	S−	S−	S−	S−
1:3,125	D1+	D3+	S−	S−	S−	S−	S−	S−	S−	S−
1:15,625	S−	D5+	S−	D5+	S−	S−	S−	S−	S−	S−
1:78,125	S−	S−	S−	S−	D5+	S−	S−	S−	D1+	S−
1:390,625	D2+	D1+	D1+	S−	D3+	D2+	D2+	D1+	D5+	D1+
Serum and organisms, no complement.	D3+	D3+	S+	S+	S+	S+	S+	D3+	D2+	D3+
Complement and organisms, no serum.	D1+	D1+	D1+	D1+	D1+	D1+	D2+	D1+	D1+	D1+
Organisms only.	D1+	D2+	D2+	D1+	D1+	D1+	D1+	D1+	D1+	D2+
Serum and complement, no organisms.	S−	S−	S−	S−	S−	S−	S−	S−	S−	S−

9 day embryos. All injections into allantoic sac. Organisms used: 10^{-6} cc. of strain 35, containing approximately 8,000 organisms. Complement: Pooled, fresh guinea pig serum. 0.1 cc. injected into allantoic sac of indicated embryos 6 hours prior to injection of serum (or serum dilution) and organisms. Preliminary *in vitro* titration of complement showed that it was not lytic itself for strain 35, and was active as complement with strain 35 and homologous serum. Serum: Rabbit serum prepared by inoculating living organisms of strain 35. Diluted as indicated with neopeptone broth. Symbols as in Table I.

matter on the composition of vaccines for use in human immunization is obvious. Passive protective tests of chick embryos, with and without complement, and *in vitro* bactericidal tests were performed in which sera prepared from strains of the several groups of Gardner and Venkatraman were used; it was shown that protection against infection with group I organisms was conferred only by antisera prepared from the homologous group, and similarly that bactericidal effect on group I organisms was exercised only by group I antisera in

TABLE IV

Group Specificity of Unabsorbed Sera

Sera	Strains																	
	VCH 5						VC 11						VC 12					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Group I antiserum prepared with strain VCH 5	S	D5	D3	S	D2	S	D1	D2	D2	D1	D1	D1	D1	D1	D1	D1	D1	D1
Group III antiserum prepared with strain VC 11	D1	D1	D1	D2	D1	D1	D1	S	D4	D5	S	S	D2	D1	D1	D1	D1	D1
Group IV antiserum prepared with strain VC 12	D1	D1	D1	D1	D1	D1	D1	D1	D1	D2	D1	D2	D4	D2	S	D4	S	D3
Control: no serum	D1	D1	D1	D1	D1	D1	D1	D2	D1	D1	D1	D1	D1	D1	D1	D2	D2	D1

9 day embryos. All inoculations made into the allantoic sac. No complement. 10^{-5} cc. of indicated bacterial culture in 0.1 cc. neopeptone broth plus 0.1 cc. indicated serum, undiluted, mixed with inoculum before injection. D = died, followed by number to indicate day following inoculation on which death occurred, S = surviving on the 5th day after inoculation.

TABLE V

Cross Protection by Unabsorbed Inaba and Ogawa Sera

Colony counts	Dose	Control (organisms alone)	Control (complement alone)	Type Ogawa antiserum (VCH 5)			Type Inaba antiserum (35)		
	10^{-1}	D1	D1	D1	D1	D1	D1	D1	D1
	10^{-2}	D1	D1	D1	D1	D3	D1	D1	D3
	10^{-3}	D1	D1	D2	D3	S	D1	S	S
	10^{-4}	D1	D1	D3	D5	S	S	S	S
	10^{-5}	D1	D1	S	S	S	S	S	S
	10^{-6}	D1	D1	S	S	S	S	S	S
244	10^{-7}	D3	D1	S	S	S	S	S	S
26	10^{-8}	D1	D1	S	S	S	S	S	S

9 day embryos. Window technic. Inocula made with indicated serial dilutions of strain VCH 5 (Type Ogawa). 0.1 cc. of indicated antiserum, undiluted, inactivated at 56°C. for 30 minutes plus dilution of organisms indicated, plus 0.05 cc. undiluted fresh guinea pig complement mixed immediately before implantation on membranes. Symbols as in Table I.

the presence of complement. Group I antisera exercised protective and bacteriolytic effects only for strains of the homologous group (Table IV).

