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CHOLERA STUDIES * †

4. Problems in Immunology

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SYNOPSIS

Relevant information regarding the numerous problems encountered in cholera immunity is dealt with in great detail in this study. Toxin production, bacterial virulence, serological reactions, and the antigenic structure of *V. cholerae* are discussed. Natural, passive, and active cholera immunity receives special attention, the authors describing the various means of vaccination as well as the evaluation of the immunity induced.

Toxin Production

Dealing with the cholera problem at a conference held in 1884 at Berlin, Koch considered this disease essentially as a toxicosis, caused by a " poison " which the causative organisms excreted. However, the experimental evidence soon procured by some workers was not in favour of the presence of such an exotoxin. Thus Nicati & Rietsch (1884), the pioneers in this field, found the filtrates of young broth cultures of *Vibrio cholerae* incapable of producing signs of toxicosis in dogs. However, an intoxication, chara terized by vomiting, dyspnoea, or general depression and paralysis of the extremities, could be produced in dogs injected intravenously with the filtrates of cholera cultures a week or more old, and led in some of the animals to death within 12 hours.

* This is the fourth of a series of studies which will be published as a monograph on cholera in separate editions in English and in French. — ED.

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Further classical experiments by Cantani (1886) showed that intraperitoneal injection of dogs with peptonized broth-cultures of *V. cholerae*, which had been sterilized by heating at 100°C, produced in these animals severe, though passing, signs of intoxication not dissimilar from those observed in human cholera. Subcutaneous injection of dogs with the same material led to less marked signs, that of peptone-free broth cultures was without effect—a difference due in Cantani's opinion to a more rapid growth as well as a more rapid death of the vibrios in peptonized broth. Generally speaking, this worker ascribed the appearance of the toxic signs he could produce in the above-described manner, to the action of an endotoxin of the *V. cholerae* which, set free after the death of the organisms, acted in a manner comparable to that of mushroom poisons.

The assumption of Cantani that the cholera vibrios, while not secreting an exotoxin, contained a potent endotoxin, was confirmed by observations of some other early workers, particularly the profound studies of Pfeiffer (1892, 1894b; see also Pfeiffer & Wassermann, 1893) and of Gamaleia (1892a).

Summarizing the results of his initial investigations, Pfeiffer (1892) stated that

" quite young, aerobically cultivated cholera growths contain a specific poisonous substance which exhibits extremely toxic effects. This primary cholera toxin stands in a very close relationship ['in sehr enger Zusammengehörigkeit'] to the bodies of the organisms, forming perhaps an integral constituent of them. The toxic substance undergoes apparently no change if the vibrios are killed with the aid of chloroform, thymol or through drying.

" Absolute alcohol, concentrated solutions of neutral salts, boiling heat decompose the poisonous substance, leaving behind secondary toxins which exert a similar physiological action but produce an identical effect only in a 10- to 20-fold dose." [Trans.]

It should be noted in this connexion that, according to Pfeiffer (1894a), in the case of the primary toxin obtained from chloroform-treated cultures of *V. cholerae*, doses varying from 2.5 mg to 5 mg per 100 g of body-weight of the experimental animals were necessary to produce death in intraperitoneally infected guinea-pigs.

As described by Pfeiffer (1892), the most conspicuous sign of cholera intoxication in these animals was an incessant drop of their body temperature which, apt to commence already 1½ to 2 hours after the toxin administration, became most marked (as low as 30°C intrarectally) in fatal cases. Hand in hand with this temperature drop the animals became prostrated; their hind extremities became paralysed, and fibrillary convulsions of the musculature could be observed. If a sufficiently high dose had been given, death usually occurred after 12-16 hours. Administration of lesser doses led to a less marked drop of the body temperature (e.g. to 34°C) and, though apt to show serious signs of intoxication, the animals became well after 24 hours.

Gamaleia (1892), whose independent work fully confirmed the findings of Pfeiffer, came to the conclusion that the labile primary cholera-toxin was

a " nucleo-albumin " which became converted through exposure to temperatures above 60°C or to strong chemicals into a more stable " nuclein ". What relationship exists between this substance and the " nucleoproteid " of the cholera vibrios prepared by Galeotti (1896) and considered by this worker to be the endotoxin of *V. cholerae*, is difficult to decide. It is important to note in this connexion that comparative tests made by Bürgers (1910) with (*a*) cholera agar-cultures treated according to Pfeiffer's methods to obtain the primary endotoxin, and (*b*) growths heated to high temperatures and consequently supposed to contain only secondary toxins, failed to show as marked differences as had been found in corresponding experiments by Pfeiffer and Gamaleia.

It is of historical interest to note that the statements made by Pfeiffer and Gamaleia met at first with considerable opposition, several workers (enumerated in the summaries of Kolle & Schürmann, 1912, Kolle & Prigge, 1928, and Kraus, 1929) maintaining that the pathogenic action of *V. cholerae* was due not to an endotoxin but to the secretion of a soluble exotoxin by the living organisms. However, as convincingly shown by Kolle & Prigge (1928), these claims, which as a rule were based upon tests with only one or a few strains—quite often of a rather doubtful nature—deserve no credence. Thus, as recently stated by Wilson & Miles (1946), it is now generally accepted that

" the cholera vibrio does not secrete a true soluble exotoxin but that it contains endotoxins which are liberated on the autolysis of the bacilli in culture or on the active disintegration of the bacilli by the cells of the animal body. The analogy that it presents with the meningococcus—another organism that readily undergoes autolysis—is very close, though the cholera vibrio is far more toxic."

Recent observations on the toxin of *V. cholerae*, as far as they fall within the scope of the present disquisition, may be summarized as follows.

Boivin et al. (1934) reported to have been able to obtain from various Gram-negative bacteria including the *V. cholerae* through extraction with trichloracetic acid in the cold an opalescent fluid, which gave specific precipitation reactions with immune sera prepared against the organisms in question and which through prolonged boiling with weak acetic acid could be split into (*a*) a nitrogen- and phosphorus-containing precipitate, and (*b*) a specific polysaccharide which remained in solution. Further studies of the " glucido-lipoid complex " obtainable through extraction with trichloracetic acid led Boivin & Mesrobeanu (1935, 1936) to the conclusion that this compound, containing the principal part of the endotoxin of the organisms in question, as well as their somatic antigen, represented their " complete antigen ", whereas the specific polysaccharide, because being incapable of producing antibodies and antitoxins upon injection into rabbits, corresponded to a residual antigen or hapten. The two workers maintained in this connexion that, when the organisms had become rough, i.e., devoid of their specific somatic antigen, they were

endowed only with a feeble toxicity due to the bacterial proteins. The latter, representing the " acid-insoluble " part of the endotoxin, were of little importance in determining the toxicity of the smooth organisms, due principally to the acid-soluble portion of the endotoxin as characterized above.

As summarized by Burrows (1944, see also Burrows et al., 1946) the findings of Boivin and his co-workers were confirmed by several subsequent observers, such as Raynal et al. (1939), Damboviceanu & Barber (1940), and also by Gallut (1943), to whose work reference is made below.

A method of separating the cholera toxin from the vibrios without the use of chemicals was described by Banerjee (1942), who took for this purpose advantage of the technique of cultivation in cellophane bags devised by Gildemeister & Neustat (1934). Slightly modifying the procedure of these workers, Banerjee used

" a long cylinder in which 2 cellophane sacs are placed, each tied tightly with string to a tube open at both ends. The length of the tubes are adjusted so that they project through the opening of the cylinder. The open ends of the glass tubes and the cylinder are plugged with cotton wool. 200 c.c. of Ramon's medium [a] is put in the cylinder outside the sacs and 50 c.c. of the same medium is put in the sacs through the glass tube. This is then sterilised in the autoclave and dialysis was allowed to proceed overnight at room temperature. In another similar apparatus oil of vaseline is put in the cylinder and also in the sacs to serve as anaerobic culture."

The culture medium in the sacs was inoculated *in situ* with one fifth of a slant of a 24-hour-old cholera culture and the apparatus was incubated at 37°C for 18 hours. The cellophane bags were then removed from the cylinders and snipped with scissors so as to empty their contents.

As was established in the course of Banerjee's work, equally good growth could be obtained if the cellophane bags were filled with normal saline instead of Ramon's broth, because satisfactory diffusion from the medium surrounding the bags took place.

To remove the vibrios from the cultivation fluids, high speed centrifugation for 20 minutes was used. Then, since the supernatant fluid was not quite free from vibrios, a current of air charged with a mixture of toluol and chloroform was passed through it.

Testing the toxicity of the fluids thus obtained, Banerjee found that —regardless of whether aerobic or anaerobic cultivation had been used—all guinea-pigs injected intraperitoneally with amounts of 2 ml died within 24 hours, whereas nearly all receiving 1 ml succumbed within 4 days. The minimum lethal dose for intravenously infected mice was 0.25 ml.

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[a] As summarized in the *Bulletin of Hygiene* (1933), Ramon's medium was prepared as follows: " One litre of water and 10 cc of pure HCl is added to 225 gm. pig's stomach which is allowed to digest at 45°C. for about 2 hours. 325 gm. of minced veal is then added and the product is stirred vigorously. After 20-22 hours of digestion heat for 20 minutes at 80-100°C. Pass through a bag-filter. Adjust to pH 8 with NaOH. Heat to 100°C. for 20 minutes. Filter through paper. Fill out in litre quantities. Autoclave at 108-110°C. for 45 minutes. Add glucose 1.5 to 2 gm. per litre and sodium acetate 5 to 10 gm. per litre. The glucose can be added before sterilization and the acetate (in the sterile condition) afterwards, or *vice-versa* the acetate first and glucose afterwards."

A rapid method for obtaining cholera toxin was described by Bernard & Gallut (1943a) thus:

The broth medium used by these two workers was that recommended by Ramon (1933) for the production of highly potent diphtheria toxin.

In 20 ml of this broth, to which 5 g of glucose and of sodium acetate had been added per litre, cholera vibrios harvested from three 18-hour-old agar cultures in Roux bottles were suspended, i.e., a quantity corresponding to the weight of 8-10 mg of desiccated organisms per ml. The suspension was then kept at 37°C. To the portions removed from it for the purpose of testing, toluene was added and prolonged centrifugation was then used to obtain a fluid free from vibrios.

It was found that toxin began to appear in the suspension after 3 hours and reached a maximum after 4 hours' incubation, when the lethal dose of the centrifugate was 0.25 ml for a guinea-pig weighing 250 g and 0.05 ml for a 15-g mouse respectively, upon intraperitoneal administration.

A still more potent toxin could be obtained by using Ramon's broth in quantities of 250-500 ml, centrifuging it after 4 hours' incubation at 37° C so as to remove the vibrios, then restoring the original glucose content (5 per mille) and the original pH (8.0) and again implanting cholera vibrios at the above-mentioned rate. By repeating these operations 10 times, a toxin was obtained, the lethal dose of which for guinea-pigs of 250 g was 0.05 ml. However, quite often the toxicity became attenuated, or even altogether disappeared, after the 5th addition of fresh vibrios. Nevertheless, as pointed out by Bernard & Gallut, even without resorting to cumulative procedures it was possible to obtain with their new method within four hours a cholera toxin, the potency of which was at least equal to that of the toxin produced by vibrios cultivated in the usual manner for 7 days in Ramon's broth without glucose.

The two workers added in a second note (1943b) that in the course of cholera-toxin preparation according to the above-described method, 99.5% of the vibrios were found to be dead after an incubation of four hours, when the pH of the medium had fallen to 5.8. In the opinion of Bernard & Gallut,

" the wholesale [massive] death [of the vibrios] under the influence of a pH of 5.8, due to the fermentation of the glucose, appeared to be the dominant factor in the rapid diffusion of the cholera toxin under the conditions of our experiences ". [Trans.]

Carrying out comparative studies with (*a*) the glucolipidic substance obtained from smooth cholera vibrios according to the method of Boivin & Mesrobeanu (1936) and (*b*) the toxin extracted after four hours according to the procedure of Bernard & Gallut (1943a), Gallut (1943) found that

(1) The content in glucolipoids in the toxin prepared according to Bernard & Gallut was higher than that obtainable from the smooth cholera vibrios themselves with the aid of Boivin & Mesrobeanu's method, usually twice as high.

(2) As suggested by a positive biuret reaction, the toxin prepared according to Bernard & Gallut contained besides the glucolipidic complex also a variable quantity of proteins and polypeptides, which could be separated from the fluid containing the non-dialysable glucolipidic complex with the aid of dialysis through highly permeable membranes.

In order to confirm whether the glucolipidic complex of the toxin was different from the glucolipidic substance extracted from the vibrios with trichloracetic acid, Gallut & Grabar (1943b) resorted to comparative

precipitin tests with a serum which had been obtained by the immunization of rabbits with the latter substance (see Gallut & Grabar, 1943a). The conclusion reached by the two workers on account of these tests was "that at an early stage of its elaboration the toxin contained a more complex antigen which was afterwards split into a more simple glucolipidic compound (like that extracted from the vibrios themselves) and a substance not precipitable with the immune sera".

Further studying the cholera toxin with the aid of ultrafiltration, Gallut & Grabar (1945) confirmed that the toxin consisted of two different substances—namely, (*a*) a simpler glucolipidic antigen and (*b*) a toxic substance which, because of small molecular size, could pass the ultrafilters. The two workers thus characterized the differences existing between these two substances:

	Glucolipidic endotoxin	*Toxic substance in ultrafiltrate*
Dimensions	80-100 mμ	4 mμ
Chemical nature	glucolipoid	probably proteid
Action of immune sera prepared either with the total toxin or with the simpler glucolipidic complex	neutralized	not neutralized
Pathogenic effect	producing congestion	producing hypothermy

More detailed studies of the hypothermy-producing component of the cholera toxin by Grabar & Gallut (1945) rendered it likely that, contrary to the previous beliefs of these workers, the substance, because found to be non-precipitable by trichloracetic acid or by sodium tungstate, was probably not of a proteid nature. The hypothermy-producing substance was found to be thermolabile and to possess no antigenic power (see also Gallut & Grabar, 1947).

Burrows (1944) used the following methods to isolate the endotoxin of *V. cholerae*: (*a*) extraction in the cold with M/2 trichloracetic acid followed by dialyzation of the neutralized extract through cellophane; (*b*) disintegration of the vibrios by high-speed grinding with sand, followed by centrifugation so as to separate the cellular debris from the toxic opalescent supernatant; (*c*) solution in 6 M urea; (*d*) digestion with pepsin for 3-5 days, followed by removal of the insoluble material through centrifugation; (*e*) extraction of lyophilized vibrios with methyl alcohol, ethyl alcohol, chloroform, or ethyl ether in a Soxhlet apparatus.

The addition of 3-5 volumes of ethyl alcohol to the trichloracetic acid extract resulted in the appearance of a flocculent precipitate (found to be a polysaccharide), while the toxin remained in solution. When the filtrate from alcoholic precipitation was concentrated by evaporation, a yellow oil separated out which, containing most of the endotoxin, had a mouse MLD (minimal lethal dose) of 0.1 ml and appeared to be a mixture of alcohol and lipids, probably similar to the substance isolated from the *V. cholerae* with the aid of trichloracetic acid extraction by Raynal et al. (1939).

While the preparations obtained in the manner just described proved unsatisfactory because of the difficulty of separating the toxic substance from the trichloracetate and the method of direct alcohol extraction of the vibrios proved inefficient, extraction of the dry (lyophilized) vibrios with alcohol or chloroform proved highly satisfactory. The crude material thus obtained was yellowish in colour. It appeared to consist of a mixture of lipids and contained in the case of alcoholic extracts considerable quantities of inorganic salts. Purification of the crude extract could be effected by successive acetone precipitation and resolution in minimal quantities of hot absolute alcohol. The white lipid material thus obtained was negative to the Molisch, Millon, and biuret tests, and was found to contain minimal values of 5% nitrogen and 0.7% phosphorus. With proper care this material, which represented about 2% of the dry weight of the vibrios, could be persistently prepared with a mouse MLD of 30 μg.

Burrows concluded from these investigations which, as will be discussed later, were amplified by important observations not falling within the scope of the present disquisition, that the endotoxin of the cholera vibrio was (1) stable to acids but unstable to alkali (N/10 NaOH at room temperature); (2) readily soluble in methyl and ethyl alcohols, chloroform and ether, but not in glycols; (3) readily dialysable; and (4) " closely related, possibly identical with a phospholipid ".

In a preliminary note referring to further studies of the cholera endotoxin in Burrow's laboratory, Freter (1953) stated that the endotoxin of cholera vibrios grown in 1% glucose-peptone water could be partially extracted in trichloracetic acid or pyridine, 70%-80% remaining in the cells. While the rapid extraction method of Bernard & Gallut gave similar results, better yields (30%-40%) could be obtained by extracting the vibrios with dilute acid at pH 3.8 for 4 hours. Freter added that

" the soluble toxin so obtained could be purified by coprecipitation with calcium phosphate or carbonate, precipitation with acetone and deionization by treatment with a mixture of anion and cation exchange resins that resulted in precipitation of inactive material ".

The purified substance, containing 4.5% N and 1.7% P, had an LD_{50} of about 0.2 mg.

As further found by Freter, the residual toxicity from vibrios treated with HCl could be brought into solution by drying with acetone and further extraction at neutral pH. The toxic extract thus obtained was not soluble at pH 3.8 and no toxic material could be extracted from the precipitate at this pH. This as well as other differences in chemical and physical behaviour seemed to indicate that the toxin of the cholera vibrio occurred in two different fractions—a conclusion which appears to be analogous to that reached by Gallut & Grabar.

However, suggestive though the observations just referred to are, the bulk of the evidence available in regard to the endotoxin of *V. cholerae*

coupled with what is known on bacterial endotoxins in general (see summary by Burrows, 1951) is more consistent with the hypothesis that the cholera endotoxin occurs as a single substance of sufficient lability to become altered by extraction and purification procedures. As a consequence, toxicity, with an original or altered pharmacological activity, might be found associated with a variety of biochemical properties, giving the appearance of several toxins and leading to divergent reports in the literature on the nature of the cholera endotoxin.

Observations on the behaviour of the better known endotoxins of the enteric bacilli, especially the dysentery bacilli, deserve great attention in this connexion. As summarized by van Heyningen (1950) and by Burrows (1951), the studies of Goebel and his colleagues have shown that toxicity lay in a relatively small basic component of the intact endotoxin of the Flexner dysentery bacillus, and that this could be prepared linked with either the polysaccharide or the polypeptide portion of the endotoxin molecule, but could not be separated in active form. Such an active moiety in this and other endotoxins was termed "TOX" by van Heyningen (1950), who suggested that the diffusible toxic substance of low molecular weight obtained by Burrows (1944) with the aid of alcohol extraction possibly represented the TOX portion of the cholera endotoxin molecule. Whether such a view on the nature of the cholera endotoxin is valid, remains for further studies to determine.

It was claimed by several observers, particularly by Kraus and his co-workers (see summary by Kraus, 1929), more recently also by Takita (1939) that—in contrast to the classical non-haemolytic *V. cholerae*—the El Tor vibrios produced in addition to an "haemotoxin" (haemolysin), as is generally accepted, also an exotoxin. However, the existence of such a separate exotoxin distinct from the haemolysin of the El Tor vibrios has been rendered rather doubtful through interesting observations of Pottevin (1913b), mentioned later (see page 1084), and of Gohar (1932a). Determining the haemolytic power of the supernatant fluids of El Tor broth-cultures which had been centrifuged after incubation for five days and testing at the same time the toxicity of these fluids by intracutaneous injection of rabbits in analogy with the method devised by Kovacs (1932), Gohar established that toxicity appeared to run parallel with the haemolytic power of the fluids. He further found that absorption of the fluids with cholesterol, suprarenal tissue, or brain tissue, i.e., with substances rich in lipoids, rendered the fluids atoxic as well as non-haemolytic, while brain tissue extracted with ether to remove as far as possible the lipoids, failed to produce these effects. The fluids became likewise atoxic if sheep erythrocytes had been haemolysed in them, and it was even found that

"if in a haemolysis experiment the last tubes containing the weakest dilutions and showing slight or no trace of haemolysis are tested for toxicity they are found to be nontoxic".

Gohar concluded from these observations that " the haemolysin and the exotoxin are probably one and the same thing". He admitted that old El Tor strains which had lost a great deal of their toxicity remained haemolytic. Presumably, however, this merely indicated a degradation of the toxin, the more so as it remained immunogenic.

Virulence

Dealing in a general manner with the problem of bacterial virulence, Wilson & Miles (1946) stated that

" the term *virulent* is sometimes used as though it were completely synonymous with *invasive* ; but this usage is unjustified by derivation and singularly inconvenient in practice. If rigidly adhered to it would necessitate the exclusion from the class of virulent bacteria of all of those organisms that exert their lethal effect by the production, in localized foci, of powerful toxins . . . It is better practice to retain the term *virulent* in its correct sense of poisonous, without any implication as to how the poisonous effect is produced, and to apply it to any organism which gives rise to a rapidly fatal infection."

Even apart from the fact that an invasion of the human body by the *V. cholerae* does not lead to a generalized infection, this broad definition of the term " virulence " is particularly adequate for the special case of this organism which, though not producing true cholera if administered to experimental animals with the aid of the usual techniques, nevertheless is apt to cause death in these animals as well as in man, if introduced in its virulent form even in a small dosage. However, while it is legitimate, therefore, to utilize the usual animal experiments, particularly intraperitoneal infection of guinea-pigs, for an assessment of the virulence of cholera strains, it must be realized that, as far as the experimental animals are concerned, virulence defined as above is an attribute not only of the classical cholera vibrio, as is the rule for man, but also of the El Tor vibrios and even some of the cholera-like vibrios.

A further but only apparent difficulty is created by the problem of the relation existing between the virulence and the toxicity of the vibrios. Some of the early workers, considering human cholera attacks the result of a toxicosis and also bearing in mind that general signs identical with those following the introduction of living cholera vibrios could be produced in experimental animals by the administration of killed organisms or even of culture filtrates, apparently thought the terms of virulence and toxicity to be interchangeable. Dungern (1895) noted in this connexion that, as shown by the observations of Pfeiffer (1894a) freshly isolated cholera vibrios were able to multiply in the peritoneal cavity of guinea-pigs if introduced in small quantities, whereas organisms grown for prolonged periods on artifical media could do so only if administered in large doses. The question arose, therefore, whether this was due to a difference in the

resistance of freshly isolated and long cultivated cholera vibrios to the bactericidal substances of the animal body or depended upon a difference in the toxicity of the strains in question, rendering them more or less able to counteract the bactericidal substances.

To answer this question, Dungern made comparative tests with (1) a freshly isolated East-Prussian cholera strain so virulent that 1/8-1/4 of a loop (0.25-0.5 mg) of 20-hour-old agar cultures was lethal for intraperitoneally infected guinea-pigs, and (2) an 8-year-old often subcultivated stock culture originally isolated from a cholera patient in Calcutta which, if administered intraperitoneally in large doses (10 mg or 20 mg), produced death from toxaemia with negative bacteriological findings, the introduced vibrios having obviously been killed.

The toxicity of these two strains, tested by intraperitoneal or intravenous injection of guinea-pigs with chloroform- or heat-killed organisms, was almost exactly identical. However, while a guinea-pig intravenously injected with 2 mg of living vibrios from the recently-isolated culture showed a rapid drop of the body temperature and died in less than 24 hours, yielding abundant growths of *V. cholerae* from the blood, spleen, liver, and the haemorrhagic peritoneal exudate, two guinea-pigs, injected with 2 mg of the Calcutta strain, survived, showing a passing slight drop of the temperature (to 35°C) in one instance, some fever (maximum 39°C) in the other. Dungern maintained that the dose of 2 mg was below the lethal one in the case of either strain but that obviously the virulent vibrios (East-Prussian strain) were able to survive and to multiply.

The conclusion reached by Dungern on account of these experiments was " that the virulence of cholera bacilli can be quite independent from their toxicity ".

The fundamental facts of cholera virulence have been elucidated through the systematic investigations of Pfeiffer (1892, 1894a, 1894b) and Pfeiffer & Wassermann (1893).

Pfeiffer established in the course of his initial work (1892) which, though carried out with a strain found afterwards to be not of a true nature, was fully confirmed by subsequent investigations with immunologically identified cholera vibrios, that in the case of living organisms the lethal dose for intraperitoneally injected guinea-pigs of about 400 g body-weight was usually about one loop, occasionally as little as ½ loop. To kill the animals by subcutaneous infection, at least 5 to 10 times higher doses were necessary. The dose necessary to cause rapid death in intraperitoneally injected guinea-pigs with chloroform- or thymol-killed cholera cultures was about three times higher than that needed in the case of living organisms—an observation supporting Pfeiffer's assumption that, though in both cases the action of the cholera toxin was the immediate cause of death, in the case of live vibrios introduced in small doses an initial multiplication of the organisms in the peritoneal cavity was an indispensable prerequisite.

Pfeiffer & Wassermann (1893) stated similarly that

" in the case of intraperitoneal injection of live cultures the excess [Plus] of toxic substances which are formed through proliferation of the vibrios in the peritoneal cavity, is most essential and, given a high virulence of the culture, can be a multiple of the amount of toxin transmitted with the originally injected bacterial substance ". [Trans.].

In fact, these two workers defined, as far as their investigations were concerned, virulence " as the capability of the vibrios in question to multiply in the guinea-pig peritoneum ".

As stated by Pfeiffer in a subsequent study on the etiology of cholera (1894a), he was able to continue work on the virulence of *V. cholerae* with numerous fresh strains isolated mostly in Germany during the recent European cholera manifestations.

According to these observations,

" the cholera cultures, regardless of whether they had been derived from most severe and rapidly fatal cholera cases or from instances of slight infectious diarrhoea, showed a remarkably uniform behaviour. In the case of intraperitoneal infection, the dosis letalis minima was invariably but part of a loop (of a total capacity of 3-4 mg culture material), 1/6 or 1/8 of a loop usually sufficing to kill the guinea-pigs. The subcutaneously infected guinea-pigs, on the contrary, showed only a feverish reaction lasting a few hours, while pigeons (infected intramuscularly with 1 loop) survived." [Trans.]

Only three of the many cultures tested showed an aberrant behaviour, killing guinea-pigs infected subcutaneously with ½-1 loop, and occasionally even pigeons. Such an extreme virulence of cholera cultures seemed so unusual that Pfeiffer seriously doubted the true nature of these three strains.

However, as pointed out by Pfeiffer (1894b), the virulence of strains subcultivated for prolonged periods was apt to become abated or even lost. The strain afterwards used by Dungern (see above) in particular had completely lost the capability of subsisting in the guinea-pig peritoneum, vibrios injected into the peritoneal cavity of normal (i.e., non-immune) animals disappearing within 20-30 minutes without evidence of marked phagocytic activity.

Gruber & Wiener (1892), who also studied the virulence of *V. cholerae*, stressed that, especially in the case of agar cultures, only quite young (i.e., 15-30 hours old) growths were fully infectious, whereas material from cultures 48 hours old or older caused as a rule only illness of varying severity, but no death, or was even altogether inactive. That the age of the growths exerted an important influence upon their virulence, seemed also indicated by the observation that intraperitoneal injection of guinea-pigs with material from the actively growing marginal portions of 48-hour-old agar cultures still proved lethal, whereas material from the centre of such growths failed to produce this effect. As claimed by Gruber & Wiener, the lost virulence of old cholera cultures could be quickly restored through subcultivation.

Flügge (1893) and Gotschlich & Weigang (1895) were not in accord with the postulation of Gruber & Wiener that the cholera vibrios were infectious only in the stage of their youthful vigour (" vollste Jugendkraft "), whereas later they lost their virulence without impairment of their capability for saprophytic growth. Paralleling determinations of the number of viable organisms in cholera cultures of different age with virulence tests

performed through intraperitoneal infection of guinea-pigs, Gotschlich & Weigang were able to show that in all instances, regardless of the age of the growths, one and the same number of viable cholera vibrios, approximately 200-300 million, represented the dosis letalis minima. They likewise established that by keeping their cultures at room temperature or in the ice-box instead of at 37°C, they could not only prolong the viability, but also preserve the virulence of the growths, cultures kept at lower or low temperatures for 2-3 days proving as virulent as those in the " full vigour of youth ". Gotschlich & Weigang concluded, therefore, that

" in one and the same culture the virulence of the individual viable organisms is of a constant size; the virulence of the culture is the resultant of the actions of the individual organisms; the changes of the virulence taking place in ageing cultures are due solely to quantitative differences in the number of viable vibrios, not to qualitative changes taking place in the individual bacilli ". [Trans.]

Acceptable though this conclusion remains as far as recently-isolated cholera vibrios are concerned, it has to be pointed out that (*a*) as shown already by Pfeiffer (1894b), the virulence of old often subcultivated strains was apt to become lost, and (*b*) as recently found, first by Shousha (1923), and generally accepted, dissociation into the rough state leads to a decrease or loss of the virulence of *V. cholerae* due to qualitative changes.

The postulate of Pfeiffer (1894a) that no parallelism existed between the virulence of different cholera strains and the severity of the disease they produced in man, has been confirmed by most subsequent observers. However, in contrast to the above-noted experiences of Pfeiffer it is now generally accepted that, regardless of the character of the manifestations which they produce in man, the virulence of different cholera strains is apt to vary within fairly wide limits. Discussing this problem, Gotschlich & Weigang stated that the unequal virulence of the various strains might be due to differences in the rate of multiplication of the growths in question or to innate racial differences, and adduced some evidence suggesting that both these possibilities were of actual importance.

As shown by some early workers, such as Haffkine (1892) and Gotschlich & Weigang (1895) and confirmed by ample further observations, passage through intraperitoneally infected guinea-pigs is an effective means to restore or, if serially repeated, even to enhance the virulence of cholera strains. Gotschlich & Weigang referred in this connexion to one strain, the minimal lethal dose of which was reduced through 7 passages directly from animal to animal from a value of over 2,600 million organisms to 900 million. Even more spectacular results were recorded by Kabeshima (1918a) in the case of an El Tor strain which had been subjected to passage through a series of 45 guinea-pigs.

Claims that the virulence of cholera strains could be enhanced by other means, e.g., through growth in diluted immune serum (Hamburger, 1903)

through symbiosis with other bacteria (Puntoni, 1913a) or through short-term exposure to a temperature of 48°C (Sulman, 1933), seem not to have been supported by further observations. Moreover, these methods do not seem to be of practical importance.

Antigenic Structure

Early observations

The scanty references made to the antigenic structure of *V. cholerae* during the period immediately following the introduction of the agglutination test as a means for the identification of this organism by Gruber & Durham (1896), are of historical interest rather than of actual importance. While, as summarized by Meinicke et al. (1906), Gruber & Durham as well as some other early observers assumed that differences in the virulence of the various cholera strains were the cause of their different agglutinability with a given immune serum, a few workers, such as Durham (1901) and Kolle & Gotschlich (1903) postulated that differences in the receptor apparatus of the organisms accounted for the discrepant serological results. This assumption was refuted by Meinicke and co-workers, who declared " that the cholera cultures possess the same receptors in about equal quantities, but that the avidity of the single receptors to the antibodies of the cholera serum differs in the various cultures ". However, Kraus et al. (1911) re-asserted that cholera strains were apt to vary in their antigenic structure and that the presence of special agglutinogens was the cause of the discrepant behaviour shown by the strains which had been isolated during the 1908 outbreak in Kamaran, Arabia. Kraus and his co-workers insisted, however, that the different behaviour of these strains could be demonstrated only with immune sera possessing a low titre. Similarly, Ohta (1914) claimed that the action of low-titre sera was different from that of sera with a high titre and that with the aid of the former the cholera vibrios could be divided into two types.

Definite proof of the existence of different serological types of *V. cholerae* was adduced through a study of over 200 strains by Kabeshima (1913), to whose observations detailed reference will be made later in this study.

Greig (1916), testing 39 more-or-less haemolytic vibrio strains isolated from Calcutta surface waters, found " that the antigenic character of vibrios isolated from water is different from that of the standard cholera vibrio as known to bacteriologists ". As shown by cross-agglutination tests with sera obtained by immunizing rabbits with these water vibrios, 32 of them fell into 6 serological groups, while 7 remained ungrouped. Included among the latter were two strains of apparently atypically behaving cholera vibrios.

Observations by Mackie & Storer (1918), afterwards confirmed and amplified by Mackie (1922), showed that " paracholera " vibrios isolated in Egypt from patients with choleraic disease, were likewise serologically distinct from true cholera vibrios.

That the last-mentioned and also some earlier observers, even though they worked with agglutinating sera which were not fully specific according to modern standards, obtained surprisingly clear-cut results, was—as Gardner & Venkatraman (1935b) aptly pointed out—due to the fact that:

" The agglutination method used by Greig, Mackie, etc., involving a low temperature (37°C) and a relatively short period of incubation (2 hours), reveals in general only 0 agglutination, and so enables a distinction to be made between the various 0 subgroups."

The validity of this contention is proved by the conclusions of Mackie (1922) who maintained that:

" [*a*] By direct agglutination tests, using plain saline emulsions and incubating at 37°C for 2 hours, the paracholera vibrios are distinctly differentiated from *V. cholerae ;*

" [*b*] *V. cholerae* antiserum exhibits apparent co-agglutination under certain conditions towards *paracholerae* A and certain similar types; this effect develops more slowly than the agglutination of the homologous organism and is of lesser degree and of lower end-titre; it is most markedly elicited when formol-broth emulsions are used and the tubes are incubated first at 55°C. "

Heat-stable (O) and heat-labile (H) antigens

While the observations recorded above indicated that the cholera vibrio possessed an antigenic structure different from that of the cholera-like vibrios, and also that the latter fell into numerous immunological groups, they failed to explain the nature of these differences and—more generally speaking—furnished beyond some rather vague speculations no clue as to the character of the antigenic make-up of the organisms.

An investigation of the latter problem was rendered possible through the classical studies of Weil & Felix (1920) on the antigenic make-up of typhoid and paratyphoid bacilli, even though the two workers concluded from some preliminary tests that, in contrast to these bacterial species, the *V. cholerae* had no double antigenic structure. Observations suggesting that this organism possessed thermolabile as well as thermostable antigens were recorded in 1921 by Miyake and by Watanabe, but these findings, published in Japanese medical journals, attracted no attention. Brutsaert (1924) supported the tentative conclusion reached by Weil & Felix, stating that:

" Our anti-cholera sera obtained by injection of Koch's vibrios heated during 2 hours at 100°C, agglutinated our different strains of cholera vibrios whether they were heated to 100°C or not, quite as well as anti-sera of 56°C prepared at the same time. As absorption tests confirmed the agglutination experiments, we state that all their antigen is thermostable." [Trans.]

Soon afterwards, however, the fine studies of Balteanu (1926) proved that the conclusion arrived at by Weil & Felix and by Brutsaert was due not to the absence of thermolabile antigens in the *V. cholerae* but merely to the difficulty of demonstrating their presence.

In the introduction to his paper, Balteanu stated that:

" The motile bacillus possesses two distinct kinds of agglutinable substances, one labile, the other stable when subjected to 100°C. or to dilute acid or absolute alcohol. The antisera contain special agglutinins corresponding to each kind of antigen. The labile factor and its agglutinins are responsible for agglutination in large, loose flocculi; the stable factor and its agglutinins for agglutination in small compact granules. The isolated flagella react as if composed entirely of labile material. The agglutinins for the labile and stable antigens are in the inverse order susceptible to heat: those for the labile antigens resist a temperature of 70°C. for 20 minutes; those for the stable are inactive after such treatment. The non-motile races of normally flagellate organisms contain only the stable antigen and their properties are limited thereby; they agglutinate only in small granules and their agglutinins are destroyed at 70°C. This is the standard scheme of serological properties which has been kept in view in studying the antigenic complex of *V. cholerae*."

In his initial studies of this problem Balteanu used, besides the polyvalent immune serum of the Lister Institute, London, sera of his own, prepared by immunization of rabbits with suspensions of a cholera culture which had been heated before injection for 30 minutes at 58°C and for 2 hours at 100°C repectively. Carrying out agglutination and absorption tests with the aid of these sera, he was able to prove that the four cholera strains studied by him possessed H as well as O antigens. However, there was sometimes a poor contrast between the floccular (H) and the granular (O) forms of reaction—obviously due to the fact that in the case of the monoflagellate *V. cholerae* the ratio of heat-labile constituents was low as compared to that met with in the proteus and paratyphoid groups. However, Balteanu was able to overcome this impasse by shaking and then centrifuging the suspensions of cholera agar-cultures, so as first to liberate the flagella and then to separate them from the bodies of the vibrios, which were mostly thrown down during centrifugation. He found that the clear fluid obtained in this manner, in which only very few bodies of vibrios but abundant flagellar material were present,

" (1) agglutinates in typical flocculi with an ordinary anticholera serum made with an emulsion of whole cholera vibrios killed at 58°C.; it no longer functions after being heated at 100°C.;

(2) When inoculated into rabbits it induces the production of a serum which makes flocculent clumps with a flagellar suspension and reacts almost exclusively with the flagellar labile constituents of an ordinary emulsion of the vibrios."

Balteanu's general conclusions, which have been confirmed by all subsequent observers, were that in *V. cholerae*:

" (1) There are ... two series of antigenic substances which may be termed stable (' O ') and labile (' H ') respectively (' Somatic ' and ' Flagellar ' antigens according

to the terminology of Th. Smith). Of these the stable elements resist a temperature of 100°C. for a considerable period while the labile elements are thereby destroyed.

(2) The agglutination of the stable constituents by themselves (as illustrated by the agglutination of steamed suspensions) takes a purely granular form; the reaction of the isolated labile elements (as shown by the agglutination of living emulsions and flagellar suspensions by ordinary immune sera previously absorbed with steamed cultures) is definitely flocculent.

(3) The combined reaction of the labile and stable constituents in living emulsions to immune sera made with ordinary cultures leads to a mixed flocculent and granular type of clumping except at the upper limit of the titre where the looser and more fluffy type is dominant."

It is of interest to add that the immotile opaque variant of *V. cholerae* met with by Balteanu (see the second of these studies [b]), behaved in the main like an " O " form, since no heat-labile " H "-agglutinable substances could be demonstrated, while the heat-stable, somatic, " O " factor was conspicuous. However, serum produced with this variant contained H agglutinins.

A further most important contribution to the knowledge on the antigenic structure of *V. cholerae* was made by Shousha (1931a, 1931b) through a study of two strains which had been isolated at the El Tor quarantine camp from pilgrims not suffering from choleraic disease and which were found to be agglutinable with one of the two available cholera-immune sera. Shousha was able to establish that the receptors which these two strains had in common with true cholera vibrios were heat-labile (flagellar) " group " receptors, whereas the somatic (O) receptors of the two suspect vibrios were quite different from the somatic antigen of *V. cholerae*. Shousha stressed, therefore, the importance of using in cholera laboratory work adequately heated suspensions of the cultures to be tested or of preparing sera by immunizing animals with heated suspensions of the organisms so as to produce sera free from H agglutinins. He also recommended with great reason that subcultures of the strains used for this purpose be issued together with the sera for the purpose of control tests.

Abdoosh (1932), examining 22 strains of true cholera vibrios, six strains labelled El Tor as well as three " paracholera " strains and 24 other cholera-like vibrios, found that none of the cholera-like vibrios possessed the same somatic antigen as *V. cholerae*. However, three of the strains labelled El Tor had the same thermostable and also the same thermolabile antigen as the classical non-haemolytic cholera vibrios, while one was agglutinated by cholera H+O serum, but not by cholera O serum. Abdoosh advised in this connexion that it was essential " to confine the term ' El Tor ' to the group of vibrios sharing with *V. cholerae* both its antigens, but differing in their being haemolytic ".

Besides the so-called El Tor strain mentioned above, two of the cholera-like vibrios were agglutinated by H+O cholera serum in the living state

[b] *Bull. Wld Hlth. Org.* 1955, **12**, 311

but not after they had been heated for two hours at 100°C. Abdoosh concluded therefore that the heat-stable antigen of *V. cholerae* was " sharply specific " for this organism and the El Tor vibrios in the strict sense. However, some cholera-like vibrios, though differing in their somatic antigens, were related to the cholera vibrios by virtue of their heat-labile antigen.

Gohar (1932b), examining 45 cholera and cholera-like strains, maintained on the contrary that the relationship of the latter to *V. cholerae* " may refer to either the flagellar or somatic antigen ". In his opinion, therefore, absorption tests were necessary in addition to agglutination tests in view of the fact that the cholera-like strains possessing the same antigens as *V. cholerae* were found incapable of absorbing all the antibodies from a cholera serum.

However, the importance of O agglutination for the laboratory diagnosis of cholera was again emphasized by Taylor (1934) and White (1934a) in reports rendered to the Office International d'Hygiène Publique in response to an inquiry into the preparation of standard agglutinating sera for this diagnostic work. The observations recorded in this connexion by Taylor indicated that smooth cholera strains could be best distinguished with the aid of heated suspensions of the organisms, while White maintained that:

" The identification of *V. cholerae* depends in fact on the O agglutinins and it seems necessary to envisage the opportunity to make the diagnostic tests with a pure ' O ' serum, i.e. with a serum raised against a vaccine heated to 100°C. or naturally devoid of flagellar antigen."

The validity of this proposal was fully confirmed by Gardner & Venkatraman (1935b), whose publication may be considered as the charter of the present knowledge on the antigenic structure of cholera and cholera-like vibrios.

Gardner & Venkatraman used for their comprehensive studies 101 cholera and cholera-like strains which according to their biochemical reactions could be divided into: (*a*) " typical " vibrios, i.e., those producing acid without gas in glucose, maltose, mannite and saccharose, giving the cholera-red reaction, and not fermenting dulcite; (*b*) " atypical " vibrios, found to be divergent in one or more of these characters, but showing a general similarity to the typical vibrios; and (*c*) " non-fermenting " vibrios, markedly different from the previous groups by failure to acidify any of the above-mentioned carbohydrates and also by an inability to produce the cholera-red reaction or to liquefy gelatin.

To test these strains serologically, Gardner & Venkatraman worked with H-O and O suspensions, and with H+O as well as with pure O sera.

Having established that, in contrast to what was the case in the Salmonella group, formol did not inhibit the 0 agglutinability of vibrio suspensions, Gardner & Venkatraman prepared their H-O suspensions by the addition of 0.2% formol and 0.2% chloroform to 24-hour-old veal-broth cultures (pH 8.0). O suspensions were obtained by placing dense harvestings from 24-hour-old agar cultures in saline for two hours into boiling water. Such prolonged heating was found to be indispensable to destroy completely the antigenic action of the H component, but is was noted that a few minutes' exposure to 95°-100°C was sufficient to remove the H agglutinability of the suspensions. Alcohol

treatment and growth of the vibrios on phenol agar were also tried to destroy the H component, but gave no satisfactory results.

H+O sera were prepared by immunizing rabbits with formolized unheated suspensions, pure O sera were manufactured with saline suspensions which, as noted above, had been exposed to boiling temperature for 2 hours.

Confirming and amplifying the observations of previous workers Gardner & Venkatraman were able to establish that:

(1) As shown by cross-agglutination tests with unheated suspensions and O sera, the " cholera group " of vibrios, i.e., the above-mentioned categories of " typical " and " atypical " vibrios, possessed a diversity of specific O antigens, six of which, because met with in more than one strain, rendered it possible to classify most, though not all, of the organisms of the group into six sub-groups. All the standard stock strains of *V. cholerae* examined and also the majority of races isolated from typical cases of epidemic cholera fell into one group, called " I ", and the same held true of the majority of the haemolytic vibrios tested, which were thus identified as El Tor vibrios in the strict sense.

(2) As demonstrated by the action of O sera on boiled suspensions, there existed in addition to the specific O antigens a common O antigen, the nature of which could not be definitely elucidated. The evidence regarding a possible extension of the non-specific O agglutination to vibrios outside the cholera group was also not conclusive, but Gardner & Venkatraman drew in this connexion attention to White's observation (1934b) on " Q " antigens which will be discussed later in this study.

(3) As shown by agglutination tests with formolized unheated broth suspensions and H-O sera, the vibrios of the cholera group possessed a common H antigen.

Gardner & Venkatraman urged on account of their experiences that for the identification of *V. cholerae* a standard subgroup " I O " serum should be used in conjunction with tests for haemolysis.

Before dealing with further investigations regarding the antigenic structure of *V. cholerae* in general, attention has to be devoted to the evidence on the existence of serological races of this organism as well as to the antigens present in dissociated vibrios.

Serological races

It is the merit of Japanese observers, first of Kabeshima (1913 ; see also Kabeshima, 1918b), to have definitely established the existence of serological races of *V. cholerae*.

As summarized by Takano et al. (1926), Kabeshima based his observations on an examination of 195 cholera strains recently isolated in Japan and Formosa, and of 19 stock strains from European laboratories. He found that according to their serological reactions these strains could be divided into a " typical " and an " atypical " group, each

of which agglutinated at high titre with homologous immune serum, but weakly with sera raised against strains of the opposite type. The presence of these two types could be confirmed with the aid of agglutinin absorption, complement fixation and bactericidal tests. Kabeshima postulated that the different behaviour of the two types was due to the presence in each of two different antigens, a principal one, responsible for the reactions with homologous sera, and an accessory one, reacting with the heterologous sera. The strains isolated during the 1912 cholera epidemic in Japan were typical in character, while those derived in the same year from Formosa belonged to the atypical group.

The existence of two serological types of *V. cholerae* was soon confirmed by several other Japanese observers (see summaries by Nobechi, 1923 and Burrows et al., 1946). Pratt (1925) also described two serological types, the presence of which, though not invariably revealed by agglutination, could always be demonstrated by cross-absorption tests.

Nobechi (1923; see also Nobechi, 1933) proved the existence of a third serological type of *V. cholerae* standing between Kabeshima's two groups, which are now usually designated as the Inaba and Ogawa types. As summarized by Nobechi (1923), the strains of his new " middle " type (now often designated as the Hikojima type),

" are agglutinated by the sera of the two other types, capable to differentiate the strains of the two types from each other, to the same titre with corresponding strains; and the middle type sera, with no exception, agglutinate all strains of the other types as well as of the middle type almost uniformly high. From the result of the agglutinin absorption test, the middle type strains studied by the author are assumed to be provided with the common antigen X, and the original type specific A, at the same time also with the varied (i.e. ' atypical ') type specific B, though the development of the last is incomplete."

In his second paper (1933), Nobechi characterized the antigenic structure of the three types thus :

Type	*Specific fraction*	*Common fraction*
Original (Kabeshima's " typical " group)	A	X
Intermediate (Hikojima)	AB	X
Variant (Kabeshima's " atypical " group)	BC	X

Aoki & Oshiro (1934) claimed that the occurrence of specific thermostable antigens and/or of unspecific, partly thermolabile antigens accounted for the differentiation of *V. cholerae* into three types. According to this concept, the vibrios of the Inaba type had only specific receptors, those of the Ogawa group only unspecific receptors, while both kinds of antigen were present in the intermediate (Hikojima) type. However, as pointed out by Burrows et al. (1946), it is difficult to correlate this assumption with what is known in regard to the H and O antigens of the cholera vibrio. In fact, the investigations of the workers quoted below leave no room for doubt that differences in the O receptor apparatus are solely responsible for the occurrence of the serological races of *V. cholerae*.

Scholtens (1933a, 1933b; 1934; 1936a, 1936b) stated in this connexion that the cholera vibrios fell immunologically into two groups, about two thirds of the strains possessing only an " A " antigen, the remainder also an additional antigen " B ". Both these antigens, met with also in the El Tor vibrios, were found to be thermostable. Identical conclusions were reached by Heiberg (1936) as far as the *V. cholerae* was concerned.

Gardner & Venkatraman stated in a preliminary communication on their above-described investigations (1935a) that they had been able to confirm through agglutination and absorption tests the existence of " original " and " variant " types not only in cholera strains from Japan but also in races from India, China, and elsewhere as well as in El Tor strains in the strict sense. They added that :

" The reality of the third or ' middle ' type is not yet fully confirmed, though some of our experiments indicate that certain of the Japanese races labelled ' middle ' type possess, as they are supposed to, both the characteristic antigens of the original and variant types. Contrary to the belief of Inouye & Kakihara (1925), these characteristic antigens are of the heat-stable or O type. They are subsidiary or additional to the main heat-stable antigen that distinguishes them all from vibrios belonging to the other sub-divisions of the cholera group."

Whether these variants of *V. cholerae* were stable or fluctuating, was in the opinion of Gardner & Venkatraman still undecided. The claims made by some of the Japanese workers that they had succeeded in transmuting strains of the variant type into the middle type through growth in immune serum, seemed not well substantiated, the less so because according to Gardner & Venkatraman (1935b) in some of the recorded instances at least such transitions had been concomitant with roughening.

In spite of these uncertainties there was, however, not the least doubt that at least two serologically distinct races of *V. cholerae* existed and Gardner & Venkatraman (1935b) urged, therefore, that the standard O sera used for cholera diagnosis should contain the subsidiary as well as the main agglutinins of the O sub-group I.

Further reference to the serological sub-groups of *V. cholerae* will be made when dealing with recent studies on the O antigens of this organism in a later section of the present study.

R and ρ antigens

That cholera vibrios which have undergone dissociation, are apt to react peculiarly in serological tests, seems to have been suggested first by attempts made by Hamburger (1903) to increase the virulence of these organisms : he noted in the course of this work that vibrios which had been grown in specific immune serum (and which, as became later clear, had thus become rough) showed spontaneous agglutination when suspended

in normal saline. This phenomenon was further studied by Kabeshima who, according to Takano et al. (1926), established already in 1913 that :

" When the cholera vibrio is cultivated in bouillon containing homologous serum, the organism becomes inagglutinable, but it acquires spontaneous agglutinability. This is due to the loss of specific receptors, and at the same time new receptors which are common to many strains are formed."