Antisera prepared from strain 35 (Type Inaba) protected chick embryos from infection by strains of both Type Inaba and Type Ogawa; and similarly

TABLE VI

Bactericidal Tests. Cross Absorption of Inaba and Ogawa Antisera

Serum	Bactericidal action on strain 35 (Type Inaba)										
	1:20	1:100	1:500	1:2,500	1: 12,500	1: 62,500	1: 312,500	1: 1,500,000	1: 7,500,000	No serum	No serum or complement
Anti 35(L) unabsorbed	−	−	−	−	−	−	−	++++	++++	++++	++++
Anti 35(L) absorbed with 41 B	−	−	−	−	−	−	++++	++++	++++		
Anti 35(L) absorbed with 35	++++	++++	++++	++++	++++	++++	++++	++++	++++		
Anti 41B(L) unabsorbed	−	−	−	−	++++	++++	++++	++++	++++		
Anti 41B(L) absorbed with 35	++++	++++	++++	++++	++++	++++	++++	++++	++++		
Anti 41B(L) absorbed with 41B	++++	++++	++++	++++	++++	++++	++++	++++	++++		
Bactericidal action on strain 41 B (Type Ogawa)											
Anti 35(L) unabsorbed	−	−	−	−	−	−	−	++++	++++	++++	++++
Anti 35(L) absorbed with 41B(L)	−	−	−	−	−	−	++++	++++	++++		
Anti 35(L) absorbed with 35	++++	++++	++++	++++	++++	++++	++++	++++	++++		
Anti 41B(L) unabsorbed	−	−	−	−	−	−	++++	++++	++++		
Anti 41B(L) absorbed with 35	−	−	−	−	−	−	++++	++++	++++		
Anti 41B(L) absorbed with 41B	++++	++++	++++	++++	++++	++++	++++	++++	++++		

The serum dilutions were made as indicated in neopeptone broth, inactivated (56° for 30 minutes) serum being used. Complement was added to all tubes to a final concentration of 1:20, except in the case of the indicated control tubes. Preliminary tests of the complement (fresh, pooled guinea pig serum) showed that the complement was not in itself lytic for the strains used in the experiment, and that it was active as complement in the presence of immune serum. The inoculum of organisms consisted in all cases of 10^{-6} cc. of a 16 hour culture in neopeptone broth of the indicated strain, diluted to 0.1 cc. in fresh neopeptone broth. ++++ = maximal growth, − = no visible growth. Readings of the tubes made after 24 hours incubation at 37°C.

antisera prepared from strain VCH5 (Type Ogawa) protected against infections by both serological types (Table V). Bactericidal tests showed corresponding results.

Reciprocal bacteriolysin absorption tests, in which sera prepared with strains 35 and 41B and absorbed with homologous and heterologous strains were used, showed that all lysins were removed by absorption with the homologous strains (Table VI). Absorption of serum prepared from strain 41B (Type Ogawa) with

packed cells of strain 35 (Type Inaba) removed the group-specific antibody which is shared by the two strains, but failed to remove the type-specific antibodies. These antibodies, like the group-specific antibodies, participate in a bacteriolytic reaction. It is apparent that two antigens in the group I vibrios give rise to bacteriolysins: a group-specific antigen and a type-specific antigen.

It is of interest to note that while strain 41B is able to remove both group-specific and type-specific bacteriolysins of the homologous strain, it is unable to absorb completely the group antibodies present in sera prepared with the heterologous strain 35.

Chick embryo–protective tests to demonstrate the partial protection conferred by the absorbed sera in the absence of complement were unsatisfactory. The amount of antibody involved in this reaction is very high compared to the lytic or protective tests in which complement is present. It is likely that the absorptions reduce, in a non-specific way, the concentration of all antibodies to an extent which prevents their action from being demonstrated in the absence of complement.

No evidence was obtained to suggest that any strain-specific antigens, as opposed to group- and type-specific antigens, were involved in protection or bacteriolysis. The number of strains investigated was small, however, and none of the strains available for study were entirely fresh in the sense of being quite recently isolated from patients, without resting on artificial media or being dried. It cannot positively be stated that no antigenic components other than the group- and type-antigens are of importance in immunity to vibrionic infections.