In a further publication (1918c) which was available to the present writer in the original, Kabeshima noted that spontaneous agglutination in 0.9% saline was shown not only by strains subcultivated repeatedly in broth containing homologous immune serum but also by 8 out of 19 old stock strains of *V. cholerae*. However, while these 8 strains remained capable of absorbing cholera agglutinins and also remained antigenic, those which had become spontaneously agglutinable in normal saline or inagglutinable with specific serum through growth in the latter, had lost their antigenicity as well as the property of agglutinin absorption—apparently because they had lost their specific receptors. Kabeshima also stated that the spontaneously agglutinating strains yielded homogenous suspensions if, instead of 0.9% saline, a 0.2% solution was used.

Shousha (1923) as well as Goyle & Gupta (1932), again studying the phenomenon of spontaneous agglutination of *V. cholerae* with a full knowledge of bacterial dissociation, confirmed the presence of profound differences shown by smooth and rough strains respectively in agglutination and agglutinin-absorption tests, but did not correlate these divergent reactions with changes in the receptor apparatus of the organisms. However, the latter problem received full attention in the studies of Yang & White and of White, which have been referred to in part in the third of these studies.[c]

As was noted there and as was also stated by White in an article on " The serological grouping of rough vibrios " (1935a), the serological specificity of the different vibrios depended on their smooth antigens. With roughening these differences tended to disappear so that forms which were quite distinct in the S state, fell into larger R agglutination groups. Transition into the ρ form led even to the disappearance of this group specificity of the rough vibrios, so that, as White (1935) put it, " the serology of the ρ vibrio variant is overwhelmingly generalized ".

In a further publication dealing with the O receptor complex of *V. cholerae* and its antibodies, White (1937c) reported on observations he had made when immunizing rabbits with polysaccharide fractions isolated from smooth cholera vibrios. These fractions were found to be actively antigenic, but the resulting sera showed a varying content of type- and group-specific agglutinins similar to that obtained in serum manufacture

[c] See *Bull. Wld Hlth Org.* 1955, **12**, 777

with whole vibrios. Besides being distinct by the range of their (type or group) specificity, the receptor groups of the smooth vibrio polysaccharides were found to be partly alkali-labile and partly resistant to alkali. Immunizing a group of rabbits with a given polysaccharide, now one, then another of these various receptor groups was found to play a dominant role in the stimulation of antibodies. As pointed out by White, this uncertainty in agglutinin response was bound to complicate attempts to standardize cholera laboratory diagnosis by issuing standard antigens for serum manufacture in local laboratories.

Summing up further experiences regarding the rough and ρ antigens of *V. cholerae*, White (1940d) stated that

" it would seem that the major component in the somatic agglutination of R and ρ vibrios is a heat-stable antigen which, though it perhaps contains protein, is at least considerably resistant to proteolytic digestion. This component carries, with certain common receptors most obviously displayed in the reaction to ρ antiserum, the differential receptors of the variants and includes the polysaccharide C β or C δ. It is possibly to be regarded as the R or ρ antigen. But the somatic agglutinating apparatus of the variants seems to present other antigenic components, probably in the main common in quality to the R and ρ forms . . . Since they appear to be totally inactivated by proteolytic enzymes, they are probably of a protein nature. Possibly they are combined with the proteolysis-resisting component in a single complex."

In White's opinion, the R and ρ agglutinating antigens furnished the " skeletal system " of the cholera vibrio and, being less hydrophile than the smooth antigen, they indirectly conditioned the spontaneous agglutinability of the R and ρ variants by " failing to counteract the hydrophobe tendency of the surface lipoids ".

" *Rugose* " *antigen*

Dealing with the immunological properties of the rugose variants of *V. cholerae*, White (1940a) stated that such races possessed a special antigen which proved to be resistant to heating in neutral solution at 100°C. Sera produced with this antigen reacted not only with the rugose variants of the O sub-group I of Gardner & Venkatraman, but also agglutinated rugose races of certain vibrios belonging to other sub-groups. As White pointed out, the reactions obtained with such heterologous strains " disclose a flaw, actual if in practice unimportant, in the doctrine of the serological specificity of the heat-stable agglutinogens of O group I vibrios ".

From rugose S, R, and ρ growths of cholera and El Tor vibrios a common, non-protein but carbohydrate-containing haptene could be isolated. This substance, which was found to be absent or inconspicuous in non-rugose strains, reacted strongly and characteristically with sera prepared from whole rugose vibrios.

Other additional somatic antigens

Q antigens. In a short preliminary note published in 1934, White stated that—notwithstanding the discrepant results recorded by some earlier workers—there existed in the cholera vibrios an alcohol-soluble antigen comparable to the Q antigen previously isolated by him from salmonellas. The total Q fraction produced from agar-grown vibrios through alcohol extraction could be divided into (1) a soluble part (Q_1) which could be separated off by treatment of the total fraction with alkalised water, and (2) an insoluble Q_2 component which could be precipitated from the residue of the total fraction with the aid of hydrochloric acid.

Making further studies of these Q proteins of *V. cholerae*, White (1935b) was led to believe that the total Q fraction was identical with the " acid-soluble A substance " isolated, with the aid of extraction methods similar to those used by him, by Linton and his co-workers (see Linton & Mitra, 1934; Linton, Mitra & Seal, 1935; Linton, Shrivastava & Mitra, 1935).

The immunological properties of the Q antigens were characterized by White (1935b) thus :

" Vibrios heated at 100°C. in saline suspension agglutinate in a generalised manner and often to a high titre with the antisera of the Q proteins of the cholera vibrio. The antibodies concerned are not inactivated by the carbohydrate fraction of *V. cholerae*. Occasional strains of vibrio react similarly in the living state with these Q (cholera) agglutinins. The antiserum of the Q_2 substance of S (smooth) *V. cholerae* seems to possess agglutinating properties additional to those of anti-Q_1 and anti-Q_2 sera, rather more specific and possibly related to the ' carbohydrate ' receptors. There is reason to believe that the Q proteins are true constituents of the living vibrio and are not serological artefacts due to reagents and heat. It seems that these substances and their antibodies are important contributors to the ' non-specific O agglutination ' of vibrios recently discussed by Gardner & Venkatraman."

Heat-labile somatic protein antigen (HLSP). As described by White (1940b), it was possible to extract from chloroform-treated young vibrio cultures with the aid of saline " a heat-coagulating antigen common to all known variant forms and seemingly derived from the deeper somatic substance ". While, accordingly, this substance took no evident part in vibrio agglutination, it showed " extremely wide cross precipitation reactions throughout, but not overstepping, the vibrio group ".

Heat-stable somatic protein antigen (HSSP). Using hot saline solutions for the extraction of chloroform-treated vibrio cultures, White (1940e) was also able to extract a heat-stable somatic protein antigen which, like the HLSP, appeared to belong to the deeply situated substances of the vibrios.

Anti-HSSP sera (prepared with the aid of rough and *rho* strains to avoid an influence of the smooth antigen) gave intense precipitation reactions with extracts of R and ρ strains of cholera and many other vibrios

and also with hot saline extracts of the smooth variants of the strains. However, the anti-HSSP sera " did not react visibly with any of the serologically active vibrio fractions ... with the exception of Cγ (the polysaccharide fraction brought into solution on proteolytic digestion of R and ρ vibrios) " (White, 1936a).

Thus, as White (1940e) summarized :

" There have now been separated from the vibrio bodies (1) a heat-labile protein antigen (H.L.S.P.), (2) a heat-stable protein antigen (H.S.S.P.) possibly associated with a haptene Cγ2, (3) an alcohol soluble 'Q protein' fraction . . ; and (4) the differential agglutinating S, R and ρ antigens with their respective polysaccharide haptenes Cα, Cβ and Cδ . . . Another haptene, Cγ1, is probably also of somatic origin, while yet another, the rugose haptene . . . has been derived from the intercellular secretion of rugose cultures. A method has been given for separating vibrio flagella. Antibodies for all these components occur or may occur in the sera of rabbits immunised with living cultures of *V. cholerae*. It is certain that vibrio cultures contain other separable serologically active and antigenic constituents and it is by no means unlikely that some of the fractions already described will prove to be mixtures."

Recent observations on the O antigens

The necessity of using, according to the recommendation of Gardner & Venkatraman (1935b), O sera for the laboratory diagnosis of cholera was fully endorsed by large-scale investigations carried out in India and recorded by Taylor (1937, 1938, 1941).

Experiences identical with those in India were made by Russo (1938) who recommended repeated (4-6) subcultivations of suitable cholera strains on agar containing 0.5% lithium chloride to obtain growths free from flagella for the preparation of pure O sera. Cultivation of the vibrios on alcohol-containing media or the use of heated suspensions were in Russo's experience less suitable to obtain H-free antigens for serum manufacture, while growth of the organisms on phenol-containing media was altogether unsuitable for this purpose. He concluded from tests with 58 strains of cholera and cholera-like vibrios that with the aid of Inaba O serum it was possible to differentiate Inaba strains from Ogawa and El Tor strains as well as from the non-agglutinable vibrios of Finkler-Prior and Deneke.

An important study, based upon an examination of 50 cholera strains, 9 true El Tor strains and 11 strains of cholera-like vibrios falling into Gardner & Venkatraman's O sub-groups II-VI, was made by Burrows et al. (1946). They subjected, for this purpose, a group of representative cholera strains to a complete analysis of their heat-stable and heat-labile antigens by reciprocal absorption tests and studied at the same time the other vibrio strains with the aid of agglutination with monospecific immune sera. Verifying the tentative O antigenic formulae thus arrived at by absorption tests with known antigens, Burrows and his co-workers found the vibrio O antigens to consist of 13 components, five of which were con-

sidered as major antigens. One of the latter, designated A, was found only in vibrios of the O sub-group I and was, therefore, regarded as the group-specific antigen. Antigen B, found in 13 out of 20 Ogawa strains, but in no Inaba strain, and antigen C, met with in all of the 25 Inaba strains examined as well as in two Ogawa strains, were considered to be type-specific. The other major O antigens showed no association with the type-specific antigens or with one another. It thus appeared that four immunological types existed within the O sub-group I, namely, type A, into which 11 of the 50 cholera strains tested fell, type AB, characteristic of the Ogawa strains, type AC, to which the Inaba vibrios belonged, and finally type ABC, inferred to correspond to the Hikojima type.

Since with the exception of the group-specific antigen A, which was met with exclusively in *V. cholerae* and the El Tor strains in the strict sense, the major antigens were also found in vibrios not belonging to O sub-group I, Burrows and his co-workers urged that the identification of vibrios falling into this class " should be based on agglutination with monospecific A anti-serum ".

Findings confirming those of Burrows et al. were recorded by Gallut (1949a, 1949b), who examined

(*a*) 49 authentic cholera strains, including 35 isolated during the 1947 epidemic in Egypt;

(*b*) 12 El Tor strains in the strict sense, partly those obtained during the 1938 Celebes outbreak;

(*c*) 21 cholera-like strains, 13 of which were of human origin and 8 isolated from water.

As summarized by Gallut, the percentage incidence of the 13 antigenic factors in cholera and true El Tor vibrios on the one hand, and in the cholera-like vibrios examined by him on the other was as follows :

Antigenic factors	*Cholera vibrios*	*Cholera-like vibrios*
A	100	0
B	32	47
C	95	14
D	45	19
E	47	9
F	26	0
G & J	1.6	19
H & M	3	4
I & K	18	9
L	47	28

Considering that, in contrast to the factor A, which had been met with only in true cholera and El Tor vibrios, the factors B and C, responsible respectively for the type-specificity of Ogawa and Inaba vibrios and jointly for that of the Hikojima type, were also present in the cholera-like

vibrios, Gallut urged the use of monospecific anti-O sera A for the laboratory diagnosis of cholera. He also recommended that, in order to avoid co-agglutinations due to the presence of factors D or E, sera specific for the factors B and C be used for type differentiation. In regard to the selection of suitable strains for vaccine manufacture he maintained that :

" It is true that the solution so far adopted of preparing vaccine from strains isolated during the epidemic against which control measures are being taken, is generally satisfactory, but the objection to it is that it is entirely lacking in precision. If the overriding necessity of having a completely polyvalent vaccine in stock seems acceptable, that is, a vaccine comprising the 13 O factors, it would however seem logical to take into account the antigenic composition of the vibrios responsible either for endemic cases or a specified epidemic. Only the complete analysis of a sufficient number of strains can furnish this indispensable information." [Trans.]

The validity of Gallut's recommendations for vaccine manufacture was denied by Sokhey & Habbu (1950c) who (*a*) compared the mouse-protective power of vaccines prepared with some of Gallut's strains possessing in part a complicated antigenic structure with that of two vaccines manufactured from Haffkine Institute strains, which had a quite simple antigenic structure, and (*b*) correlated these findings with determinations of the virulence of the strains in question for white mice. The conclusion reached was that the protective power of a cholera vaccine depended not upon the complexity of the antigenic structure of the strains used for their manufacture but upon the virulence of the strains chosen. Sokhey & Habbu suggested in this connexion

" that the complexity of the antigenic structure observed by Gallut might be due to the degeneration of the strains from age, because they were found to have no virulence to white mice".

Kauffmann (1950) and Singh & Ahuja (1950), approaching the problem with the aid of serological methods, were also unable to confirm the results of Burrows and of Gallut.

The main conclusion reached by Kauffmann after an examination of 41 strains sent to him by Gallut as well as of six additional cholera strains was that " the occurrence of new types or variants within the O group I that were claimed to be characterized by the antigens D, E, F, G, H, I, J, K, L, and M could not be demonstrated ". However, while noting this statement, the present writer finds it impossible to share Kauffmann's opinion that " technical errors in the planning and estimation of the serologic examination " accounted for the apparent occurrence of these antigens.

Kauffmann considered a polyvalent O serum, prepared with the aid of Inaba as well as Ogawa strains as most suitable for the identification of *V. cholerae* and recommended for the differentiation of the two types of strains a serum obtained by absorption of a polyvalent or an Ogawa serum by an Inaba strain. He also advocated the manufacture of polyvalent

cholera vaccines with the aid of Inaba and Ogawa strains without giving attention to the serological subtypes as Gallut had urged.

Singh & Ahuja (1950) thus summarized the experiences gained through a serological and biochemical investigation of (*a*) 96 cholera, El Tor, and cholera-like strains of their own, and (*b*) 49 strains put at their disposal by Burrows and by Gallut:

" We are sceptical of the claim of Burrows et al. (*loc. cit.*) that a new type of cholera vibrio has been discovered, namely one containing cholera group-specific antigen only. This has not been confirmed by using the type representative material supplied to us by Burrows and by Gallut.

" Of several thousand strains of *V. cholerae* tested by us with cholera ' O ' serum (containing group-specific *plus* type-specific agglutinins) not one strain has yet been encountered which subsequently did not agglutinate with type-specific serum, either Ogawa or Inaba. Type ' A ' serum supplied by Gallut has been found by us to be a non-differential serum containing cholera group-specific ' A ' *plus* type-specific ' C ' agglutinins. It is the same type of diagnostic reagent as is normally used for the preliminary identification of *V. cholerae* and is in no way superior in its diagnostic properties to the non-differential serum used at present in India.

" Antisera raised against so-called ' A ' type vibrios—Burrows and Gallut types—were tested against strains of cholera vibrios including freshly isolated and old laboratory cultures. Not a single strain showed positive agglutination. In the light of our experience in India we are of opinion that the existence of a new type of cholera vibrio—containing antigen ' A ' only—has not been established, nor have we been able to confirm the presence of cholera type-specific ' B ' and ' C ' antigens in non-cholera vibrios."

In contrast to the last-mentioned observers, Wahba (1951), re-examining the 1947 Egyptian strains formerly tested by Gallut (1949) but excluding those which showed abnormal features (i.e., loss of agglutinability, spontaneous agglutination, positive results with Millon's reagent, or thermolability), confirmed the multiplicity of the antigenic factors demonstrated in *V. cholerae* by Burrows et al. and by Gallut. It is noteworthy, however, that Wahba found the antigenic formulae of the strains examined by him " not completely stable ". He stated in particular that (*a*) the C factor was apt to disappear rapidly in aging cultures and was not demonstrable in formolized suspensions; (*b*) the factors D and E, which had been found to be absent in a number of the strains in 1948, were now present, while the L factor, previously demonstrated in several of the strains, had become absent. It was also noted that, while the results of agglutination tests became manifest after four hours as far as the major antigenic factors A-E were concerned, agglutination of the minor factors took place more slowly, becoming manifest only on the following morning.

Evaluating the results obtained by Wahba, it must be kept in mind that he worked exclusively with old strains. Thus, as pointed out with great reason by the reviewer of Wahba's article in the *Tropical Diseases Bulletin*, his paper " does not appear to help in clearing up the point at issue, which could best be settled by the examination of freshly isolated strains of *V. cholerae* ".

Recent observations on the serological races of V. cholerae

As noted before, Burrows et al. (1946), agreeing with the views, but changing the symbols adopted by White (1937c), stated that the antigens B and C of the cholera vibrio were type-specific, while the antigen A was group-specific. The antigenic structure of the three serological types of *V. cholerae* was, therefore, as follows:

	Antigenic structure according to:	
Type	*White*	*Burrows et al.*
Inaba	A X	A C
Hikojima	A B X	A B C
Ogawa	B X	A B

The classification proposed by Burrows et al. was also adopted by Gallut (1949a, 1949b) and by Kauffmann (1950), to whose postulations reference will be made below.

Kabeshima (1918b) had claimed that he had succeeded in transmuting his " variant " (Ogawa) strain into the " original " (Inaba) type by cultivation in homologous serum and also by inoculation into the gall-bladder of rabbits. Whether he also observed transmutations in the reverse direction, seems uncertain. In analogy with Kabeshima's experiences, Nobechi (1923) was able to transmute through cultivation in homologous serum two " variant " strains into the " middle " (Hikojima) type. It is of great interest that observations recorded by Shrivastava & White in 1947 lent support to these early statements of Kabeshima and Nobechi.

Shrivastava & White recorded that they had been able

(*a*) to obtain in the case of 10 cholera and 3 El Tor strains of the Ogawa type through cultivation in Ogawa monospecific serum races which were indistinguishable from Inaba type strains; and

(*b*) to change, with the aid of monospecific Inaba serum, 4 out of 8 cholera strains which, though predominantly Inaba-like in serological reactions, possessed also an Ogawa factor, into the Ogawa type.

It proved impossible, however, to produce serological changes other than roughening in 5 cholera and 3 El Tor strains of the strict Inaba type through cultivation in homologous monospecific serum.

Discussing the significance of these observations, Shrivastava & White tentatively postulated:

" That the Ogawa serological complex represents the known acme of elaboration of the specific somatic antigen of *V. cholerae*.

" That this antigen is subject to degradation presumably by failure of the organism to synthesize certain chemical groupings.

" That this change is expressed serologically as a positive modification in the detail of antigen and not merely as a factorial loss.

" That from this debased Inaba antigen there is no easy return to the Ogawa state by a revival of lost synthetic power, the only escape from the interference of specific anti-

bodies being in the ' rough ' change, i.e. entire failure to synthesize the specific complex with resultant unmasking of the ' R ' antigen."

The views on the antigenic structure of the serological types of *V. cholerae* expressed by Joya (1950) do not seem significant, because in the opinion of this worker thermolabile antigens played a more important role in the serological differentiation of Inaba and Hikojima strains than the thermostable antigens. This is not in accord with the generally accepted doctrine.

In the opinion of Kauffmann (1950), Hikojima and Ogawa strains, because they proved identical in cross-absorption tests, had to be considered as one common type. He proposed, therefore, that a distinction should be made merely between two types of *V. cholerae*, namely the Inaba type (A C) and the Ogawa-Hikojima type with the antigenic formula A B (C). Kauffmann maintained in this connexion that the C antigen, though present in small amounts in the Ogawa vibrios, was not well developed in strains grown at 37° C, so that these were incapable of completely absorbing Inaba sera. However, if the strains were grown at 20° C, they completely or almost completely absorbed Inaba sera. In Kauffmann's opinion this was the case because at 20° C the B antigen of the Ogawa strains developed less abundantly and was thus incapable of inhibiting the development or " disponibility " of the C antigen.

It is important to note that Gallut (1953b), studying two cholera and two El Tor strains with the aid of single-cell cultivation, came to the conclusion that the Hikojima type, even though it showed sometimes a tendency to change into the Ogawa type, was a valid race of *V. cholerae*. Since Gallut was unable to find C antigen in Ogawa strains even if these had been cultivated at 20° C, he refuted Kauffmann's proposal to classify these and the Hikojima strains in a common group, as the latter worker had proposed.

As shown by these researches, the problem of the somatic antigens of *V. cholerae* is still far from being fully solved. It is, therefore, not surprising to find that within recent years the optimistic attitude adopted in this respect immediately after the publication of Gardner & Venkatraman's basic observations has been replaced not rarely by one of doubt. Thus, as stated by White in a report rendered in 1948, many experienced cholera workers in India " for one reason or another cling to the view that vibrios other than *V. cholerae* may from time to time contribute to cholera ".

The validity of this view, which implies that the system of cholera laboratory diagnosis adopted on the basis of Gardner & Venkatraman's findings on the somatic antigens of *V. cholerae* is not sufficiently comprehensive, was thoroughly discussed during joint meetings of the Cholera Advisory Committee of the Indian Council of Medical Research and the WHO Expert Committee on Cholera (1952), held at New Delhi in 1951. Though the speaker introducing this subject went so far as to consider Gardner

& Venkatraman's characterization of the *V. cholerae* merely " a preconceived notion ", in the opinion of the WHO experts,

" the gaps still existing in the knowledge on this subject did not detract from the practical value of the tests adopted for the laboratory diagnosis of the infection. Endorsing this opinion, the committee reached the conclusion that the present definition of the cholera vibrio, though incomplete, was sufficient for practical purposes." [Page 6].

Acceptable though this conclusion remains, there is a most vital need for further research on the *V. cholerae* O antigen. This ought to include (1) studies on the antigenic stability of the organism under varied conditions of culture and storage; (2) further inquiry into the O antigen complex, especially as regards concealed and blocking antigens; and (3) the application of both old and new information to the study of the O antigen complex of vibrio strains of precisely known history, and with a minimal number of transplants intervening between isolation and study.

Recent experiences on the H antigens

Vassiliadis (1936a) found that treatment of cholera-vibrio suspensions with chloroform (1.5 ml per 10 ml of suspension), while leading to a considerable reduction of the O agglutinability of the organisms, markedly increased their H agglutinability. More than that, some strains of cholera-like vibrios, which were not agglutinable with H + O cholera-immune serum in their original state, showed considerable H agglutinability after they had been treated with chloroform. As stated by Vassiliadis in a second paper (1936b), this difference was probably due to a removal of lipoids inhibiting H agglutination by the chloroform. The reduction of O agglutinability through the action of this reagent was presumably due to the dissolution of lipoids necessary to bring about agglutination.

A method for isolating the flagellar fraction of vibrios was described by White (1940c) who recommended using for this purpose R or ρ cultures on account of their freedom from the smooth specific antigen.

The procedure was started by adding chloroform to a dense saline suspension of the vibrios and then stirring so as to separate the flagella from the bacterial bodies. The extract obtained by centrifugation of the chloroform-treated growths after dispersal in saline was treated with an equal volume of saturated ammonium-sulfate solution. After 24 hours' contact with the precipitant the flagella could be collected and washed with the aid of brisk centrifugation.

The preparations obtained in this manner proved suitable in dilution for H agglutination tests, while dense suspensions could be used for the absorption of flagellar agglutinins. Injection of the preparations into rabbits stimulated the production of apparently pure flagellar agglutinins.

Following up the work of Gardner & Venkatraman (1935), who had shown that vibrios possessing different specific O antigens had the same H

antigen, Taylor et al. (1937) tested 558 strains of cholera-like vibrios with different sera, including an Inaba H + O serum and a serum manufactured with a chemically not fully defined but mainly protein-containing extract of Inaba vibrios. The interesting fact was established that, while these two sera agglutinated, besides *V. cholerae* strains, also many of the cholera-like strains, this held true only of those giving a cholera-red reaction.

Ahuja & Singh (1939), making further studies on 219 vibrio strains which were not agglutinated with specific cholera O serum (sub-group I of Gardner & Venkatraman), found that 35.5% of these cholera-like strains possessed H antigens partially or completely identical with the H antigen of *V. cholerae*. As shown by cross-absorption tests carried out with 10 of these strains and pure H sera (prepared by absorption of sera raised against living suspensions of these strains with massive doses of heat-killed cultures of the homologous vibrios), the cholera-like vibrios agglutinable with cholera H + O serum apparently fell into three groups, namely,

(1) those possessing an H antigen identical with that of *V. cholerae* ;

(2) strains, the major portion of whose H antigen was identical with that of the cholera vibrios; and

(3) strains possessing besides a major individual H fraction a minor one identical with the H antigen of *V. cholerae*.

The cholera-like strains which were inagglutinable with cholera H + O serum, possessed mainly individual H antigens, though some showed a partial H relationship among themselves.

A detailed analysis of the H antigens of *V. cholerae* was made by Burrows et al. (1946) through reciprocal absorption tests carried out on a representative group of 10 cholera strains with the aid of H + O sera which had been absorbed with their homologous O antigens. The H antigenic structure of these strains was found to be of a complexity similar to that of the O antigens, but only ten components of the H antigen could be detected, one of which was common to all of the strains. Apparently no correlation existed between the variant distribution of the individual H and O antigens respectively.

A further study, carried out with agglutination tests only on a larger group of vibrios, including, besides cholera and El Tor strains, strains of cholera-like organisms, indicated " an apparently random distribution of the H antigens in both cholera and non-cholera vibrios ".

Kauffmann (1950) stated that he had been unable to confirm the existence of sub-types of the H antigen.

Specially prepared antigens

Basu et al. (1940) stated that they had obtained a thermostable antigen by (*a*) immersing a cellophane or collodion bag filled with sterile normal

saline in a growing culture of *V. cholerae* in peptone solution, and (*b*) filtering the contents of the bag after five days' incubation through a Chamberland L_3 candle.

The diffusate obtained in this manner contained carbohydrate substances but practically no protein. Injected into rabbits, it gave rise to specific agglutinins and precipitins and also protected these animals against lethal doses of *V. cholerae*.

According to a report published in 1947, Feigina et al. obtained through tryptic digestion of cholera vibrios an antigenic complex which, however, was far less active than the glucido-lipoid antigen of Boivin & Mesrobeanu. Injected into rabbits, the tryptic digest stimulated the appearance of agglutinins but not of precipitins. Hydrolysis of the digest, the antigenicity of which seemed to be due to the presence of peptides, led to the separation of nitrogen-containing substances and the loss of antigenic power.

Chemical constitution of the antigens

An early attempt to extract the antigens of *V. cholerae* with the aid of alcohol was made by Levaditi & Mutermilch (1908). The residue of their extracts, obtained through centrifugation and evaporation of the supernatant, proved to be antigenic both in complement-fixation tests and in rabbit experiments, and conferred active immunity to guinea-pigs. It was apparently thermostable.

Since the validity of these findings was doubted by Prausnitz (1911), a further and thorough study of this matter was made by Landsteiner & Levine (1926, 1927).

These two workers obtained by extraction of saline-washed cholera vibrios with hot 75% alcohol and further extraction with ether and hot absolute alcohol a solution, the sediment of which, separated off after cooling (*a*) reacted in high dilution (1:500,000) in precipitin tests with cholera-immune serum, and (*b*) acted as an antigen when injected into rabbits.

While this crude substance gave both protein and carbohydrate reactions, the white powder obtained through purification with alcohol and other reagents was almost protein-free and no more antigenic, but continued to give precipitin reactions up to the above-mentioned titre and positive carbohydrate tests.

In Landsteiner & Levine's opinion, these findings were compatible with the assumption that the crude extracts contained an antigenic complex consisting of protein and a specifically precipitable but non-immunizing complex carbohydrate substance, which probably belonged to the class of " residue " (residual) antigens.

Investigations to demonstrate the presence of such residual antigens (haptens) in cholera-like as well as in cholera vibrios were made by Jermoljewa & Bujanowskaya (1930). They extracted for this purpose the washings of 24-hour-old agar cultures, after digestion with caustic potash, with

acetic acid and alcohol. The substances thus obtained were protein-free but gave reactions proving the presence of carbohydrates. The extracts gave precipitin reactions with cholera-immune serum but were not antigenic when injected into rabbits, unless gelatin or normal pig-serum had been administered simultaneously.

Linton (1932) extracted a carbohydrate-like fraction from cholera and cholera-like vibrios with the aid of the following technique :

" The organisms were sown on Roux bottles and incubated for 48 hours. The growth was then washed off in normal saline, and the solution brought to an acidity of N/20 with glacial acetic acid ... The bacterial mass was boiled on a sandbath under a reflux condenser until coagulation occurred. The coagulated mass was allowed to cool ... and then run several times through a Sharples supercentrifuge until a semi-opaque brownish solution was obtained. This solution was precipitated with three volumes of 90% alcohol and placed in the icebox overnight. The heavy precipitate was pipetted off, separated from the alcohol as completely as possible by centrifuging, and taken up in 200 or 300 cc. of water. Insoluble matter was discarded and the solution again precipitated with alcohol. As before, the precipitate was freed from any remaining insoluble matter, and dissolved in 100 cc. of water, where it formed a clear, brown-tinged solution, with a faint but unmistakable biuret reaction. It was strongly acidified with glacial acetic acid, and boiled. After cooling, the dark brown flocculum which had appeared was centrifuged off, and the supernatant fluid, which was now biuret negative, was precipitated with three volumes of alcohol. The precipitate was dried, weighed and dissolved in approximately N/20 NaOH to make a 1% solution."

As Linton added, the solutions thus obtained, while giving negative biuret, Millon's, and xanthoproteic reactions, proved positive with Molisch's reagent even at extremely high dilution and, after boiling with dilute acids, were found capable of reducing Fehling's reagent. In cross-precipitation tests the carbohydrate fractions obtained with the aid of the method described above from cholera-like as well as from cholera vibrios gave positive results not only with their homologous immune sera but with all sera tested which comprised besides five raised against *V. cholerae* one manufactured with a water vibrio. Thus, as Linton put it, the carbohydrate fractions, " if not identical, are at least closely related in the agglutinating and non-agglutinating vibrios " (i.e., in cholera and cholera-like vibrios). The carbohydrate fractions obtained in an identical manner from typhoid and dysentery (Flexner) strains failed to react with any of the six above-mentioned sera.

The results of studies on the immuno-chemistry of the vibrio group continued by Linton and his co-workers, which already have received preliminary attention in the third of these studies, will be further dealt with later on.

As noted earlier in this study, Boivin and his collaborators obtained with the aid of trichloracetic acid, from cholera vibrios as well as from other species of Gram-negative bacteria, extracts stated by these workers to represent the " total antigens " of the organisms in question. According

to Boivin & Mesrobeanu (1935), the substances in question corresponded chemically to a complex combination of specific polysaccharides with fatty acids. Exposure of the complete antigens to heating in a weakly acid medium led to the separation of an insoluble portion containing the fatty acids from the polysaccharides, which remained in solution. The latter, which represented the residual antigens of the organisms, could be solidified by precipitation with alcohol or acetone. If redissolved, the residual antigens produced solutions which, in contrast to those made from the total antigens, were non-opalescent and weakly dialysable as well as non-antigenic.

On account of the rather fragile nature of the complete antigens, it was possible to split off the specific polysaccharides without trichloracetic extraction directly from the intact organisms by the use of " brutal " methods, such as heating of the bacteria in an acid medium. The same result could be produced by the action of the " diastases " of the organisms.

Damboviceanu & Barber (1940), carrying out chemical analyses of the trichloracetic acid extracts of five cholera strains, confirmed that the complete antigen of *V. cholerae* was a glucido-lipoid complex which contained amino-nitrogen and phosphorus. The extracts did not give a biuret reaction and also failed to reduce Fehling's solution, but gave a feebly positive Molisch reaction.

Reviewing the experiences of Boivin and his co-workers as well as of subsequent observers in regard to the trichloracetic acid extraction of *V. cholerae*, Burrows et al. (1946) insisted that none of these workers

" demonstrated either the biochemical homogeneity of these preparations or their postulated identity with the O antigen of the vibrios by cross absorption experiments, nor have the preparations been subjected to immunological analysis".

Burrows and co-workers also laid stress upon the fact that the purified substances obtained by Burrows (1944) with the aid of organic solvents, which were found to consist of phospholipid and additional nitrogenous material, gave a negative Molisch reaction.

It has to be added that Linton, Shrivastava, Seal & Mookerji (1938) recorded that they had separated from a cholera strain, which had been isolated in the early stage of an outbreak, a glucolipid fraction. They deduced from this observation that the presence of such a complex might be characteristic for an epidemic type of the organism. Apparently, however, no further observations confirming this assumption have been made.

Attention has been drawn in the third of these studies to the observations of Linton and his co-workers (see Linton, 1940, 1942), who were able

(*a*) by racemization with dilute alkali solutions to demonstrate the presence of two types of protein [d] in the vibrios, the first of which was usually present in *V. cholerae*; and

[d] These proteins have been further studied by Mitra (1938) who, though finding marked differences in the respective structure of their molecules, admitted " that it is impossible with the present data to say whether proteins I and II represent two different entities or whether they are mixtures of several proteins ".

(*b*) also to show the existence of three types of vibrio polysaccharides, most cholera strains being found to possess those of type I (galactose and an aldobionic acid consisting of galactose and glycuronic acid), but a considerable minority showing type II polysaccharides, in which arabinose instead of galactose was found to be combined with an aldobionic acid of the same composition as in the type I polysaccharides.

As maintained by Burrows et al. (1946) and also admitted by Shrivastava, one of Linton's principal co-workers, in a 1951 summary, the relation of these polysaccharide types to the H and O antigenic structure of the cholera vibrios and to their differentiation in serological races is not clear. It is important to note in this connexion that, as summarized by Shrivastava (1951), the polysaccharide fractions initially isolated by Linton and his colleagues were found to be serologically inactive and that those prepared later by Shrivastava & Seal (1937) and by Linton, Shrivastava & Seal (1938) and Linton, Shrivastava, Seal & Mookerji (1938), though giving precipitin reactions with suitable sera raised against intact vibrios, were found to possess no antigenic properties, thus falling into the category of haptens.

As will be gathered from statements already made in the present studies, in the course of his immunological investigations White was also able to make observations on the chemical character of the various vibrio antigens demonstrated by him. The following supplementary statements have to be made in this connexion:

(1) *Non-protein carbohydrate-containing specific substances*

As stated by White (1936b, see also 1936a),

"The protein-free polysaccharide specific substance was prepared in a suitable manner by digesting vibrios washed in alcohol and boiled in saline with 1% papain at pH 5.5 for 7 to 8 hours at 90°C., centrifuging the mixture and precipitating the active substance from the supernatant with alcohol, extracting the active material from this precipitate with a saturated aqueous solution of picric acid and then reprecipitating it with alcohol. Picric acid was removed by reprecipitation with alcohol."

As mentioned already, White (1937c) found these preparations actively antigenic.

(2) *Q antigens*

Describing the chemical properties of the protein Q antigens, White (1934b) stated that both the Q_1 and the Q_2 antigens gave a positive biuret test, the former antigen reacting less intensely than the Q_2. Both precipitated with Millon's solution, the colour of the precipitate turning to pink or red at room temperature. In contrast to Q_2, the Q_1 antigen was soluble in dilute hydrochloric acid. Both antigens proved to be readily soluble in alcohol in the presence of HCl. The solvent action of acetic acid was considerably less marked.

(3) *Other special antigens*

Basic chemical reactions shown by the other special vibrio antigens which White (1940b, 1940c, 1940e) described, may be tabulated thus:

	Tests		
Antigen	*Biuret*	*Millon*	*Molisch*
Heat-labile somatic protein antigen . . .	intensely positive	intensely positive	definitely positive
Heat-stable somatic protein antigen . . .	strongly positive	strongly positive	strongly positive
Flagellar antigen . . .	intensely positive	imperfect	weakly positive

Note: The rugose hapten isolated by White (1940a) gave a negative biuret reaction but an intensely positive Molisch reaction.

Important as these and the previously-discussed related observations of White are, they merely characterize the chemical composition of the vibrio antigens in a general manner. In fact, as stated by White (1937c), it was impossible to decide whether his specific carbohydrate-containing antigen was strictly a polysaccharide or a polysaccharide-containing complex of the type described by Boivin and collaborators and also by other workers.

Shrivastava et al. (1948) tried, for further studies on the immunochemistry of *V. cholerae*, in addition to the above-described method of White (1936b) the following two methods:

" [a] The centrifugate of the growth for 72 hours in papain-digested mutton broth is concentrated *in vacuo* at a temperature of 40°C. to 45°C. and the concentrate worked for the isolation of polysaccharides [Shrivastava & Seal, 1937]. Protein is removed from the precipitate by shaking it with chloroform and butyl and amyl alcohol."

" [b] Phenol method [Palmer & Gerlough, 1940], in which the acetone-dried growth of the bacteria is treated with 90 per cent phenol. This dissolves away the protein and liberates the polysaccharides."

The final product obtained in bulk from an Inaba strain of *V. cholerae* with the aid of the last-mentioned method, which was found most suitable, proved soluble in distilled water and 0.85% saline, biuret-negative, and Molisch-positive in a dilution of 1:100,000. The nitrogen and acetyl group contents were 7.7% and 2.1% respectively. The substance reacted up to a titre of 1:200,000 with immune sera prepared against both Inaba and Ogawa sub-types of *V. cholerae* and, administered subcutaneously to white mice in two doses of 0.2 ml each at weekly intervals, conferred to the animals thus treated a high degree of immunity against intraperitoneal infection with mucinized suspensions of *V. cholerae* (Inaba sub-type).

Singh et al. reported in 1950 upon further studies of the *V. cholerae* polysaccharides, isolated through phenol treatment of acetone-dried growths and subsequent precipitation with 95% alcohol. It was possible to obtain with the aid of this method fractions of high antigenicity from Ogawa as

well as from Inaba sub-type strains. As was established in the course of this work, polysaccharide fractions which precipitated at high titre with cholera-immune serum, did not necessarily confer a high degree of protection to mice. Another interesting finding was that intravenous injection of a polysaccharide complex isolated from an Inaba strain into guinea-pigs which had been passively immunized with mono-specific or non-differential cholera-immune serum, did not produce signs of anaphylaxis.

In contrast to the observations recorded above, Sato et al. (1950) found that the polysaccharide fractions isolated by them from Inaba and Ogawa strains of *V. cholerae* were non-antigenic. Possibly, however, this was the result of de-acetylation, due to the alkali treatment which had been used for extraction.

Purified, polysaccharide-free protein fractions, which were also tested, took, in the opinion of Sato et al., " a prominent part in the type-specific antigenicity in complement fixation tests and agglutinin absorption tests ". These are rather surprising results, needing confirmation.

Krejci et al. (1949) reported that they had separated with the aid of electrophoresis from Inaba and Ogawa cholera strains three main constituents, of which two (" X " and " B ") showed antigenic activity. It appeared that the heat-labile antigens were associated with the X constituents, the heat-stable O sub-group I antigen with the B constituents. These were found to contain polysaccharides in combination with proteins. The X constituents appeared to consist mainly of proteins or of lipids.

Serological Reactions

Early observations

Profound studies on cholera immunity led Pfeiffer (1895a) to the recommendation of a serological method for the differential identification of *V. cholerae*. He thus described the technique of this test which is now known under the name of " Pfeiffer's reaction ":

" As a rule I use cholera serum, the titre [e] of which is at least 0.001, and take of it for each test 0.01, i.e., ten times the minimal effective dose. One loop of the culture to be tested is mixed with 1 ml broth and the above-mentioned serum dose, and injected intraperitoneally into young guinea-pigs of 200-300 g . . . The syringe used for this purpose is provided with a blunt canula. The resistant corium is split with the aid of scissors and the blunt end of the canula penetrates thus quite easily into the peritoneal cavity. After 20 minutes I remove with the aid of glass capillaries droplets of the peritoneal contents for examination in hanging drop and stained preparations. If after that

e Pfeiffer (1895) designated as titre of a cholera-immune serum " that minimal quantity of serum which, if injected together with the cholera dose in 1 ml broth into the peritoneum of young guinea-pigs of 200 g, just suffices to lyse 2 mg of the normal virus within 1 hour ".

As " normal virus " he designated cholera cultures possessing a virulence sufficient to kill guinea-pigs within 24 hours, if injected intraperitoneally in doses of 1/5-1/10 loop (0.4-0.2 mg) of a 20-hour-old agar culture.

time still numerous well-preserved and motile vibrios are present in the peritoneal cavity, the reaction is negative and cholera bacteria are, therefore, absent. If on the contrary after 20 minutes in the exudate the injected comma bacilli are found to be changed into granula, among which only quite few and immotile vibrios are noted, two possibilities are given: (1) the test culture is devoid of pathogenic properties and thus rapidly destroyed even in normal animals, or (2) true cholera vibrios are present, which are lysed by the specific bactericidal substances (positive reaction)." [Trans.]

To decide this issue, a control guinea-pig was used, which received intraperitoneally 1 loop of the culture to be tested + 0.01 ml normal serum in 1 ml of broth. If droplets of this mixture removed from the peritoneal cavity after 20 minutes showed the presence of viable organisms, the diagnosis of cholera was confirmed. If, on the contrary, the organisms in the exudate of the control animal had disappeared, the result of the test was doubtful in so far as avirulent cholera vibrios as well as cholera-like vibrios were apt to be affected by the normal serum. Pfeiffer recommended testing under these circumstances the antigenicity of the culture in question through immunization of guinea-pigs and stated that it had been possible to confirm in this indirect manner the true nature of an old avirulent Calcutta strain of *V. cholerae*. Pfeiffer admitted that thus the results of his test were bound to be more reliable the higher the virulence of the cultures under examination was, but added that " as a rule it is absolutely impossible not to come to a decision ". In his opinion, an application of the above-described test was indicated only in the case of atypically-behaving strains from stools and of water vibrios, whereas the hitherto adopted methods sufficed to arrive at a reliable diagnosis in most instances.

As had been noted already, Gruber & Durham introduced in 1896 the expedient and therefore generally-applicable method of agglutination for the laboratory diagnosis of cholera.[f] Achard & Bensaude claimed in the following year that advantage could also be taken of this method by testing the sera of cholera patients with known cholera cultures.

Kraus (1897) noted that, as had been previously shown in the case of the typhoid bacillus, cholera vibrios remained agglutinable with specific serum after they had been killed by heating at 56°C. He also made the important observation that the addition of specific immune sera to germ-free filtrates of cholera broth-cultures led to the formation of precipitates. As proved by controls with normal sera as well as with sera raised against other bacteria, this was, like agglutination, a strictly specific test. Since identical reactions could be produced with the juice obtained by exposure of a

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f Bordet had already noted in 1895 that, in contrast to normal rabbit serum, the sera of rabbits immunized against cholera first immobilized and then promptly agglomerated *V. cholerae* and that, unlike the bactericidal power, this property was not lost when the sera were heated to 55°-60°C. Nevertheless, as summarized by Fitzgerald & Fraser (1928), Gruber & Durham " for the first time described the agglutination reaction as a separate and distinct characteristic of immune sera ". This statement is also valid as far as the observations recorded in 1896 by Pfeiffer & Vagedes are concerned, because these two workers considered the phenomenon of agglutination of cholera vibrios observed by them to be due to the causes producing Pfeiffer's reaction and, in contrast to Gruber & Durham, referred merely in a tentative manner to the practical value of agglutination tests.

mixture of cholera vibrios and glass dust to a pressure of 300 atmospheres, the precipitinogens appeared to form part of the bodies of the organisms instead of being excreted by them.

Though, as summarized by Hetsch (1912, 1928), the question of the interrelations existing between the above-described antibodies as well as the problem of their immunological importance soon became the subject of considerable dissensions, early practical experiences, in the first line the large-scale investigations of Kolle et al. (1903) endorsed the specificity and consequently the diagnostic importance of the reactions concerned, particularly of the agglutination test.

A somewhat divergent opinion was expressed by Friedberger & Luerssen (1905) who claimed that the usefulness of the latter method was limited in so far as in the case of freshly-isolated strains the vibrios showed after an incubation at 37°C for 6-8 hours spontaneous agglutination (" pseudo-agglutination ") in normal saline. Since, however, in actual practice agglutination tests are made after longer intervals (at an average after about 18 hours) when, as admitted by Friedberger & Luerssen, spontaneous agglutination had become absent, their observations would be of little importance even if generally valid. However, as shown by subsequent investigations, especially by the experiences of Kabeshima (1918c) who examined 160 freshly-isolated cholera strains in this respect with negative results, the phenomenon of pseudoagglutination, observed by Friedberger & Luerssen in the case of only 11 strains, must be rare.

As already alluded to in the third of these studies, the discovery of the El Tor vibrios (Gotschlich 1905, 1906) which, though giving serological reactions identical with those of the classical *V. cholerae*, appeared to be different from the latter on account of their apathogenicity and their haemolytic properties, led to most serious dissensions in regard to the specificity of the serological tests. Ruffer (1907), one of the protagonists of the school claiming a separate status for the El Tor vibrio, went so far as to conclude

" That it is not advisable to trust to the agglutination test only in the bacteriological diagnosis of cholera. The test is useful but not specific. "

The German workers on the other hand (see summary by Kolle & Schürmann, 1912), who denied the existence of qualitative differences between the cholera and El Tor vibrios, continued to maintain that

" the system of cholera diagnosis, which is based largely upon the immunological reactions, still rests upon a fully secure scientific foundation and has as well proved its practical value ". [Trans.]

Though it remains legitimate to evaluate the differences existing between the classical cholera vibrio and the *V. El Tor* either in favour of a unicity of both or to support the concept of their separate standing, modern

investigations have left no room for doubt that, as far as their basic serological reactions are concerned, the two organisms do not differ. Evidence in this respect, additional to that furnished in the preceding section of the present study, will be brought forward in the following pages.

Further investigations on Pfeiffer's reaction and bacteriolysis

As shown by investigations of Baumgarten (1921), it is possible to use mice in place of guinea-pigs when performing Pfeiffer's test. However, as noted by Hetsch (1928), the results obtained with the aid of this modification were not as uniform as those with Pfeiffer's original technique, presumably because complement, which is indispensable for bringing about the reaction, is not as plentiful in mice as in guinea-pigs.

According to the summary of Hetsch (1928) the outstanding value of Pfeiffer's test carried out in the classical manner was confirmed by numerous observers. He noted in this connexion that, while cholera-immune sera failed to react with cholera-like vibrios, the latter reacted typically with sera manufactured with homologous or serologically identical organisms. Vice versa, the immune sera raised in this manner gave negative tests with cholera vibrios.

Harvey (1929), though admitting that Pfeiffer's reaction had been largely superseded by agglutination tests, upheld its value " in difficult sporadic cases of cholera-like disease ". In view of the availability of the highly-specific O sera it is a moot point whether this statement is still valid at present.

In the course of his investigations, Pfeiffer devoted attention to the question of to what extent his reaction could be produced outside the living organism.

Pfeiffer (1894b) established in this connexion that, if a broth suspension of cholera vibrios mixed with a dose of potent immune-serum was injected into the peritoneal cavity of freshly-killed guinea-pigs and the carcases were kept in the incubator, marked bacteriolysis took place for the first 20 minutes but did not progress further.

As Pfeiffer observed in 1895, dilutions of cholera-immune serum in broth, which proved highly vibriolytic when injected into the peritoneal cavity of guinea-pigs, exerted in vitro no bactericidal action on cholera vibrios and even formed a suitable substrate for their multiplication. If, however, a 1% dilution of cholera-immune serum in broth was injected intraperitoneally and droplets of the peritoneal contents of such animals removed after 20 minutes were seeded with cholera vibrios, bacteriolysis took place but often did not become complete.[g]

In vitro bacteriolytic tests, to be used side by side with, or in place of, Pfeiffer's reaction, have been recommended by several authors.

Thus Serkowski (1906) mixed various saline dilutions of the sera to be tested with constant quantities of cholera vibrios and of complement (normal serum) and, after

[g] This method was also used by Bordet (1895). Craster (1914), giving priority to the latter worker, stated to have utilized " Bordet's test " to demonstrate bacteriolysis in vitro side by side with Pfeiffer's reaction for a study of cholera-like vibrios.

incubation at 37°C for 4-6 hours, used the mixed material for pouring agar plates, which were kept in the incubator for 24 hours. The absence of growth, or the number of colonies which had by then developed, indicated to what extent the serum dilutions in question possessed bacteriolytic properties.

Using a similar technique, Amako (1909) added to two parts of the various serum dilutions to be tested 1 part of a suspension of cholera vibrios and 1 part of normal rabbit serum diluted 1:10. The tubes were kept at 37°C for 1 hour, then smears were made and stained with dilute carbol fuchsine, in order to determine to what degree bacteriolysis had taken place. At the same time amounts of 0.01 ml from each tube were used to pour gelatine-agar plates. Colonial counts were made after an incubation at 37°C for 24 hours.

Prausnitz & Hille (1924), besides confirming the above-mentioned observations of Pfeiffer, also found it possible to reproduce the phenomenon of bacteriolysis in vitro with the aid of adequately-graduated amounts of immune serum and complement, particularly if fresh complement was added from time to time. Bacteriolysis became still more marked if also limited amounts of an exudate, which had been obtained through intraperitoneal injection of a guinea-pig with sterile broth, were added to the tubes.