DISCUSSION

Infection of chick embryos by many bacterial species has been studied since Goodpasture and Anderson (8) demonstrated the usefulness of the embryos for that purpose. No previous report has appeared of infections with *V. cholerae*. The absence of complement in hens' eggs prior to hatching makes them particularly suited to a study of immune phenomena in which complement is or is not required, since complement can be added or withheld at will. Buddingh and Polk (9) protected chick embryos from meningococcal infections by means of antisera. They stated that complement was not necessary for protection. In a series of papers, Weil and his coworkers (10–13) have reported their findings on chick embryo infections with typhoid and Shiga organisms. They were able to protect the embryos with immune sera, but no mention is made of the rôle of complement in the protection they observed, and they do not state whether or not their antisera were inactivated.

In the case of the cholera vibrios it is quite likely that the antibodies which are responsible for the "partial" protection without complement and for the complete protection with complement, are identical, the difference in effect

observed when complement is or is not added being a quantitative difference. If this is the case, complement then might be regarded as a catalyzing agent, acting to accelerate an immune reaction which, to a much smaller degree, occurs without complement and can be demonstrated under special circumstances such as those described here. Ward and Enders (14) made a similar suggestion for the rôle of complement in phagocytosis, and presented a striking demonstration of its accelerating effect.

The mechanism by which chick embryos are killed by vibrios is not understood, and attempts to extract from vibrios a lethal toxin have so far met with failure. Suspensions of vibrios killed by minimal exposures to heat (10 minutes at 56°C.) are not toxic when injected into the allantoic sac, even in large quantities. Simple, cell-free, distilled-water extracts have a low and irregular toxicity, although they are highly antigenic and produce on injection into rabbits antibodies which correspond quite closely in their activities to those of group I which are stimulated by inoculation of the intact cells. These observations suggest that the toxic substance of the vibrios is not necessarily identical or even closely associated with the O antigen; *i.e.*, a "complete antigen" in the sense of Boivin, which is toxic. However, the studies which have been made are too rudimentary to allow a definitive statement on the subject at the present time.

The vaccine which is commonly prepared for active human immunization against cholera contains two strains: an Inaba strain and an Ogawa strain. The reason for including strains of the two types is largely empirical, and is is based on the assumption that immunity to cholera is type-specific. The bacteriolytic and embryo-protective tests reported here show that, while type-specific antibodies do have lytic and protective activity, there is shared by both types of group I a common antigen which alone is able to protect against infection with strains of both types and to produce lysis of them. The wisdom or the necessity of including representatives of both types is subject to question, therefore, although it cannot be said from the work here that other antigens may not be involved in the way, for instance, that the Vi antigen of typhoid organisms is important in those strains which contain it.

The concept of serological types in the cholera vibrios arose from agglutination and agglutinin absorption studies. The situation in the case of the vibrios of group I is quite different from that present in certain other bacteria (notably the streptococcus and the pneumococcus) in which the type-specific antigen alone is primarily responsible for the stimulation of antibodies which lead to animal protection. In the case of the vibrios, the group as well as the type antigens are active in the production of such antibodies.

SUMMARY

Developing chick embryos are highly susceptible to infection with strains of *V. cholerae* representing Gardner and Venkatraman's 6 groups and the types Inaba and Ogawa. There is a moderate decrease in susceptibility with ad-

vancing age of the embryo. The influence of dosage on survival rates is not marked, probably because a minimal dose, consisting of a very few organisms, is sufficient to produce death rapidly.

Passive protection of a low order is conferred on the embryos by the introduction of inactivated specific immune serum at the time of inoculation of vibrios. This protective influence is enormously enhanced by the previous or simultaneous administration of guinea pig complement.

The antigens of group I organisms which give rise to embryo-protective and bacteriolytic antibodies are dual in character. One antigen is shared by all members of the group and is productive of antibodies which will protect against infections with all strains of the group, of whatever type. The other antigen is type-specific, and its antibodies are protective and lytic only for organisms of the homologous type.

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