An in vitro test for bacteriolysis with the aid of peptone water has been described by Kiribayashi (1931b). As summarized in the *Tropical Diseases Bulletin* (1932), his technique was as follows :

" A loopful of a 20-hour agar culture of test vibrio is suspended in peptone water (peptone 3; sodium chloride 5; dist. water 1,000) of pH 7.6 which is isotonic with the serum components of the test. A comparison is made by setting up two sets of dilution mixtures, the one containing inactivated immune serum, complement and suspension of test organism and the other, a control, containing inactivated rabbit serum, complement and suspension. Specific bacteriolysis is indicated after 3-5 hours by the absence of turbidity in the first set of mixture and positive turbidity in the control set."

Ahuja (1951) and Singh & Ahuja (1951), noting that fresh guinea-pig serum exerted a marked vibriocidal effect on rough or partially rough cholera vibrios, while leaving smooth vibrios unaffected, recommended the following test for the detection of roughness in *V. cholerae* strains :

An 18-hour-old peptone-water culture of the strain to be tested was diluted one hundred-fold with the same medium. One part of this dilution was mixed with two parts of 1 in 2 complement (diluent peptone-water). The mixture was incubated for 4 hours at 37°C. The initial inoculum and the 4 hours' growth in the presence of complement were then sampled by plating 3-mm loops on agar plates without spreading, and results were read after incubation at 37°C for 18 hours.

It was found that rough or partly rough cholera vibrios either failed to grow or grew to a greatly reduced degree in the presence of complement, an effect which was inhibited by heating the mixtures at 56°C. Since 20% of the guinea-pig sera tested did not exert this effect, it was essential to make preliminary tests with known smooth strains.

Interesting as these findings are, it has to be noted that Gallut (1953a) did not consider the above-described test as fully reliable for the detection of rough cholera strains.

It may be conveniently added that, as recorded by Popescu (1924), the washed blood-platelets of cholera-immunized rabbits produced not only agglutination but also lysis of cholera vibrios. It is also of interest that, according to the experiences of Pacheco & Peres (1940), mucin reduced

or even inhibited the vibriolytic action of cholera-immune sera (inactivated by heating al 55°-56°C) in the presence of complement. The differences between the lytic action of cholera-immune sera and that produced by bacteriophage were studied by MacNeal et al. (1937). In contrast to the former, bacteriophage lysis was transmissible in series and rapidly led to variations in the size and form of the vibrios.

General agreement exists that bacteriolysins are present in the sera of normal persons at rather low titres only. Papamarku (1917) noted in this connexion that while some previous observers met usually with titres varying from 0.1-0.75 in the sera of their controls, he found that 3 out of 16 such normal individuals had bactericidal titres of 0.05, while the others had titres below this figure.

As summarized by Svenson (1909), the presence of bacteriolysins in the sera of cholera patients or convalescents was demonstrated by several early workers, such as Lazarus (1892), Metchnikoff (1893), Pfeiffer (1894a), and Amako (1909), who as a rule resorted to animal experiments, especially Pfeiffer's test, but occasionally relied only upon in vitro tests. As found by these and other observers, the bactericidal titre of such sera (i.e., the minimal amount still protecting guinea-pigs against intraperitoneal cholera infection) varied considerably and was, according to Metchnikoff, even in the case of past severe infection sometimes not higher than that of normal sera. In the experience of this worker, during the acute phase of the disease bactericidal substances were present in but small amounts in the sera of not more than 45% of the sufferers. However, as established by him and all other observers, the bacteriolytic titre rose during convalescence, to become usually maximal during the second and fourth week. In the experience of some, but not of all workers quoted by Svenson, the protective value of the convalescent sera was not higher than that of normal sera after 6 weeks.

Svenson himself was able to demonstrate the presence of bacteriolysins in 89% of the 27 convalescents examined by him within the second to fourth week after onset of the disease. There was no parallelism between the presence of bacteriolysins and that of agglutinins, found in the sera of only about one third of these persons. In view of the fact that Pfeiffer's test was negative in the case of some convalescents who had survived severe cholera attacks, Svenson concluded that the appearance of bacteriolysins, because frequent, was a characteristic sign of recovery, but did not fully account for it (". . . eine Begleiterscheinung, die sehr häufig bei der Genesung beobachtet wird und ein charakteristisches Symptom derselben ist, mit derselben aber nicht unbedingt identifiziert werden darf").

Referring to experiences made in regard to the persistence of bacteriolysins in the sera of persons who had recovered from cholera, Papamarku (1917) stressed that obviously these immune bodies were apt to become inconspicuous or even absent at a time when the individuals in question

were still fully immune against the disease. A case in point was that of Pfeiffer, whose serum showed no specific immunizing properties already three months after he had been affected by cholera.

Further noteworthy observations on the presence of bacteriolysins in the sera of cholera patients or convalescents may thus be summarized :

Shiiba & Oyama (1920) demonstrated the presence of bacteriolysins in the sera of 97 convalescents with the aid of the Neisser and Wechsberg test. As a rule, though not invariably, the results thus obtained ran parallel with those of agglutination tests.

Tagami & Watanabe (1920), also applying Neisser and Wechsberg's method, obtained positive results in 89% of the 91 sera tested. Bacteriolytic tests were found to yield results earlier than agglutination tests, becoming positive in 80% to 85% of the cases in 1 to 3 weeks. The bacteriolytic titres decreased after the third week, only half of the sera still proving positive after one month. In the experience of these two workers, " the agglutination and bactericidal reactions do not run parallel, but sometimes quite oppositely ".

Ukil (1928), studying 30 convalescent sera collected from cholera patients in Calcutta, 25 of which agglutinated the causative organisms at titres ranging from 1:100 to 1:1,000, found that 18 of these sera possessed marked bacteriolytic properties, while seven produced less marked, and five weak reactions in tests made in vitro.

Continuing such tests (platings from tubes containing a mixture of two drops of the convalescent serum under test, 4 drops of a suspension of *V. cholerae* containing 2,000 million organisms per ml, 2 drops of 50% complement and 0.6 ml normal saline, which had been incubated at 37°C for 4 hours), Ukil & Guha Thakurta (1930) found that the bacteriolytic properties of the convalescent sera increased progressively, reaching their maximum at the time when the faeces of the individuals tested no more yielded positive cultures, i.e., usually 1-3 weeks after onset of the disease.

As far as it was possible to continue the observations, it appeared that the bacteriolytic properties of the convalescent sera remained manifest for several weeks. The presence of bacteriolysins was confirmed by rabbit experiments, a dose of 0.5-1 ml intravenously as a rule protecting the animals against intravenous injection of a lethal dose of *V. cholerae*. It was found that 85% of the convalescent sera tested agglutinated cholera vibrios at titres ranging from 1:100 to 1:3,200.

Attempts to demonstrate the presence of specific bacteriolysins in the sera of cholera patients with the aid of a modified Pfeiffer test gave, according to a statement made in the 1941 report of the Indian Research Fund Association, no fully satisfactory results. Though sometimes present early in the disease, bacteriolysins could be demonstrated in but 33% of the sera examined. In a majority, the bacteriolysins showed no type specificity.

Observations on the presence of bacteriolysins in the sera of healthy cholera carriers are not numerous. Massaglia (1911) claimed that bacteriolysins as well as agglutinins were present in the sera of such individuals in the same amounts as in the sera of convalescents, and postulated, therefore, that the freedom of the carriers from manifest signs of cholera was due to the presence of immune bodies in their blood. De Bonis (1912), because unable to demonstrate bacteriolysins (or agglutinins) in the sera of healthy carriers even within the days immediately following the isolation of *V. cholerae* from their faeces, reached on the contrary the conclusion that the freedom of the carriers from clinical manifestations of the infection could not be the result of a general

immunity but must have depended upon other factors, possibly a local immunity.

Levi della Vida (1913), though able to demonstrate the presence of agglutinins in all but 9 of the 48 convalescent and healthy carriers examined by him, found bacteriolysins less regularly demonstrable, so that they were absent from some of the well-agglutinating samples. On the other hand, bacteriolysins were never detected in sera devoid of agglutinating power.

Sano (1921) found like de Bonis that the sera of healthy cholera carriers contained practically no immune bodies, while Toguchi (1919), as quoted by Takano et al. (1926), recorded that " the immunological reactions of the carriers are not uniform, and in some cases they are not stronger than those of the healthy person ". In accord with de Bonis, Toguchi assumed that the absence of clinical manifestations of cholera in the carriers must " be explained on some basis other than the immunological reactions of the blood serum ". A similar opinion was expressed by Satake (1926) who was able to demonstrate the presence of bacteriolysins in but one out of 5 cholera carriers and apparently found agglutinins in none of them.

Classical investigations by Kolle (1896, 1897) showed that administration of killed cholera vaccines in a single high dose (1/10 of a culture) to 17 individuals led to a most marked increase of the originally almost invariably low bacteriolytic titre of their sera. As far as could be established, the increase of the titres became manifest already 6-10 days after vaccination and was still demonstrable for periods up to about 12 months (350 days) after the vaccine had been administered.

While several of the subsequent observers maintained in agreement with Kolle that a rise of the bactericidal titres did not become demonstrable before the fifth day after vaccination, some noted an earlier increase of the bacteriolysins. Balteano & Lupu (1914), examining two individuals vaccinated for the first time with doses of 1 ml, spoke in this connexion of the fourth day after administration of the prophylactic. Aaser (1910) drew attention to three persons in whom a considerable rise of the bacteriolytic titres (twice to 0.01, once even to 0.003) had been noted already on the third day after vaccination. Ahuja & Singh (1948), examining 21 persons once inoculated with 1 ml of cholera vaccine, likewise established that the sera of these individuals showed vibriocidal properties after three days.

It is unanimously stated that the initial appearance of bacteriolysins in the sera of cholera-vaccinated persons is followed by a further increase of the bacteriolytic titres. However, the statements as to when their maxima are reached and as to how long the titres remain at this level vary considerably. Thus according to Ahuja & Singh (1948) the maximum of the bacteriolytic titres was attained about the 8th day after vaccination and a fall of the titres became manifest by the 30th day. Sano (1921) noted that the bactericidal titre in the sera of cholera-vaccinated persons reached an

acme in three weeks, while Balteano & Lupu (1914) found that in two persons vaccinated against cholera for the first time the bacteriolytic titre became maximal not sooner than after 56 days.

Though it is generally agreed that the drop of the bactericidal titres setting in after their maximum had been reached and persisted for some time is gradual, observations regarding the length of the period during which bacteriolysins remain demonstrable in the sera of cholera-vaccinated individuals at increased titres gave divergent results. As noted already, Kolle found in some of the persons vaccinated by him conspicuous bactericidal titres even after a period of about 12 months. Sano (1921) noted that these titres fell as low as they were in normal blood-sera within 10 months. Hetsch (1928), summarizing the observations made in this respect during the first World War maintained that as a rule the increase of the bacteriolytic titres persisted not longer than 7-8 months. In the experience of Ahuja & Singh (1948), the level of the bacteriolysins in the sera of cholera-vaccinated individuals was by the 100th day already but slightly higher than before immunization. Papamarku (1917), examining 60 persons at varying times after cholera vaccination, noted that Pfeiffer tests, made with the sera collected from 31 of these individuals between the 11th and 134th day after immunization were positive in 61%, whereas an identical result could be obtained in only 8% of 28 persons tested during a period of from 146 days to about 300 days after vaccination.

The few observations made in regard to changes in the bactericidal titres as a result of re-vaccinations against cholera gave strikingly divergent results. Balteano & Lupu (1914), while maintaining that in the two persons who had received only a single dose of cholera vaccine, a temporary drop of the immune bodies, including the bacteriolysins, was noticeable, stated that in those persons who had received one or two further doses at weekly intervals, no such " negative phase " was present, the bacteriolytic titres rising rapidly and reaching a maximum of 1:150 which was maintained up to the end of the observation period of three months. Karwatzki (1906b) found that the bactericidal power of the sera of 11 individuals which did not greatly increase after the first administration of cholera vaccine, was most markedly enhanced after a second dose had been given five days later. Ahuja & Singh (1948) stated on the contrary that injection of a booster dose six months after the initial cholera vaccination did " not increase the vibriocidal power of the serum to any marked extent compared to the effect produced by the primary stimulus ". Aaser (1910) even noted in the case of one person who had been re-vaccinated with 2 ml of cholera vaccine 20 days after administration of an equally-high initial dose, a rapidly setting-in, but temporary drop of the bactericidal power. It is of great interest that Papamarku (1917), re-vaccinating guinea-pigs three weeks after an initial administration of cholera vaccine, also noted such a drop of bactericidal power, but established that nevertheless most of the animals

in question resisted during the persistence of this " negative phase " intraperitoneal challenge with lethal doses of *V. cholerae.*

Haemolysins

Supplementing the information already furnished in the third of these studies, it has first to be noted that, though giving consistently negative results in standard haemolytic tests, according to some workers the classical cholera vibrios were able to lyse goat and sheep red blood-corpuscles under peculiar conditions. The following observations have to be recorded in this connexion :

Doorenbos (1932) claimed that it was possible to transform through bacteriophage action non-haemolytic cholera vibrios into haemolytic vibrios which thus showed the properties of *V. El Tor.* Having also found that 24-hour-old cultures of non-haemolytic cholera vibrios possessed anti-haemolytic properties, being capable of inhibiting haemolysis of sheep erythrocytes through El Tor vibrios, Doorenbos reached the conclusion that " the presence or absence of haemolytic properties depends solely upon the proportion of haemolytic and anti-haemolytic elements present in the strain in question".

Reporting in a later article upon the examination of 12 cholera strains which had been isolated one to two months previously at Calcutta from the dead bodies of cholera victims and had been forwarded to Alexandria, Doorenbos (1936a) stated that all these strains were incapable of producing haemolysis of sheep erythrocytes after cultivation for 24 hours, but that after incubation for only 8 hours, 4 of the strains exerted a marked and 5 a feeble haemolytic action.

Doorenbos (1936b) further maintained that, in analogy with the findings made in the case of *V. cholerae,* young (6-8 hours old) El Tor cultures showed more marked haemolytic properties than those grown for 24 hours. He also claimed the existence of strains which, on account of their feeble haemolytic properties, stood half-way between the classical *V. cholerae* and the true El Tor vibrios.

Vassiliadis (1935b) stated that cholera vibrios which had been cultivated in broth containing 5 per mille glucose, showed in contrast to those grown in ordinary broth haemolytic properties for sheep erythrocytes and also recorded (1935a) that two originally non-haemolytic cholera strains produced marked haemolysis after they had been passed three times through ordinary broth and subcultivated for a fourth time in glucose broth. Vassiliadis further stated (1935b) that he had been able to produce through immunization of rabbits not only with El Tor vibrios but also when using instead a non-haemolytic cholera strain for this purpose, anti-haemolysins inhibiting the haemolytic properties of filtrates from El Tor cultures. He also claimed that the cholera-immune serum routinely used in his laboratory for agglutination tests neutralized the El Tor haemolysins at the same titre as anti-haemolytic El Tor sera. Vassiliadis concluded from these observations that a non-active haemolytic antigen was present in the classical *V. cholerae.*

Van Loghem (1925), studying 14 cholera and 4 El Tor strains, reached the conclusion that a haemolysin was produced by the latter organisms which reacted like an *exotoxin* : it was soon demonstrable in culture filtrates, was thermolabile and, injected into rabbits, produced an anti-haemolysin, as previously shown by Kraus & Přibram (1906). The haemolysin of *V. cholerae* appeared much later in the cultures and had the character of

an *endotoxin*, being neither thermolabile nor antigenic. It was possible to demonstrate the presence of such an endotoxin also in old El Tor cultures.

Van Loghem recorded in a further communication (1926) that bacteriophage action hastened the liberation of the cholera endohaemolysin : whereas in filtrates of cultures, which had been acted upon by bacteriophage, marked haemolysis was demonstrable in 5-6 days, in the filtrates of cultures subject to autolysis only, this phenomenon became apparent not sooner than after 8 or 9 days.

The observations of van Loghem were confirmed and amplified through interesting studies of Bernard et al. (1939a, 1939b, 1939c).

Bernard et al. (1939a) were able to extract with the aid of ammonium sulfate from 3-day-old El Tor agar cultures and even from 24-hour-old broth cultures or saline suspensions a substance which, re-dissolved in normal saline, proved strongly haemolytic for sheep erythrocytes. It was not possible to extract such an haemolysin from cholera cultures, but a feebly haemolytic substance could be extracted from saline suspensions or broth cultures of *V. cholerae* which had shown evidence of haemolysis after incubation for 5 and 9 days respectively.

In their second note (1939b) Bernard et al. recorded that addition of suitable quantities of El Tor or cholera vibrios to a 2.5% suspension of sheep blood-corpuscles led to the production of a violet colour. Due apparently to a reduction, this phenomenon could not be produced by vibrios which had been killed by heat (56°C) or by alcohol.

Bernard et al. maintained in this connexion that haemolysis of *V. El Tor* was facilitated by an optimum relation between the number of organisms and that of erythrocytes, the violet decolouration remaining absent in this case. Both an excess and too small an amount of blood corpuscles retarded haemolysis. The violet decoloration was marked in the former case, but disappeared as soon as lysis took place.

In a third communication (1939c) Bernard et al. stated that

(1) the exohaemolysin of *V. El Tor* was inactivated by heating for 1 hour at 56°C or for 5 minutes at 100°C, was destroyed by ether or formol and neutralized by cholesterol, but not affected by toluene and activated by lecithin (egg yolk);

(2) the endohaemolysin of *V. cholerae* acted slowly, producing the brown colour of methaemoglobin, was destroyed by 5 minutes heating at 100°C, neither inhibited by cholesterol nor activated by lecithin;

(3) mixtures made in varying proportions of (*a*) the endohaemolytic substance of *V. cholerae* inactivated by heating, and (*b*) the active exohaemolysin of the El Tor vibrio were apt to inhibit the haemolytic action of the latter organism. Haemolysis was apt to take place, if an excess of El Tor haemolysin was used, but led to the production of methaemoglobin.

Bernard et al. reached the tentative conclusion that cholera and El Tor vibrios possessed a common haemolysin which was free in the latter organisms, combined with a neutralizing substance in *V. cholerae*. They noted that with the aid of acetone one could extract from both organisms a haemolytic substance which was soluble in ether and warm alcohol, insoluble in benzene, and thermostable when heated for 10 minutes at 100°C. Emulsified in saline at a pH of 7.2 to 8.0, it haemolysed living vibrios.

This substance, which consisted of water-insoluble fatty acids, appeared to play no role in the usual phenomena of haemolysis.

In analogy with the above findings, Read et al. (1942), through an exhaustive investigation of 62 strains of classical cholera, El Tor and cholera-like strains already referred to in the third of these studies, reached the following conclusions :

" [a] The strains can be divided into two groups: the ' early haemolytic ' and the ' late haemolytic ' organisms, corresponding to Greig-positive and Greig-negative organisms. In the former group, haemolysis is usually complete within a few minutes to 24 hours, is not markedly affected by performing the test at 12°C or under reduced oxygen tension and production of the haemolysin is not affected by exclusion of oxygen. In the latter group, haemolysis is usually partial, hardly occurs in 24 hours and is abolished when the test is performed at 12°C or under restricted oxygen supply.

" [b] Antihaemolytic sera prepared from Greig-positive organisms have a definite specific neutralizing effect on the haemolysin of the early haemolytic group. No similar effect has been demonstrated in the late haemolytic group."

In the opinion of Read et al., the " early " haemolysins were most likely identical with van Loghem's exohaemolysin, the " late " haemolysins with the haemodigestive ferment described by this worker.

Investigating recently three El Tor strains, one identical strain from Celebes, and 10 strains of water vibrios, all but two of which fell into Heiberg's carbohydrate group I, Brück & Brandis (1953) found that all these growths produced a soluble haemolysin, demonstrable in Berkefeld filtrates, but not in Seitz filtrates of 3-day-old broth cultures. Though thermolabile upon prolonged heating at 50°C, application of this temperature for a time sufficiently long to kill the vibrios (e.g., for 15 minutes) did not inhibit the action of this haemolysin for sheep erythrocytes. Likewise, the haemolysin was not inhibited by ultrasonic vibration sufficiently intense to kill the vibrios.

As already referred to in the third of these studies, Zimmermann (1934) was able to establish that most of the classical cholera strains examined by him were capable of producing thermolabile haemolysins for human erythrocytes, whereas the El Tor haemolysins were active not only against these but also against sheep red blood-corpuscles. As noted, these findings have been recently confirmed by De et al. (1954) through an examination of 27 cholera, 2 El Tor, and 14 cholera-like strains. It was found in the course of this investigation that calcium, while inhibiting the haemolytic activity of El Tor and cholera-like vibrios, was essential for that of *V. cholerae*.

When trying to evaluate the above-discussed observations in conjunction with those described in the third of these studies, one may claim that, as far as their behaviour in blood-containing media is concerned, not merely quantitative, but distinctly qualitative differences exist between the classical cholera and the El Tor vibrios. Since, however, the *V. cholerae* is to some extent endowed with haemolytic properties, it appears to be at the same

time more a question of personal bias than of factual evidence whether, as far as their behaviour in blood-containing media is concerned, it is justified to place the two organisms into two different species instead of considering them as variants of one species.

Agglutination

(A) *Identification of suspect strains*

As can be gathered from the publication of Gruber & Durham (1896), these pioneer workers used for the identification of suspect strains with the aid of agglutination tests sera prepared by intraperitoneal immunization of guinea-pigs with killed cholera cultures. Dilutions of these sera in broth and broth suspensions of the organisms to be tested were used for both microscopic and macroscopic examinations. To carry out the former, drops of the diluted sera and of the suspensions were mixed on coverglasses and the latter were mounted on hollow slides. Macroscopic observations (checked if necessary under magnification) were made by mixing 0.5-ml quantities of the diluted test-sera and of the vibrio suspensions. Moreover, a microscopic preliminary test (" Vorprobe ") was recommended for which, instead of drops of suspensions made from pure agar cultures, loopfuls taken from the top-most layer of the primary stool cultures in fluid media were used after the presence of motile organisms had been ascertained. As stated by Gruber & Durham, it was possible under favourable conditions to obtain in this manner a fairly conclusive result within 6 to 10 hours.

Systematic studies in the Berlin Institute for Infectious Diseases led to a considerable refinement and a standardization of the agglutination technique. As summarized by Kolle & Gotschlich (1903), it was found necessary to use for the manufacture of immune sera rabbits or donkeys in preference to horses or goats, the normal serum of which agglutinated cholera vibrios at a higher titre (1:40-1:50) than the rabbit and donkey sera, the titres of which were 1:10 and 1:20 respectively. It was also indispensable to use instead of broth, apt to give inconstant results in successive tests, normal saline for the dilution of the sera and for suspension of the test organisms. Though, as stated in an instruction for the laboratory diagnosis of cholera by Koch et al., originally issued in 1902 and revised in 1904 and 1907 (see text reprinted by Kolle & Schürmann, 1912), it was permissible to use hanging drop preparations (inspected at low magnification and not under oil immersion) as well as macroscopic tube tests for this work, results obtained with the former method could be considered as final only if quite clear-cut (" über allem Zweifel eindeutig "—Kolle & Gotschlich, 1903).

A modification of the agglutination technique introduced soon after these studies was the use of rapid slide tests for the preliminary identifica-

tion of cholera-suspect colonies or growths. As Costa (1912) claimed, without furnishing a reference, Salimbeni was the first to take advantage of this expedient and now amply used procedure in cholera-diagnostic work. To judge from a remark made by Sierakowski (1920a), an early recommendation of this method was also made by Bujwid.

Like Gruber & Durham, Dunbar (1905) suggested a rapid method for the serological identification of *V. cholerae*, according to which mucous particles of suspect stools, emulsified in drops of peptone water on coverslips, were mixed with drops of diluted (1:500) high-titre cholera serum or, for the purpose of control, with normal rabbit serum, diluted 1:50. As claimed by Dunbar, in positive cases the motility of the vibrios was soon inhibited by the specific serum or it was even possible to observe agglutination. However, as stated by Kolle & Schürmann, tests made in the Berlin Institute for Infectious Diseases failed to confirm the value of this method. It was possible to obtain occasional positive results through agglutination tests with drops which had been removed from the peptone-water cultures used for preliminary enrichment after 5 hours' incubation, i.e., by a method analogous to the " Vorprobe " of Gruber & Durham. Similar procedures were also recommended by several subsequent workers without reference to the original method of these two observers.

An alternate method recommended by Bandi (1910) for the rapid laboratory diagnosis of cholera was preliminarily to add adequate amounts of specific serum to 5 ml peptone-water samples filled into special drawn-out test tubes. If these were then inoculated with cholera-suspect stools and incubated at 37°C, according to Bandi's observations in positive cases clumps of agglutinated vibrios could be seen in the lowest part of the tubes after 2-7 hours.[h]

The validity of Bandi's method was endorsed by several observers, and recently by Cossery (1951), who stated:

> " I have used Bandi's test routinely for the examination of cholera-suspect vibrios since 1918.
>
> During this long time I have used it in many thousands of cases. It has always given me a fairly satisfactory result if the two following conditions are observed:
>
> (1) A high-titre serum is used, of which the final dilution in the peptone solution is about 1:100;
>
> (2) The tubes are not shaken while in the incubator or while getting them out for reading."

Parallel tests made by Ghosal & Paul (1951, 1952) with the aid of Bandi's method and with two now available highly-specific media which, as will

[h] It is of historical interest that Achard & Bensaude (1897), besides making direct agglutination tests, used a method similar to that later recommended by Bandi to demonstrate the presence of agglutinins in the sera of cholera patients by cultivating cholera vibrios in 10 drops of broth to which 1 drop of serum of the patient in question had been added. As noted later, an analogous method was also applied by Ransom & Kitashima in 1898.

be described in a later instalment of these studies, give fully satisfactory results if used for direct platings from cholera-suspect stools, failed to confirm the outstanding value of the former test. As established under experimental conditions, Bandi's test was useful in the diagnosis of cholera when *V. cholerae* preponderated in the stools, whereas a preponderance of coliform organisms exerted an adverse effect upon the results. In the actual examination of 285 stool samples the cultural method was found to give 38% more positive results than Bandi's method.

Considering this evidence, the WHO Expert Committee on Cholera (1952)

" came to the conclusion that Bandi's test did not give sufficiently reliable results to recommend its adoption for the laboratory diagnosis of cholera ". [Page 4]

Studying 81 cholera and 31 El Tor strains, Gispen (1937, 1939) found that the O agglutinability of alkaline saline suspensions of the former organisms which had been heated for three hours at 56° C, became as a rule inhibited or at least markedly reduced, whereas El Tor suspensions did not exhibit such a thermolability. The agglutinability of the cholera vibrios could be restored by prolonged heating or by the addition of broth or peptone to the heated suspensions. In Gispen's opinion the differences in agglutinability observed by him could be ascribed to the presence of different proteins in the cholera and El Tor vibrios respectively according to the observations made by Linton (1935).[i]

De Moor (1939) expressed doubts regarding the diagnostic value of Gispen's reaction, stating that it

" may show up striking differences in a great number of cholera and El Tor strains, but the estimation of it in a given case is more difficult than Gispen pretends. Gispen mentions cholera strains that present the phenomenon either not or less distinctly . . . It appeared here that cholera strains which one time had become in- or hardly agglutinable, would another time lose hardly any of their agglutinability in the O-cholera antisera Inaba or Ogawa."

As recorded in 1948 by de Moor's assistant, Tanamal, a difference between cholera and El Tor vibrios could be demonstrated by using a potent cholera O-serum, free from preservatives and diluted 1:200, to which 0.3% of sodium carbonate had been added. Cholera vibrios, if added to such a serum in a dense suspension in distilled water, did not become agglutinated, while the agglutinability of El Tor vibrios was not impaired by addition of the chemical.

It is of interest to add that, as also noted by Tanamal, cholera vibrios added in a dense suspension to a 0.5% solution of sodium bicarbonate, to which after 15 minutes

[i] As stated in a footnote to an article by de Moor (1949), F. H. Meyer claimed in a thesis published at Amsterdam in 1939 to have observed differences in agglutinability identical with those recorded by Gispen, when treating suspensions of cholera and El Tor vibrios with 2% chloroform.

an equal volume of 0.5% mercuric chloride was added, became precipitated, whereas El Tor vibrios remained in suspension.

Sharing the opinion of Gispen, de Moor (1949) ascribed the above-mentioned differences between cholera and El Tor vibrios to the different nature of their protein fractions.

(B) *Tests with human sera*

As noted already, Achard & Bensaude reported soon after Gruber & Durham had recommended agglutination tests for the identification of unknown cholera strains, that this method could be used as well to demonstrate with the aid of known cholera cultures the presence of agglutinins in the sera of cholera patients.

The initial observations of these two workers have been followed up and amplified by numerous investigators, who have devoted attention to the presence of agglutinins not only in the sera of cholera patients and convalescents but also in normal persons, including those who had been vaccinated against cholera, and in carriers. The results of these studies may thus be summarized:

(1) *Normal (cholera-free) individuals.* As summarized by Karwatzki (1906b), and Greig (1915), in the experience of the earlier workers the normal sera of cholera-free and non-vaccinated individuals almost invariably agglutinated the *V. cholerae*, if at all, at titres not exceeding 1:10 or at most 1:20. Exceptions to this rule have remained extremely rare, Krishnan & Dutta (1950) being apparently the only observers definitely stating that they had observed once a higher titre (1:80) in a " normal " group of 18 persons tested before they received cholera vaccination. Three other members of this group showed agglutinating titres of 1:10 only, while the sera of the 14 failed to agglutinate either Inaba or Ogawa suspensions.

(2) *Cholera patients and convalescents.* The following of the fairly numerous observations on the presence of agglutinins in the sera of cholera patients or convalescents deserve attention for the purposes of the present disquisition.

Achard & Bensaude, reporting in 1897 on a total of 14 observations, stated that they had found agglutinins in 13 of these instances—12 times during the stage of attack (first to sixth day), when the agglutination titres ranged from 1:25 to 1:50, and once in a convalescent examined for the first time on the 28th day after the onset of cholera and then showing a titre of 1:120.

The first studies on somewhat larger groups of cholera patients or convalescents seem to have been published in 1909 by Amako, Kopp and Svenson.

Amako (1909) tested the sera of 58 cholera-affected individuals with the aid of tube tests, adding 2 mg respectively of a cholera culture to 3 ml of the various serum dilutions, and reading the results after an incubation at 37°C for 3 hours. While results during the first week after onset were negative, the agglutination titres became maximal during the second week and then decreased. The titres ranged in slight cases from 1:40 to 1:80, in

moderately severe cases from 1:20 to 1:640, in severe cases from 1:160 to 1:640. No agglutination was observed in cases ending fatally or in comatous patients.

Kopp (1909), studying 32 patients admitted to a hospital in St. Petersburg, Russia, obtained positive results with agglutination tests in 26, the titres usually ranging from 1:10 to 1:50, but rarely reaching 1:100 and usually becoming maximal in the second or third week after onset. No relation seemed to exist between the character of the attacks and the height reached by the agglutination titres.

Svenson (1909), testing 37 sera of cholera patients or convalescents with a technique similar to that of Amako, but taking readings already after incubation for one hour and again after the tubes had been kept at room temperature overnight, obtained only 13 positive results. The agglutination titres, which seemed not to be influenced by the character of the cholera attacks, remained invariably low, reaching or approaching 1:50 in 8 instances, 1:25 in 5 instances. Positive results were obtained with specimens taken between the fifth and 60th day after onset of the disease, but only in rare instances before the 10th day. Evaluating his own and Kopp's results, Svenson emphasized that the agglutinatory power of the sera from cholera patients or convalescents " is apt to be low and frequently not different from that of normal human sera ".

In contrast to this postulation of Svenson, some other of the earlier workers observed occasionally that the sera of cholera convalescents agglutinated *V. cholerae* at higher titres, the maxima recorded by Liveriato (1914) and Kabelik (1915) being 1:5,000.

Salimbeni (1915), examining 27 cholera patients or convalescents, laid stress upon the fact that the sera of six of these individuals, who could be tested during or immediately after the attack, had neither agglutinating nor protective properties. He concluded, therefore, that recovery from cholera took place before antibodies had appeared in the blood-stream. However, though this postulation deserves attention, it has to be kept in mind that some of the workers mentioned above as well as some quoted below noted in part an increased agglutinin response during or soon after the acute stage of the disease.

Commencing important observations on the presence of specific agglutinins in the sera of cholera patients or convalescents, Greig (1913a) found that two convalescents who continued to harbour cholera vibrios in their stools for considerable periods, also produced positive agglutination reactions with their sera, whereas results of the latter test were negative in convalescents whose stools had become free from *V. cholerae*.

In 1915, Greig published the results of an examination of 363 sera derived mainly from cholera patients and convalescents, and to a lesser extent also from individuals in the stools of whom no *V. cholerae* had been found. To carry out agglutination tests with these sera, mixtures were made in capillary tubes of equal amounts of the serum dilutions to be tested and of suspensions of cholera vibrios in 0.85% saline, the results being read after an incubation at 37°C for two hours. It was established through comparative tests that results of the agglutination tests were identical regardless of whether standard cholera strains or the homologous vibrios isolated from the convalescents in question were used.

The outcome of Greig's large-scale study may thus be summarized:

Nature of sera tested	*Number of sera*	*Result of agglutination tests*
From fatal cholera cases	64	Agglutinins absent in about half of these individuals even though some of them survived for 4 to 12 days. In the 23 positive cases agglutinins appeared comparatively late and almost invariably the titres did not exceed 1:40.
From recovering cholera patients	210	Agglutinins appeared rapidly, in some instances as early as the second or third day of illness, and became as a rule well marked by the sixth day from onset. The titres, which varied from 1:400 to 1:1,000, remained high up to the 17th day. As far as could be gathered from scanty observations, a drop became marked about the 20th day.
Patients from whose stools both cholera and cholera-like vibrios had been isolated	18	Agglutination was positive only with *V. cholerae* but not with the cholera-like vibrios in question.
Individuals in whose stools only cholera-like vibrios had been found	35	Agglutination tests with the homologous cholera-like strains were negative but in some instances positive results were obtained with *V. cholerae*, the individuals in question having obviously suffered from an unrecognized cholera attack.
Individuals in whose stools no vibrios had been found	36	Apparently for the reason stated above, some positive results were obtained in agglutination tests with *V. cholerae*, the titres at which reactions took place in this and the preceding group never exceeding, and but exceptionally reaching, 1:100.

Though realizing that agglutination tests were of little, if any, value for the diagnosis of acute cholera attacks, Greig stressed on the basis of the above-recorded observations the importance of this method for a retrospective diagnosis of the disease.

Shiiba & Oyama (1920), making agglutination tests with the sera of 97 convalescents, found that as a rule a higher titre was reached after 2-3 weeks in the Japanese convalescents (1:80 to 1:640) than in the Chinese tested, in whom the titres ranged from 1:40 to 1:160. Only 7 out of the 97 persons composing this series had titres above 1 : 1,000 with a maximum of 1:5,120 in one instance.

Tagami & Watanabe (1920), testing a series of 91 cholera convalescents, found agglutination to become positive in 87%, usually between the third and tenth day after the onset of illness, rarely later up to the 15th day. The agglutination titres ranged usually from 1:100 to 1:400, but exceptionally high titres up to 1 : 10,000 were said to have been met with occa-

sionally. Individually, the highest titres were usually reached in 1-2 weeks, then a decrease set in, which was gradual at first but became more rapid after a week so that as a rule agglutination became negative one month after onset of the disease.

As referred to before, Ukil (1928) found that 25 out of the 30 cholera convalescent sera examined by him reacted positively in agglutination tests, the maximal titres being 1:100 in six instances, 1:500 in nine, and 1:1,000 in ten. Supplementing this information in 1930, Ukil & Guha Thakurta stated that 15% of their convalescent sera failed to agglutinate *V. cholerae*, while 20% reacted at titres below 1:800, 45% at titres ranging from 1:800 to 1:1,600, and 20% at a titre of 1:3,200.

The studies on the agglutination reactions observable in cholera patients and convalescents by Pasricha et al. (1939) are of particular value because, in contrast to the previous investigators, these workers could base their observations on the recent experiences made in regard to the antigenic structure of *V. cholerae* and the importance of the O antigens in immunological reactions.

Pasricha et al. used for their agglutination tests, made in Dryer's tubes, suspensions of young cholera cultures to which 0.2% formol had been added as H antigens, and boiled saline suspensions of *V. cholerae* as O antigens. Final readings were taken after the tubes had been kept for 18 hours at 55° C in a water bath.

Results of H and O agglutination tests made in this manner with the sera of 175 cholera patients (bacteriologically confirmed and non-fatal cases) were recorded by Pasricha et al. as follows:

Day of illness	*Number examined*	*Number with cholera agglutinins*	*Percentage showing H agglutinins*	*O agglutinins*
1st	19	0	0	0
2nd	13	0	0	0
3rd	17	0	0	0
4th	8	4	50	12
5th	8	4	50	37
6th	10	5	50	50
7th	13	8	61	46
8th	20	18	90	55
9th	24	18	71	60
10th	23	17	74	57
11th	9	8	90	66
12th	11	9	81	73
Total	175	91	52	

It will be gathered from this table that

(1) No agglutinins could be demonstrated in the sera of 49 patients of this series examined during the first three days of illness.

(2) Becoming first manifest in the patients seen on the fourth day of illness, agglutinins were present in an on-the-whole increasing number of the sera from the seventh day of illness onwards.

(3) O agglutinins appeared more tardily and were throughout the observation period present in the sera in a lesser percentage than the H agglutinins.

It is in accord with this last observation that, as shown elsewhere in the article by Pasricha et al., the maximal titre at which O agglutination took place was 1:320 as against 1:640 in the case of H agglutination and that the average titres of O agglutination were considerably lower than those of H agglutination.

Further interesting points elicited by these workers were that:

(*a*) In contrast to the postulation of Greig, agglutinins were found to be better developed for the homologous strains (isolated from the faeces of the patients in question) than for the standard Inaba strain of *V. cholerae* used, agglutinins for the latter being absent in 11 of the 58 sera examined in this respect.

(*b*) However, in 9 bacteriologically-proven cases agglutinins, almost exclusively such of the H type, were found for the standard strain, and no agglutinins for the homologous strains—a phenomenon for which "no satisfactory explanation" could be advanced.

(*c*) In conformity with Greig's findings, in patients showing both cholera and cholera-like vibrios in their stools, agglutinins were demonstrable only for *V. cholerae*. Such agglutinins were also found in a few patients harbouring only cholera-like vibrios in their stools as well as in some patients with clinical signs of cholera but negative bacteriological findings.

In regard to the last-mentioned point, it is, however, important to add that, according to further observations recorded in the 1941 report of the Indian Research Fund Association, in some instances where both cholera and cholera-like vibrios had been isolated from the stools, agglutinins for both kinds of organisms could be demonstrated in the sera of the patients in question.

The important aim of the investigations of Yacob & Chaudhri (1945) was to establish for how long agglutinins persisted in the sera of cholera convalescents—a subject which, in spite of its great importance for a retrospective diagnosis of the disease, had received but little attention in the past owing to the difficulty of observing the convalescents for prolonged periods. Yacob & Chaudhri procured for this purpose sera of persons who had suffered from cholera during epidemics taking place in the three Indian localities in question some months before.

Describing their technique, the two workers stated that they mixed 1/25, 1/50, 1/100, 1/150 and 1/200 dilutions of their sera in normal saline in Dreyer agglutination tubes with equal amounts of a suspension of live cholera vibrios, which had been found to react positively with an Inaba O serum. The tubes were kept in the water bath at 56°C for 2 hours and then transferred to a refrigerator, readings being taken 24 hours after setting up the test. Results were confirmed by slide agglutination tests.

The findings made by Yacob & Chaudhri may be summarized as follows:

Locality	*Interval between end of outbreak and date of test*	*Number of specimens examined*	*found positive*	*Titres*
Kasur town	about 2 months	11	6[a]	1:50 to 1:150
Narli village	102 days	11	6	1:25 to 1:150
Lahore city	about 3 months	15	1[b]	1:100

[a] The interval elapsing between the dates of attack and of serological examination varied from 60 to 64 days.

[b] Tested 113 days after the time of attack.

Yacob & Chaudhri concluded, therefore, " that agglutinins can persist in the blood of recovered cases of cholera up to a period of three-and-a-half months and possibly more "—a postulation supported by the observations of some earlier workers, such as Kabelík (1915).

(3) *Healthy carriers.* While a number of observers, such as de Bonis (1912) and Sano (1921), found specific agglutinins to be absent or practically absent in the sera of healthy cholera carriers tested by them, positive findings have been recorded in this respect by some other workers, for instance—as has been mentioned earlier in this study—by Massaglia (1911) and Levi della Vida (1913), and also in a few instances by Shiiba & Oyama (1920). As quoted by Takano et al. (1926), Sakai (1917) found that one third of the 84 sera of healthy carriers tested by him agglutinated *V. cholerae* at titres over 1:200, the highest titre observed in this series being 1:2,000. As a rule, the individual carriers showed maximal titres from four to nine days after detection of cholera vibrios in their stools, but sometimes the highest titre was reached already on the day of the first positive findings in the faeces or as late as almost two weeks afterwards. Agglutination became negative on the average within 15 days but the reaction could remain positive for 32 days. There was no definite correlation between the length of vibrio excretion by the carriers (which varied from one to 21 days with an average of eight days) and the height of the titres, but as a rule these were highest in the carriers with the longest excretion periods.

(4) *Vaccinated subjects.* After early observations by Bertarelli (1905), Karwatzki (1906b), and Serkowski (1906) had shown that cholera vaccination led to a marked agglutination response in the sera of the individuals concerned, this problem was further studied by numerous observers. The following of the earlier findings recorded in this respect deserve particular attention:

Balteano & Lupu (1914) concluded from rather limited experiences that:

" [a] In individuals who had received a single injection, agglutinating power becomes manifest already after 24 hours (1/20). 3 days afterwards this falls to 1/10, to raise again at the end of 48 hours. It reaches a maximum (1/130) after 24 days, then decreases gradually to 1/100 and remains at this level still two months after injection;

"[b] A similar evolution takes place in individuals who received 2 or 3 injections, except that then the titre reaches 1/150 and remains at this level for 7 days before it begins to decrease."

It has to be added that soldiers in the field who had been vaccinated, retained an agglutinating power of 1/40 for 3½ months. At the end of 5 months their titre was not higher than 1/20-1/30.

Castelli (1917) noted that, while administration of the usual cholera vaccines as well as that of cholera nucleoproteid led to an increase of the bacteriolytic titre in the sera of the vaccinated, an agglutinin response was observable only if the usual vaccines had been administered, but not after nucleoproteid had been given.

Sierakowski (1920a), studying a total of 259 individuals, to whom differently-prepared cholera vaccines had been administered, established that differences in the methods of killing these products exerted a marked influence on the agglutinin levels observable in the various groups after vaccination. He further found that (*a*) in three persons tested before and 48 hours and 96 hours, respectively, after vaccination, higher agglutination titres became manifest within two days after the vaccine had been given; (*b*) in a group of 13 persons the agglutination titres, which had reached values observable in normal persons 6 months after the administration of two vaccine doses, were found to have risen 5-7 days after two booster doses had been given, without, however, reaching higher values than after the original two injections; and (*c*) 6 months after administration of the booster doses the agglutination titres had sunk once more to levels observable in non-vaccinated individuals.

In the experience of Sano (1921), the agglutination titres in cholera-vaccinated persons reached a maximum in 3 weeks and fell in 10 months to the levels found in non-vaccinated controls.

Hetsch (1928), summarizing the results of these and other observations made during and soon after the first World War, stated that though as a rule an agglutinin response was elicited in the sera of cholera-vaccinated individuals, which became maximal after 2-4 weeks and remained manifest for 6-10 months, or according to some workers only for 3-4 months, the titres found varied considerably. Hetsch was inclined to ascribe these divergent results on the one hand to variations in the dosage in which the vaccines had been given and to the number of injections administered, on the other hand to differences in the technique of the agglutination tests. However, the observations of Sierakowski (1920) and further experiences referred to later in this study leave no room for doubt that differences in the antigenic value of the various cholera vaccines exert a profound influence on the agglutinin response following their administration. It is certain at the same time that the height of the titres is markedly influenced by differences in the agglutination technique. Particularly, as stressed by Ionesco-Mihaiesti & Ciuca (1916) and confirmed by further experiences, fully satisfactory, and therefore comparable, results can be hoped for only when live cholera vibrios and not killed antigens are used for agglutination tests with the sera of the vaccinated.

Recent contributions to the knowledge on the problem presently under review were as follows:

Griffitts (1944), to whose observations on the appearance of mouse-protective bodies in the sera of cholera-vaccinated individuals reference will be made later in this study, also noted that agglutinins appeared in these sera one week after vaccination, remained

at high titre (1:180 to 1:1,620) for 1-2 weeks and then diminished, the majority of the sera reacting at low titre 6 months, one year and 18 months after vaccination.

Eisele et al. (1946), testing the sera of (*a*) 7 individuals who had been inoculated twice with a standard cholera vaccine, and (*b*) 27 persons injected twice with different doses of an experimental vaccine which contained mainly the O antigen of *V. cholerae*, found that specific agglutinins became demonstrable at an equally high titre in both groups and also noted that administration of larger doses of the experimental vaccine did not lead to an increase of the agglutination titres.

Observations made by Gohar & Makkawi (1947) during the 1947 Egyptian outbreak showed that the sera of individuals who had been given a vaccine prepared from the autochthonous Inaba-sub-type cholera strain, agglutinated this as well as classical Inaba strains to a titre of 1:160, and Ogawa strains to half that titre (1:80).

In the course of interesting investigations, to which further reference will be made later in this study, Singer et al. (1948b) studied the presence of agglutinins in the sera of 211 cholera-vaccinated individuals 10 days after completion of the immunization. Administration of four different types of vaccines led to a fairly high agglutinin response, the titres often reaching, and sometimes exceeding, 1:320. While the methods of producing these various vaccines did not seem to exert an appreciable effect on the titres which were attained, the agglutinin content of the sera from subjects vaccinated by the intracutaneous route was found to be significantly higher than was the case in the subcutaneously vaccinated individuals.

Ahuja & Singh (1948), to whose observations on 21 persons given 1 ml of cholera vaccine reference has been made before, found that, in marked contrast to the rise of the bactericidal titre in these individuals, the agglutination titres did not become considerably heightened, the maxima reached on the 10th day after vaccination being 1:125 to 1:250 as compared with a pre-vaccination level of 1:25.

Erdim (1951), examining the sera of 67 individuals 8 weeks after they had been given two doses of a cholera vaccine containing 8,000 million of organisms per ml, found even lower agglutination titres with a maximum of 1:160 in 46 of these individuals, 21 giving negative results.

Observations by Brounst & Maroun (1949) showed that

(*a*) in a group of 371 individuals, inoculated once with 2,000 million of an Inaba-Ogawa cholera vaccine and tested 30-60 days afterwards with formolized suspensions (2 per mille) of *V. cholerae*, agglutination titres ranging from 1:25 to 1:200 were demonstrable six times only, 365 of these sera failing to produce agglutination even at a titre of 1:25;

(*b*) 95 of these individuals, who received 50 days after the first vaccination two booster doses totalling 12,000 million *V. cholerae* and whose sera were tested as described above, gave similarly disappointing results, agglutination at titres ranging from 1:25 to 1:100 being found only in 11 instances;

(*c*) somewhat better results were obtained when suspensions of live cholera vibrios, cultivated for 24 hours at 37°C, were used instead of formolized suspensions for the tests: the sera of 20 individuals, vaccinated and examined as the persons composing group (*b*), yielded 7 positives (i.e., 35%), the titres ranging from 1:25 to 1:200.

Further comparative tests by Gallut & Brounst (1949) with the sera of 18 individuals, vaccinated and examined as those of the above groups (*b*) and (*c*), confirmed that, except when testing unknown cholera strains with the aid of high titre rabbit sera, it was not permissible to use formolized *V. cholerae* suspensions for agglutination tests. While obtaining as unfavourable results with the latter suspensions as Brounst & Maroun, Gallut & Brounst recorded 55% and 77% positive results when testing their 18 human sera with live suspensions of an Inaba and an Ogawa strain respectively, the titres ranging from 1:20 to 1:500.

It will be noted that these findings fully support the conclusion reached in 1916 by Ionesco-Mihaiesti & Ciuca.

(5) *Test-vaccination for retrospective cholera diagnosis.* At a meeting of the Joint OIHP/WHO Study-Group on Cholera held in 1949, Krishnan & Dutta (1950) recorded the following results obtained when administering 1 ml cholera vaccine to the individuals enumerated below and testing the agglutination titre of their sera two weeks afterwards:

		Number tested	*Agglutination negative*	*Titres of positive agglutination* 1/20 or less	1/18 or less	1/320 or less	1/1280 or less	1/2560
Normal group	A	18	14	3	1	.	.	.
	B	16	2	3	10	1	.	.
Inoculated group	A	132	58	31	41	2	.	.
	B	119	6	11	44	58	.	.
Cholera group [convalescents]	A	17	.	.	4	9	3	1
	B	4	.	.	.	1	3	.

A = *before* inoculation.
B = *after* inoculation.

Bearing in mind the scantiness of the evidence available in regard to the group of cholera convalescents, the Study Group recommended further observations in order to decide whether this method of test vaccination might be a useful means for the retrospective diagnosis of cholera. As was reported at the first session of the WHO Expert Committee on Cholera in 1951, further investigations had not confirmed the usefulness of the above method for the retrospective diagnosis of individual cholera cases. It was considered possible, however, that the procedure might be helpful in establishing the nature of infection in *groups* of people who, giving a history of diarrhoea and vomiting, were suspected to have recently suffered from cholera. The conclusion arrived at by the Committee was

" that the method thus far had not given results of definite practical value. Should work in this direction be continued, attention ought to be given to the possible role of cross-reactions due to the presence of brucella or salmonella infections. For this reason and on general grounds great attention ought to be paid to the advisability of using in addition vibriocidal tests for the retrospective diagnosis of cholera." [Page 5]

The usefulness of the method of test vaccination for the retrospective diagnosis of cholera in groups of suspects was recently upheld by Lahiri & Dutta (1954), who drew attention to the following figures:

		Number tested	*Percentage negative*	*Percentage positive at 1 : 160 or less*	*Percentage positive at 1 : 320 or above*
Bacteriologically confirmed cholera cases	A	94	2	46	52
	B	29	0	28	72
Clinically diagnosed cholera cases	A	26	15	50	35
	B	10	0	50	50

A = *before* test vaccination.
B = *after* test vaccination.

(C) *Paragglutination and co-agglutination*

As summarized by Sierakowski (1920b) in an important study devoted to the problem of "Mitagglutination" in cholera, Karwatzki (1906a) reported that he had met with some cholera-like vibrios which agglutinated at fairly high titres with cholera-immune serum, but could be distinguished from *V. cholerae* with the aid of Pfeiffer's reaction—results which are not surprising when the inadequacy of the sera then available for agglutination tests is considered.

Sierakowski himself found among the numerous vibrio strains isolated by him during the 1914-15 cholera outbreak in Galicia and Poland six strains of water vibrios and one derived from the faeces of a cholera convalescent, which showed morphological, cultural, and biochemical properties compatible with those of *V. cholerae* and agglutinated at fairly high titres with sera obtained by cholera immunization of horses, in two instances also with specific rabbit sera. However, while one of these seven strains reacted like *V. cholerae* in complement-fixation tests, none gave a specific Pfeiffer reaction. Moreover, the sera raised against the seven strains did not agglutinate cholera vibrios and, while the latter were found to be capable of absorbing the agglutinins for the seven strains from cholera-immune sera, the reverse did not hold true, the suspect vibrios leaving the cholera agglutinins intact, if allowed to absorb cholera-immune sera.

Trying to interpret these findings, Sierakowski postulated that the agglutinating sera contained besides main agglutinins a series of minor agglutinins ("Mitagglutinine"), capable of acting upon heterologous bacterial species. The latter, when brought into contact with the agglutinating sera, absorbed only the minor agglutinins, whereas the homologous organisms absorbed the main agglutinins as well. Sierakowski emphasized, therefore, the importance of agglutinin-absorption tests for a differentiation of cholera-like vibrios showing some serological relationship with *V. cholerae* from the latter organisms. It has to be stressed, however, that in the case of his as well as of Karwatzki's strains equally clear-cut differences from *V. cholerae* were revealed by Pfeiffer's reaction.

Observations indicating that organisms belonging to heterogenous bacterial genera may be agglutinated by cholera-immune sera seem to have been made first by three German workers in 1916:

Meggendorfer (1916) isolated from the faeces of a healthy soldier a large motile bacillus which was agglutinated to full titre by cholera-immune serum, but whose homologous serum exerted no action on *V. cholerae*. Results of agglutination tests with cholera-immune serum remained unchanged after the bacillus in question had been subcultivated 74 times. In view of these findings, Meggendorfer stressed the necessity of verifying the vibrio nature of the strains to be serologically tested in cholera-diagnostic work.

As quoted by Meggendorfer, Quadflieg (1916) cultivated from the stools of a cholera suspect an *E. coli* strain which, though not reacted upon immediately, was after 4 hours agglutinated by cholera-immune serum at titres nearly corresponding to those found in the case of *V. cholerae*.

Examining roughly 1,000 stool-specimens, Messerschmidt (1916) found in about 20—partly in association with cholera vibrios—bacteria belonging to the *E. coli*, *Proteus*, or *Sarcina* groups, which became agglutinated at full titre by cholera-immune sera and

retained this property after repeated subcultivation, as far as observed for 4 months. Like Meggendorfer, he stressed the importance of a preliminary smear examination of the growths to be tested serologically, as prescribed in the official German instructions for cholera diagnosis.

Following up an earlier observation by Wong (1936) on two individuals who, to treat eye diseases, had been injected intravenously with cholera-typhoid vaccine, Wong & Chow (1937)

(*a*) demonstrated the presence of group agglutinins for *V. cholerae* and *Brucella abortus* in rabbits immunized by the subcutaneous or the intravenous route with either species or with cholera-typhoid vaccine;

(*b*) injecting six human subjects either intravenously or subcutaneously with this vaccine, found that while the sera of all six individuals agglutinated cholera vibrios at titres of maximally 1:160, 4 of the sera also agglutinated *Br. abortus* at titres of maximally 1:40.

The validity of these initial findings was fully confirmed through observations on larger groups of cholera-vaccinated individuals by Eisele et al. (1946, 1947, 1948) and by Erdim (1951). That the Brucella agglutinins developing in the sera of such persons—often at higher titres than observed by Wong & Chow—are apt to persist for considerable periods is well illustrated by the records of Eisele et al. (1947) who demonstrated the presence of these agglutinins in 27% of the members of a group tested 18 to 28 months after cholera vaccination.

Following up and amplifying laboratory studies by Eisele et al. (1946), McCullough et al. (1948) reported that :

" Reciprocal agglutinin-absorption tests on antiserums for *V. comma* and the brucella species (*abortus, suis, melitensis*) showed conclusively that the antigen shared by these groups of organisms is an H antigen of *Vibrio comma.* This antigen is present in all three species of brucella with minor qualitative and quantitive differences."

In strict contrast to this conclusion, Gallut (1950) stated that the cholera vibrio and the Brucella species possessed common O antigens, found to correspond to the antigenic factors C and D of *V. cholerae* for the strain of *Br. suis* tested, and to the factor D only in case of the two *Br. melitensis* strains examined. Recording the experiences he made when testing these brucella strains with cholera-immune sera, Gallut noted that

" It is known that for cholera diagnosis agglutination tests must be made with unheated vibrio suspensions, preferably those of living vibrios [Gallut & Brounst, 1949]. By this technique the El Tor vibrios cannot be differentiated from the authentic cholera vibrios. However, their different chemical composition (protein I of Linton for the *V. cholerae*, protein II for *V. El Tor*), produces differences in their serological behaviour after heating for 3 hours at 56°C. We submitted *Br. abortus* suspensions . . . to such heating and found that these organisms behave like true cholera vibrios and not like El Tor vibrios. This has been confirmed by the use of a serum raised against the protein of *V. El Tor* which failed to agglutinate *Br. abortus suis.*" [Trans.]

In a further paper, Gallut (1953c) stated that he had studied the question of whether a pure A-type cholera-immune serum could be obtained by the

absorption of a serum raised against the Inaba variant of *V. cholerae* (which according to him had the antigenic formula A C) with *Br. suis*. It was found that the latter organisms were incapable of fully absorbing the C antibody of Inaba serum.

In their valuable study on the laboratory aspects of the 1947 cholera-outbreak in Egypt, Gohar & Makkawi (1948) noted that on several occasions suspicious colonies which gave positive results in slide agglutination tests with a cholera O serum, were ultimately identified as *B. faecalis alkaligenes*. Following up these observations, Gohar & Makkawi confirmed not only that *B. faecalis alkaligenes* strains were agglutinated at a titre of 1:25 by cholera-O serum but established also that *V. cholerae* was reacted upon at the same titre by immune sera raised against *faecalis alkaligenes* strains. However, when absorption tests were made, neither these organisms nor cholera vibrios were found capable of completely absorbing the agglutinins from the heterologous sera. It appeared, therefore, that the organisms concerned " merely shared an O-antigenic fraction ". Gohar & Makkawi also referred to previous observations by one of them which had shown that *V. cholerae* shared an H-antigenic fraction with *Salmonella enteritidis*.

A further study of the antigenic relationship existing between the Inaba strains of *V. cholerae* isolated in Egypt and *S. enteritidis* was made by Felsenfeld (1948). He concluded from agglutination and agglutinin absorption tests made with 3 Egyptian cholera strains and various Salmonella strains, respectively with Inaba H-O and Salmonella O and H sera, that the *V. cholerae* strains contained fractions of the Salmonella antigens I, XII and g.

A more general investigation of the serological relations existing between *V. cholerae* and common Enterobacteriaceae was undertaken by Felsenfeld et al. (1951). Making slide agglutination tests with cholera H-O sera, they obtained the following results with various faecal strains isolated at Chicago:

Organism	*Number tested*	*Number positive in agglutination tests with H-O sera raised against V. cholerae*	
		Inaba	*Ogawa*
E. coli	70	11	2
A. aerogenes	134	26	1
Paracolobactrum	5	1	0
Proteus	29	2	0
Pseudomonas	55	2	0
Faecalis alkaligenes	6	0	0
Streptococcus	16	7	2
Total	315	49	5

Absorbing Inaba H-O serum with *Brucella melitensis*, *Br. suis*, *S. enteritidis*, *E. coli*, *A. aerogenes*, and *P. morgani* strains, Felsenfeld and co-workers found

" a fraction of the cholera ' H ' antigen to be identical with the factor common to the ' g.m. ' antigen of Salmonellae and a part of the Brucella flagellar antigen, while a

common ' O ' antigen component was found to be part of the I. IX. XII antigen of Salmonellae ".

Changes in agglutinability

In order to do full justice to the problem of whether agglutinability by the usually available specific sera is an unalterable characteristic of *V. cholerae*, permanently distinguishing this organism from the cholera-like vibrios, it is necessary to scrutinize not only whether authentic cholera vibrios may lose their agglutinability by the above-mentioned sera, but also whether agglutinability by such sera may be acquired by vibrios initially giving negative reactions in this respect.

Loss of agglutinability. Apart from Bordet, who claimed in 1896 that animal passage could render the cholera vibrio inagglutinable with specific serum, Ransom & Kitashima (1898) seem to have been the first workers to have devoted attention to the subject presently under review. They noted that, in contrast to its agar-grown parent strain, a sub-strain of *V. cholerae*, which had been passed twenty times through broth containing 1% cholera-immune serum, exhibited feeble or even no agglutinating power, if incubated for 24 hours in 10-ml amounts of the same medium with a specific serum content of 1%-2%.

The original observation of Ransom & Kitashima that growth in the presence of its homologous serum is apt to impair or to inhibit the agglutinability of *V. cholerae*, has been repeatedly confirmed by subsequent observers. There can be no doubt that a transition into the rough state is responsible for this well authenticated alteration.

Some workers in Russia, particularly Zlatogoroff (1909, 1911) and Horowitz (1911), seem to have been the first to assert that *V. cholerae* was apt to lose its specific agglutinability in the human intestine or in water supplies, and a few other observers such as Barrenscheen (1909) and Puntoni (1913b) confirmed the findings made in the latter respect by Zlatogoroff. However, several other investigators, such as Haendel & Woithe (1910), Köhlisch (1910), Wankel (1912), and Bindi (1913), though working in part with the strains and even with the diagnostic serum of the Russian observers, were unable to confirm the findings of the latter. Stamm (1914) found that several of the cholera strains tested by him no longer became agglutinated with cholera-immune serum after they had been repeatedly passed through water, while others remained unaltered even after more frequent passages. The strains which were no longer agglutinated, were almost without exception still immunogenic, i.e., capable of producing immune sera positively reacting with cholera vibrios. Since in Stamm's opinion variations of *V. cholerae*, including changes in agglutinability, were not easily effected

under natural conditions but, once they had taken place, were apt to be permanent, he declared that

" consequently it is impossible to explain the rise, cessation and recrudescence of cholera epidemics through the hypothesis of a transmutation of the cholera vibrios into saprophytic variants and *vice-versa* ".

Similar views were vigorously expressed by the orthodox German school, Kolle (1909b) even maintaining that any " non-agglutinable " vibrio found in human stools was not a cholera vibrio. It has to be noted, however, that this extreme view was reached at a time when the phenomena of dissociation were still unknown. Indeed, it would seem that Finkelstein (1931) was the first worker inclined to ascribe the loss of agglutinability by *V. cholerae* after a sojourn in water to a transition from the smooth into the rough state.

Finding that, if cholera stools were added to Indian tanks (water reservoirs), *V. cholerae* invariably lost its agglutinability with specific serum within 16-20 hours, Tomb & Maitra (1926) were led to consider the " agglutinating " faecal vibrios and the " non-agglutinating " organisms met with in the tanks, and also in human carriers, as identical in character. The far-reaching conclusions these two workers drew in strict contrast to Stamm's views from these and related observations, will be discussed later on.

Brahmachari (1927, 1929), to whose work also further reference will be made below, maintained like Tomb & Maitra that passage through water rendered the cholera vibrios inagglutinable with specific serum and also claimed that intravenous injection of rabbits with *V. cholerae* led to the appearance of inagglutinable vibrios in the stools of the animals—a result which in view of the ubiquity of cholera-like vibrios in India ought to be considered as a *post hoc* rather than a *propter hoc* phenomenon.

Minervin (1931), in order to study the changes *V. cholerae* was apt to undergo when introduced into specifically immunized animals, injected typical cholera vibrios into the testicles of cholera-immune rabbits and excised these organs 3-10 days later. In three instances it was possible to isolate from the excised testicles cultures of vibrios which had obviously undergone roughening. Agglutination tests with these passage strains gave at first negative results, but it was possible to restore agglutinability in one instance through repeated subcultivation, and in the other two through animal passage (intravenous injection of rabbits).

D'Hérelle et al. (1930) observed some instances of inagglutinability with specific-immune serum in the case of cholera vibrios isolated from the faeces of convalescents, and suggested that this change was the result of bacteriophage action. Referring to these observations, Vardon (1940) expressed the opinion that the corresponding changes noted by Tomb & Maitra (1926) in the case of faecal vibrios exhibited in water tanks might

have been due to the same cause. Morison (1932), while admitting that " the bacteriophage has something to do with the production of rough from smooth vibrios and *vice versa* ", added that he had " been unable in the case of cholera to make agglutinating vibrios inagglutinable by growing them in the presence of bacteriophage ". However, Doorenbos (1932) adduced evidence to show that the presence of bacteriophage was apt to alter the agglutinability of cholera vibrios and it would seem that Morison (1935) obtained the same result when using instead of pure-line bacteriophage-strains combinations of different races.

Yang (1935), keeping cholera vibrios derived from one strain in the dark at room temperature in raw, candle-filtered, and autoclaved water samples respectively, found that the agglutinability of the organisms became lost in the raw and filtered river-water samples as well as in the raw canal- and well-water samples after periods ranging from 21 to 28 days.

The present writer, on the contrary, when studying the survival of cholera vibrios in numerous filtered and autoclaved samples of Shanghai surface-waters kept under conditions identical to those used by Yang, was never able to observe a loss of agglutinability of the test organisms, even though some of the specimens could be watched for a period of almost a year. Vibrios which were not agglutinable with cholera-diagnostic sera could be isolated not rarely side by side with *V. cholerae* from the stools of patients. Since, however, cholera-like vibrios abounded in the surface waters and were occasionally met with in the stools of healthy individuals at times when cholera was absent from Shanghai, it was not possible to consider the occurrence of " inagglutinable " organisms in the cholera stools as significant. This was in accord with previous observations by Crendiropoulo (1912) who adduced evidence to show that the apparent replacement of *V. cholerae* by inagglutinable organisms in the stools of carriers was really the result of an initial co-existence of cholera and cholera-like vibrios in the intestines of these individuals.

(2) *Acquisition of agglutinability.* Zlatogoroff, apparently the first worker who paid systematic attention to the subject presently under review, claimed in 1909 that he had succeeded in rendering 10 out of 18 water vibrio strains, originally found to give negative results in agglutination tests, agglutinable with cholera-immune serum. The methods he used for this purpose were (*a*) frequently repeated subcultivation, resulting in the appearance of agglutinable vibrios in the 54th generation; (*b*) subcultivation alternating with intraperitoneal passage of the strains through guinea-pigs; and (*c*) combination of the latter procedure with the simultaneous injection of killed typhoid or *E. coli* cultures or of living streptococci so as to increase the virulence of the growths. Evaluating his results, which he stated that he had confirmed with the aid of Pfeiffer's reaction in one instance, through

complement-fixation tests eight times, Zlatogoroff pointed to the importance of the water vibrios as a potential source of cholera infection.

Reporting on further investigations, Zlatogoroff (1911) stated that he had been successful in restoring the agglutinability of *V. cholerae* lost in the human intestine not only with the aid of the above-mentioned methods, but sometimes also by subjecting the serologically altered vibrios, after they had been suspended in cholera-immune serum diluted with normal horse serum, to repeated freezing and thawing. The method of simply passing the vibrios through diluted cholera-immune serum seemed unreliable because of the possibility of its leading to spontaneous agglutination.

Zlatogoroff concluded from his observations that

"each vibrio which is isolated from the faeces during an epidemic or at its onset, even if it does not agglutinate, should cause suspicion of cholera for the reason that the agglutinability of the cholera vibrios is very changeable ". [Trans.]

Horowitz (1911) who found besides intense subcultivation symbiosis with *Sarcina lutea* effective in restoring the agglutinability of *V. cholerae* lost in the human intestine, reached an identical conclusion.[j]

Several other workers, such as Köhlisch (1910), McLaughlin & Whitmore (1910), Freifeld (1912), and Wankel (1912) were not able to confirm the validity of the above-recorded findings, the last mentioned observer stating that

" even though the techniques recommended by Horowitz and Zlatogoroff have been followed most painstakingly, it was not possible to transmute even a single of the ten strains from Petersburg into an authentic cholera strain ". [Trans.]

A further noteworthy observation made by Douglas (1921) concerned a " paracholera " vibrio strain which after repeated subculture on artificial media had acquired agglutinability with cholera-immune serum. However, since suspensions of this organism were found incapable of absorbing the agglutinins for *V. cholerae* from the immune serum, the positive reactions it gave in agglutination tests were apparently not of a specific nature. It served as a corollary for this assumption that a serum raised against the paracholera vibrio failed to agglutinate *V. cholerae* even at a titre of 1:100. These findings well illustrate that claims regarding the appearance of agglutinability in vibrios have to be interpreted with great caution, unless they have been supported by further and thorough serological examinations of the supposedly transmuted strains.

Tomb & Maitra (1927), though admitting that the few attempts they had made in the laboratory to render initially inagglutinable vibrios agglutinable with cholera-immune sera had given " inconclusive and inconstant " results, laid great stress upon the fact that they had invariably been able to

j It should be noted that Puntoni (1913a) also claimed good success when growing a strain of " inagglutinable " vibrios five times in succession in the presence of two organisms isolated from the air.

effect a transmutation of cholera vibrios into an inagglutinable form in their water-tank experiments and also upon observations regarding the frequency of organisms of the latter type in human faeces as well as in the tanks. They felt entitled to conclude from this evidence

" that the non-agglutinating vibrio ... takes on the agglutinating characteristic under certain biochemico-physical conditions in the human intestine the nature of which are at present unknown, and in this mutation or epidemic form is the cause of epidemic cholera, since it is not unreasonable to assume that a characteristic so unstable may be as easily acquired as lost ".

Claims as fargoing in their implications as those of Tomb & Maitra were made by Brahmachari (1927, 1929) and by Pasricha et al. (1931, 1933).

The former worker reported in 1927 that not less than 40 out of 68 strains of vibrios which had been isolated in an endemic area in Calcutta from patients showing clinical signs of cholera, healthy persons, or water tanks, and which initially did not become agglutinated with cholera-immune serum, gave positive results in agglutination tests after they had been kept for six months, particularly if they had been frequently subcultivated.

Brahmachari (1929) also claimed that he had been able to restore through animal passage the agglutinability of cholera vibrios which had become spontaneously lost in the intestine of an intravenously infected guinea-pig.

Testing the action of cholera bacteriophages on 355 cholera-like strains, Pasricha et al. (1931) found that " the secondary ' phage-resistant colonies that develop after the action of cholera bacteriophages ... in some experiments are agglutinable by cholera high-titre serum. They completely absorb the agglutinins from a cholera-type serum and produce a serum which agglutinates cholera vibrios in very high dilutions ".

Pasricha et al. admitted that this acquired agglutinability was difficult to maintain on subculture, requiring repeated plating and selection of the best agglutinating colonies. They felt certain, nevertheless, that a large proportion of the vibrios found in cholera-affected places, which differed from *V. cholerae* only in their serological reactions, were mutant forms of the latter organism and played " a great part in the aetiology of the disease ".

In their second paper (1933), Pasricha et al. maintained that an action identical with that of the cholera phages was exerted by *vibriophages*, which were capable of lysing only cholera-like but not true cholera vibrios. They claimed in this connexion to have obtained the following results from experimenting with 56 strains of recently isolated cholera-like vibrios:

Remained serologically unchanged after bacteriophage action	32
Became agglutinable after action of *cholera* phage	11
Became agglutinable after action of *vibriophage*	13
Total	56

Yang (1935), subjecting four water vibrio strains which were originally inagglutinable with H + O cholera-immune serum, to daily subculture in plain broth and sheep-serum broth respectively, found that all samples except one subcultivated in sheep-serum broth became rather suddenly agglutinable with the specific serum after intervals ranging from two to six days.

Summarizing the results of a study on vibrios isolated from non-cholera sources in India, Taylor & Ahuja (1935a) stated that:

" A vibrio isolated from water in an area widely removed from places where cholera is endemic and which had been free from cholera for a number of years, was inagglutinable when first received, but in a period of six months' sub-culture in the laboratory developed all the biological characters of an authentic cholera vibrio, including H and O agglutination to full titre, and was indistinguishable from a cholera strain when quantitative and qualitative tests were applied."

While stressing that this strain differed in chemical structure from typical strains of *V. cholerae*, Taylor & Ahuja stated that the epidemiological significance of vibrios agglutinable with cholera-immune serum, yet chemically differing from typical Group I cholera-vibrios, was not clear, it having not yet been determined whether the vibrios of an aberrant chemical structure might be cholerigenic.

In a second paper (1935b), Taylor & Ahuja stated that they had been able to produce through a series of intraperitoneal passages in mice agglutinable variants giving the H and O reactions of typical *V. cholerae* strains in the case of three formerly inagglutinable vibrios, namely, (1) a strain of *V. metchnikovi;* (2) a water vibrio isolated three years previously in Calcutta; and (3) a vibrio obtained in an endemic area of Bengal from a healthy person. In the case of the two last mentioned strains the acquisition of agglutinability was accompanied by a shift in chemical constitution.

Aptly summarizing observations in point made by Linton, Shrivastava & Mitra (1935; see also Linton, 1935) White (1937b) stated that:

" From a first plating of cholera stool two colonies were picked off yielding, respectively, a typical culture of *V. cholerae* termed ' Rangoon smooth ', and a vibrio race, termed ' Rangoon rough 1,' held to be a rough derivative of *V. cholerae* and showing no serological nor antigenic relationship with that organism. From ' Rangoon rough 1 ' there was isolated a race, ' Rangoon rough 2,' growing in convoluted colonies and serologically distinct from ' Rangoon smooth ' and ' Rangoon rough 1.' Next there was separated from ' Rangoon rough 2 ' a fourth race, ' Rangoon rough 2a,' described as smooth-rough, serologically intermediate between ' Rangoon smooth ' and ' Rangoon rough 2 '; and finally from this a fifth race, ' Rangoon smooth recovered,' in which the distinctive serology of *V. cholerae* was completely restored."

These shifts in agglutinability were stated to have been accompanied by shifts in the chemical composition of the successively isolated strains: while " Rangoon smooth " and " Rangoon rough 1 " were found to belong to the chemical Group I of Linton et al., " Rangoon rough 2 " showed the chemical characteristics of Group V, and " Rangoon smooth recovered " those of Group VI.

Commenting upon these modifications, Linton (1935) declared that either they might have resulted from successive changes in the molecular arrangement of the vibrio proteins and carbohydrates, or all the different variants might have been present in the originally isolated strain. However,

in a further paper Linton, Seal & Mitra (1938) stated that they had obtained from a strain of "Rangoon rough 2," cultivated from a single cell, through 10 daily transfers in 0.5% glucose broth a variant which was inagglutinable with "Rangoon rough 2" immune serum, but was agglutinable with the serum raised against the original smooth Rangoon strain and was otherwise as well indistinguishable from the latter, showing the chemical constitution of Group I. The serological results obtained with H + O sera were confirmed through agglutination tests with an Inaba O serum which, while not producing a reaction with the "Rangoon rough 2" strain, agglutinated the smooth variant obtained from this at titres up to 1:2,500.

Discussing these findings, Linton et al. stated that:

"Although almost nothing is known about the internal arrangement of the vibrios, it may perhaps be permissible to suggest that each of them possesses the enzymic equipment capable of synthesizing the various proteins and polysaccharides which are found in the whole group."

Yu (1940) recorded that he had been able to render 16 out of 20 water vibrio strains, which had been isolated at the time of a cholera outbreak in Shanghai from the Whangpoo river, partially agglutinable with cholera O serum by passing the organisms suspended in mucin five times in succession by the intraperitoneal route directly from guinea-pig to guinea-pig. He felt entitled to conclude from this observation that such transformations might also take place under the influence of mucin in the human intestine, particularly in the case of gastro-intestinal disturbances, when mucoid substances were apt to be abundant. It has to be emphasized, however, that Yu's strains had initially shown a trace of agglutinability with cholera O serum. Hence, as maintained by Gallut (1951), these organisms possibly contained minor O-antigenic factors apt to react with non-specific components of the O serum used. Be this as it may, Gallut (1951) obtained entirely negative results when repeating Yu's experiments with seven Egyptian water-vibrio strains, which were apparently similar in their initial serological properties to the strains of the latter worker. While Gallut's strains did not acquire any specific agglutinability when being passed in mucin suspension ten times from mouse to mouse, they lost after the third to the sixth passage the agglutinability with their homologous serum, so that mucin seemed to degrade rather than to enhance the serological properties of the organisms. Some modifications of the chemical properties became noticeable in the course of the passages but these occurred also in the vibrios passed in mucin-free suspensions through control animals and even in the cultures of the strains kept in stock.

As will be perceived from above-recorded statements, numerous workers have claimed success in restoring the lost specific agglutinability of cholera vibrios or even in transmuting cholera-like vibrios, which originally failed to react positively in agglutination tests with cholera-immune sera, into

organisms behaving in this repect like *V. cholerae*. However, in view of the technique adopted by them, the results recorded by most of these workers have to be viewed with great scepticism. They often failed to confirm their findings through adequate agglutinin-absorption and cross-agglutination tests. Further, it is frequently impossible to rule out the possibility that the strains with which the experiments were started, were not of a uniform composition but contained, besides a large number of organisms reacting negatively in specific agglutination tests an initially unrecognized minority of true cholera vibrios. Most important finally, it must be kept in mind that with a very few exceptions the observations referred to above have been made with not fully specific H + O sera.

It is under these circumstances not surprising to find that, whenever some workers reported that they had brought about a serological transmutation of vibrios, others, even though using identical methods, failed to substantiate these claims. It has been stated already in this connexion that several investigators, repeating the experiments of Zlatogoroff and of Horowitz, were unable to confirm the findings of these two observers. It has likewise been noted that, history repeating itself, Gallut (1951) recently obtained strictly negative results when checking the validity of claims to similar those of Zlatogoroff and Horowitz, made by Yu in 1940. The papers read by Tomb & Maitra and by Brahmachari in 1927 were also much criticized, Pandit for instance stating that, though he had kept cholera-like vibrios isolated from water supplies in India for over two years, he had failed to note the change in the agglutinability of such strains claimed to be frequent by Brahmachari.

A most determined stand against what he called the " legend " of serological transmutability of vibrios was taken by White (1937b). Discussing in particular the above-mentioned observations of Linton et al., White maintained that since

" ' Rangoon smooth ' and ' Rangoon rough 1 ' were derived from two colonies in a first plating of stool, belief in their genetic connection is a matter of pure assumption ".

White's main objection against the validity not only of the claims made by Linton et al. but also that of the results recorded by Taylor & Ahuja (1935b) was based upon tests he made with a special type of cholera bacteriophage, the LL phage (White, 1937a). He stressed that, both in the case of the Rangoon series and in that of Taylor & Ahuja's strains, cultures found to be infected with this phage were alleged to have been derived from growths in which it was absent. Discussing this discrepancy, White said that:

" Various hypotheses may be improvised to fit the facts: genesis of bacteriophage *de novo* ; mutation of vibrio phage or vibrio phages unknown, collaterally with that of the vibrio itself; but the simple and obvious indication is that the alleged mutant cultures are not derived from the parents presented."

Generally speaking, White maintained that:

" There is, I believe, not only insufficient evidence on which to base a theory of vibrionic transmutability such as is at present current, but definite evidence against acceptance of such alleged instances of change as have been discussed."

Referring again to the Rangoon strains, White (1940a) stated that:

" The negative results obtained with extracts of a rugose derivative of ' Rangoon rough 1,' which is a smooth culture with the serology of *V. metchnikovi*, and of the capsulated culture ' Rangoon rough 2 ' support my contention . . . that these strains have no immediate relation to nor derivation from their alleged parent, the cholera strain ' Rangoon smooth.' "

In view of White's objections it is difficult to assert the validity even of Taylor & Ahuja's observations. However, even if one could admit the possibility that under highly artificial conditions the agglutinatory properties of vibrios might become changed, there is no convincing evidence to show that such transmutations take place under natural conditions and that consequently cholera-like vibrios or cholera vibrios which had lost their agglutinability with the usual specific sera, form a reservoir from which epidemics may be produced *de novo*. It is significant to note that Seal (1935), one of Linton's principal co-workers, discussing in particular the variations brought about by bacteriophage in the laboratory, considered it an open question whether such changes in the character of vibrios " do also occur in nature or inside the human system ".

A peremptory statement made in this respect by d'Hérelle (1927), when discussing Tomb & Maitra's claims, was that he could

" not agree with the possibility of the regression from non-agglutinating to agglutinating. In our quarantine station of Tor, during the last fifty years, hundreds of thousands of pilgrims harbouring non-agglutinating vibrios in their intestine have passed through the station on their way towards the North, and not a *single* case of cholera has been discovered amongst them nor has an outbreak of cholera ever occurred north of Tor. We must conclude that, in Nature, the regression from non-agglutinating to agglutinating vibrios does not take place and that carriers of such non-agglutinating vibrios are harmless and never the origin of an outbreak of cholera. To say that non-agglutinating vibrios may be the cause of the epidemicity is a mere hypothesis, but to show that a Mecca pilgrim carrier of non-agglutinating vibrios has never been the cause of an epidemic, that is a fact."

Gallut (1951), commenting on the significance of Yu's claims, similarly stressed that observations on the incidence of cholera in Shanghai did not support the idea of a causative role played by the water vibrios in the origin of the epidemics.

The validity of this statement is fully supported by the observations the present writer had opportunities to make in most cholera-affected parts of China during about a score of years. Though water vibrios were found to abound everywhere, not a single cholera outbreak was seen which was not found to have been due either to an importation of the infection or

to its continued sporadic occurrence in man. In this sense, therefore, one is certainly entitled to consider the alleged serological transmutability of vibrios as a myth.

Haemo-agglutination

As summarized by Doorenbos (1932), he observed in 1931 that, when a few drops of a suspension of sheep erythrocytes were added to a saline suspension of recently isolated El Tor vibrios, after a few minutes the colour of the blood changed to violet and at the same time the blood corpuscles began to become agglutinated in the form of small flocculi which rapidly sank to the bottom of the tubes. Further studying this phenomenon, Doorenbos established that

(*a*) the reaction took place at 37°C as well as at 0°;

(*b*) the haemo-agglutinins were inactivated by heating the suspensions for 5 minutes at 64°C;

(*c*) the haemo-agglutinins were absorbed by red blood-corpuscles;

(*d*) some strains possessed the property of haemo-agglutination only during a short period of their development, the phenomenon suddenly appearing after a few hours' incubation and disappearing as quickly when the cultures became older;

(*e*) the haemo-agglutinins inhibited the action of the haemolysins and vice versa.

Doorenbos added that seven Syrian strains, recently isolated from carriers who had arrived from a cholera-affected area (Iraq), produced a marked haemo-agglutination only in guinea-pig blood suspensions, and a feeble reaction if goat blood was used. Haemolysis tests with these strains gave more clear-cut results, proving positive with guinea-pig blood, and negative with goat blood.

Panayotatou (1931), using guinea-pig blood for such tests, had satisfactory results with four strains from Basra, haemo-agglutination becoming manifest before haemolysis became apparent. She recommended using for the former tests 4- to 6-hour-old cultures, suspending the vibrios in broth diluted 1:10, 1:100, and 1:1,000 respectively with normal saline and reading the results after an incubation at 37°C for 15 minutes. She confirmed that haemo-agglutination disappeared as soon as haematolysis set in.

While in the opinion of Panayotatou haemo-agglutination tests appeared to be of value for the laboratory diagnosis of cholera, Cantacuzène (1933), finding that identical reactions were given by various cholera-like vibrios, considered such tests to be without practical importance.

Gallut & Brumpt (1944) explored the possibility of whether in cholera-diagnostic work advantage might be taken of the method of "haemo-agglutination" of Brumpt (1941), for which, instead of the serum, the whole blood of the patients was used for rapid tests on slides or gelatinized paper. Besides working with formolized H+O suspensions, Gallut & Brumpt also

made tests with O suspensions of *V. cholerae*, prepared by suspending alcohol-killed vibrios after centrifugation in 10% sodium citrate solution and adding one drop of a 1% methylene blue solution per ml. The rabbits, whose blood was tested, had been immunized either with H+O or with O antigens. It was found that O as well as H+O haemo-agglutination took place within a few minutes or even seconds at ordinary temperature, while tests with the blood of normal animals or of healthy human beings gave negative results. Gallut & Brumpt therefore recommended this method, which had proved satisfactory in the case of other infections, such as typhoid, paratyphoid, and typhus, for the diagnosis of cholera. So far, however, no practical advantage seems to have been taken of this recommendation.

Acid agglutination

It being proposed, for the convenience of record, to deal with acid agglutination tests at the present juncture, it has first to be noted that Beniasch (1912) and Sgalitzer (1914) found this method unsuitable for a differentiation of cholera and cholera-like vibrios. However, Vercellana (1926) recorded that, when tested with lactic acid, cholera-like vibrios became invariably and rapidly agglutinated in the form of large and stable flocculi, whereas cholera vibrios, if reacting at all, became agglutinated at lower dilutions only in the form of small and unstable flocculi. Damboviceanu (1933), testing 63 strains of *V. cholerae* and 34 cholera-like strains with the aid of acid agglutination tests, found that more than half (58%) of the latter reacted like the cholera vibrios. However, she expressed the opinion that tests of this kind might be of value for a distinction of cholera-like strains which were descendants of *V. cholerae* from such having no genetic relation with this organism.

Precipitin reactions

Though, as noted earlier in this study, Kraus (1897) drew attention early to the precipitin reactions taking place when filtrates or extracts of cholera cultures were brought in contact with specific immune-sera, this method was not adopted for the purposes of routine laboratory diagnosis in view of the close correspondence found to exist between the results it gave and those obtainable with the more expedient agglutination tests.[k] Observations proving this rule have been made under various conditions, for instance by Balteano & Lupu (1914) when using the sera of cholera-vaccinated individuals for parallel agglutination and precipitin tests, and by Dambo-

[k] As quoted by Hetsch (1928), two Japanese observers, Fukuhara & Ota, noting that extracts of typical cholera stools gave specific precipitin reactions with cholera-immune sera, recommended the use of such tests for the purposes of practical laboratory diagnosis. However, as stated by Hetsch, no advantage has been taken of this theoretically interesting proposal.

viceanu et al. (1934) who applied both these methods to study the immunogenic properties of the residual antigen extracted from *V. cholerae* with the aid of trichloracetic acid. It is also noteworthy that Gallut (1950), making precipitin tests with the glucolipidic extracts of *Br. suis* and cholera-immune sera on the one hand, with the corresponding extracts of cholera vibrios and various anti-brucella sera on the other, obtained results identical with those of analogous agglutination tests. However, Shrivastava & Seal (1937, see also Linton, Seal & Mitra, 1938) recorded that by making precipitation reactions with the vibrio polysaccharides isolated by them and various immune sera, it was possible to distinguish between cholera and El Tor vibrios which, though behaving identically in O agglutination tests, showed a different chemical constitution.

Shrivastava & Seal (1937) determined the precipitin reactions given by polysaccharides isolated from (1) a Group I Inaba strain of *V. cholerae* and (2) an Inaba variant belonging to the chemical Group VI of Linton et al. with the aid of immune sera representative of each of the six groups into which the vibrios were divisible on the basis of their chemical constitution. It was found that in the case of the typical Inaba strain, positive reactions were obtained only with Group I immune sera, while the Inaba variant reacted only with the two Group VI immune sera used. Three sera raised against El Tor vibrios falling into the chemical Groups IV or V failed to react with either of the two above-mentioned polysaccharides.

Continuing these studies with polysaccharides prepared from 23 vibrio strains belonging to different chemical groups, the haemolytic properties of which were not stated, Linton, Seal & Mitra (1938) reached the general conclusion that

> " precipitin reactions between the polysaccharides and antisera to the whole organisms indicate that in general the serology expresses the underlying chemical pattern of these organisms and indicates the same groups as the chemical analysis ".

It also deserves attention that Gallut & Grabar (1943a) noted the presence of marked differences even among vibrios belonging to one and the same serological group, when quantitatively assessing with the aid of nitrogen determinations the precipitations, which resulted from the action of fixed amounts of various immune sera upon variable amounts of the glucolipidic antigens of the organisms. They suggested that application of this method might be of value in defining the serological characteristics of the vibrios.

A profound study of the serological properties of *V. kadiköj*, a " haemotoxic " (haemolytic) vibrio belonging to the El Tor group, by Eisler & Kovacs (1926) showed that no relationship existed between the precipitinogen of this organism and its toxin, which was merely apt to become adsorbed to the flocculi produced by the action of the precipitating immune sera. A further important result of these studies was that Eisler & Kovacs were able to demonstrate the presence of two components of the precipitinogen, a thermolabile and coagulable one, which was adsorbable to animal charcoal, and a thermostable component resistant to boiling heat.

It is of importance to refer at the present juncture also to attempts to distinguish between cholera and cholera-like vibrios by precipitation tests

with concentrated salt solutions. Liefmann (1913), following up casual observations made in this respect by Porges (1906) with ammonium sulfate, preferred for his work magnesium sulfate, used not only in varying concentrations for tube tests, but also for slide tests. For the latter purpose, loopfuls of the cultures to be examined were thoroughly mixed with drops of concentrated magnesium sulfate solution and also—to guard against wrong positives through spontaneous agglutination of the vibrios—to drops of normal saline placed on slides, results being read immediately.

Liefmann obtained in this manner precipitations in the case of 12 out of 14 cholera strains, whereas out of 9 cholera-like strains 8 gave entirely negative results. In tube tests 30 out of 40 cholera strains were well precipitated with 90% magnesium sulfate, 6 gave weaker and 4 negative results. Out of 20 cholera-like strains, only one reacted strongly, while the strain reacting positively in the slide tests produced a trace of precipitation; the other 18 strains gave negative results.

Greig (1913b), repeating such tests with a larger material, found that out of 176 cholera strains 164 were completely salted out, 12 only in traces, whereas out of 41 vibrio strains not agglutinable with cholera-immune serum only 6 showed a strongly positive reaction, 8 reacted weakly and 27 were not at all affected. Thus, as concluded by Greig, a close but not an absolute parallelism existed between these and agglutination tests. It follows that the method of salt precipitation does not furnish fully reliable results.

Complement-fixation tests

As can be gathered from the summaries of Köhlisch (1910), Kolle & Schürmann (1912), and Hetsch (1912), the early application of complement-fixation tests for the purposes of cholera laboratory diagnosis (1906-7) soon led to considerable debates. Apart from the question of whether with the aid of this method a differentiation could be made between cholera and cholera-like vibrios, it became at once a hotly contested point whether cholera and El Tor vibrios reacted identically or differently when tested in this manner with cholera-immune sera.

Advocating the latter opinion, Markl (1906) maintained that he had observed complete complement fixation when testing classical cholera vibrios with the aid of a cholera-immune serum, but only partial fixation in analogous tests with El Tor strains—a difference which he ascribed to differences in the receptor apparatus of these two categories of strains.

Ruffer and Crendiropoulo (see Ruffer, 1907), obtaining negative results in complement-fixation tests with El Tor vibrios, which reacted like the classical cholera strains in agglutination tests with cholera-immune serum, felt entitled to deny the specificity of the latter method.

There can be no doubt, however, that the technique used by these workers for their complement-fixation tests was unsatisfactory, particularly

because they used suspensions of living organisms as antigens. As shown by Neufeld & Haendel (1907), it was impossible to obtain in this manner reliable results with haemolytic vibrios, because as a rule haemolysis became manifest after one hour in all tubes into which culture material of such organisms had been embodied. If, on the contrary, El Tor suspensions killed by half an hour's exposure to 70°C were used, results comparable to those with non-haemolytic strains could be obtained.[1] Neufeld & Haendel established in this manner that " the El-Tor-vibrios also in complement fixation tests with specific cholera sera react like true cholera bacilli ".

Though a few subsequent workers again advocated views similar to those of Markl and of Ruffer, the validity of the findings of Neufeld & Haendel, which were soon confirmed by Besche & Kon (1909), is now generally accepted. Indeed, in view of the fact that no, or at least no marked, differences exist in the antigenic structure of cholera and El Tor vibrios respectively, it is impossible to assume that these two categories of organisms react differently in properly performed complement-fixation tests.

In addition to the above-described controversy, some of the early workers, for instance Schütze (1907), Neufeld & Haendel (1908), Baerthlein (1912), Michiels (1913), and Pottevin (1913a) expressed doubts as to the usefulness of complement-fixation tests for a differentiation of cholera and cholera-like strains.

However, the full specificity of the complement-fixation tests and the parallelism of their results with those obtainable with the aid of the agglutination method has been asserted by numerous other cholera workers, e.g., by Ballner & Reibmayr (1907), Bocchia (1911), Feldmann (1917), Kabeshima (1918a), and Mackie (1922), while Koshland & Burrows (1950) even came to the conclusion that the agglutinating and complement-fixing antibodies to the vibrio O antigen were closely similar, if not identical.

It has to be admitted that the practical value of complement-fixation tests in cholera-diagnostic work is limited, but this is due merely to the tediousness of the method and to the special technical requirements involved (Bocchia, 1911). Nevertheless, hand in hand with Pfeiffer's reaction or, in the case of avirulent strains, even in place of the latter method, complement-fixation tests might still prove of value in dealing with atypical cholera strains.

It is of interest to add that in place of live organisms or preferably of killed culture materials, some special antigens have been used for cholera-diagnostic complement-fixation tests. Thus Rondoni (1910) established that the nucleoproteid of *V. cholerae* was fully satisfactory in this respect and that *vice-versa* subcutaneous injection of rabbits with this material produced sera containing complement-fixing antibodies. Kutscher &

[1] To use cholera vibrios killed by moderate heat (60°C) as antigens in complement-fixation tests, was also recommended in 1907 by Weil. Some Japanese cholera workers, such as Uyeda (1924) and Fujimori (1928), laid stress upon the use of well-boiled antigens so as to abolish the inhibitory action of a supposed " impedin ".

Schaefer (1916) proved through tests with rabbit-immune sera that cholera vaccines formed suitable antigens for complement-fixation tests. Proposals have been made by a few workers, first apparently by Nedrigailoff (1909), to expedite cholera-diagnostic work by directly using the patients' faeces as antigens in complement-fixation tests.

Nedrigailoff (1909) put fluid cholera stools into tall cylindrical glasses and used after sedimentation the supernatant as antigen. Complement-fixation tests performed instead with Berkefeld-candle filtrates of cholera stools gave almost completely negative results. Since the same held true if fresh broth cultures or suspensions of fresh agar cultures of *V. cholerae* were passed through the candles, whereas the filtrates of old cholera cultures proved to be suitable antigens in complement fixation tests, Nedrigailoff postulated that —in contrast to the latter materials—cholera faeces contained no endotoxins.

Tokunaga (1911), carrying out complement-fixation tests with the faeces of cholera patients and cholera-immune sera, obtained positive results with 79% of his specimens. Faeces of cholera carriers gave, on the contrary, invariably negative results.

It was claimed by Amako & Kojima (1912) that, if the supernatant of typical cholera stools was used as antigen in complement-fixation tests, a diagnosis could be arrived at in 7-8 hours. Atypical stools containing only few vibrios had little or no antigenic value and it was necessary in such cases to use the upper layer of 6- to 10-hour-old peptone-water cultures made from these stools in order to obtain good results in complement-fixation tests.

In view of the technical difficulties involved, it is not surprising to find that no large-scale advantage has been taken of the above-described method of cholera stool examination even in the past. At present, when highly specific media are available for a rapid direct isolation of *V. cholerae* from the stools, it is no more possible to ascribe any practical value to it.

As stated by Svenson, some preliminary investigations by a Russian worker, Tuschinsky (1909), gave reason to hope that diagnostic advantage might be taken of complement-fixation tests with known cholera antigens and sera of patients with signs of choleraic disease. Amako & Kojima (1912), who further studied this possibility, used as antigens the combined washings of 3 or 4 agar-grown cholera strains. Making complement-fixation tests with human sera, they obtained positive results in 15 out of 34 mild cholera cases, in the case of 20 out of 28 patients with moderate to severe attacks of the disease and in 5 out of 17 cholera carriers. Complement-fixation tests with the sera of 2 patients with fulminant cholera and with those of 3 individuals with signs of cholera typhoid gave negative results. Commenting on these findings, Amako & Kojima stressed the necessity of using polyvalent antigens of known activity, because—as has been generally acknowledged—different cholera strains may give variously marked results in complement-fixation tests.

In view of this evidence, it is undeniable that such tests might be used for establishing the diagnosis of cholera currently or perhaps rather retrospectively, the more so because Yoshino (1922) established that complement-fixation tests with known cholera antigens and normal human sera (as

well as with normal rabbit or horse sera) invariably gave negative results. Still, for practical reasons it appears more expedient to use agglutination tests, possibly also in vitro bactericidal tests, in preference to complement-fixation tests for this purpose.

Balteano & Lupu (1914) found that complement-fixing antibodies appeared in persons who had been vaccinated once against cholera 14 days after the injection, and after 9 days in those who had in the meanwhile received a second vaccine dose. In the former group the complement-fixing property of the serum reached its maximum five days after it had become manifest and disappeared within two months as against three months in the case of the twice or thrice vaccinated.

Schöbl & Andaya (1925), who also performed complement-fixation tests with the sera of cholera-vaccinated persons, used for this purpose rather small vaccine doses, 28 of the individuals in question receiving one dose of 500 million, 5 a single dose of 1,000 million and 7 two doses of 500 and 1,000 million respectively. Nevertheless, as revealed by tests made one week or 12 days after vaccination, complement-fixing antibodies became demonstrable practically always, the only exceptions being two individuals who had received a single dose of 500 million and were tested one week later. As shown by repeated tests, the complement-fixing antibodies were apt to persist for 6-10 months. Both the length of persistence and the titres reached seemed to depend to a higher degree upon the number of the injections given than upon the amounts of vaccine administered.

Phagocytosis tests

Though profound studies by Neufeld & Hüne (1906, 1907) adduced evidence that specific bacteriotropic substances or, as they are usually called, opsonins, rendering the vibrios liable to phagocytosis, were present in cholera-immune sera, and further investigations by Neufeld & Haendel (1907) showed that El Tor vibrios were analogously influenced by the cholera tropins, but few attempts have been made to utilize methods based upon these observations for the purposes of practical cholera diagnosis.

Schütze (1909), who seems to have been the first to devote attention to such tests, evolved a technique of his own, described as follows:

An exudate rich in leucocytes was produced by injecting guinea-pigs intraperitoneally with 10 ml of broth in which 2 g of aleuronat had been suspended, and puncturing the peritoneal cavity of the animals 8 hours later. The exudate thus obtained was suspended in normal saline in centrifuge tubes and twice washed in such saline with the aid of centrifugation, the resulting sediment, suspended in 4-5 ml of normal saline, being used for the tests.

To perform these, 1 ml of the leucocytic suspension was mixed in centrifuge tubes with equal amounts of (*a*) cholera-immune serum inactivated by heating for 20 minutes at 54°C, and (*b*) broth cultures of the vibrios to be tested. The mixtures were kept at 37°C for 10 minutes, then centrifuged for ½ hour. After the sediment had been twice

washed in normal saline, it was used for the preparation of smears which, after heat-fixation, were stained for 3-5 minutes with alkaline methylene blue solution and then examined under the microscope in order to assess the degree of phagocytosis.

Carrying out such tests with a cholera and an El Tor strain as well as with three strains of cholera-like vibrios (Metchnikoff I and II, and Finkler-Prior), Schütze established that the former two vibrios were phagocytosed under the influence of cholera or El Tor immune sera to a considerably higher degree than the cholera-like vibrios, which were not at all ingested by the leucocytes when 1:50 dilutions of cholera-immune serum were used. Analogously, if a serum raised against *V. metchnikovi I* was used, almost no phagocytosis of the heterologous strains resulted. However, no such differences were apparent, if instead a serum raised against *V. metchnikovi II* was used in a dilution of 1:20. Schütze postulated, therefore, that the specificity of the vibrio opsonins was not absolute and that, though he had been able to distinguish with the aid of his method between cholera and cholera-like vibrios, this procedure alone should not be used for the purposes of differential diagnosis, agglutination tests, supplemented by Pfeiffer's reaction, remaining the " main criteria " for this purpose.

Amako (1909), studying the opsonic properties of the sera obtained from 58 cholera patients or convalescents, came to the following conclusions:

" 1) According to my tests with cholera vibrios, normal sera and particularly cholera convalescent sera showed marked opsonic effects.

" 2) If fresh undiluted convalescent serum was used, the cholera vibrios were lysed extracellularly so that opsonic effects were not noticeable; if, however, serum dilutions were used, one could observe a clear ['deutliche'] opsonic action . . .

" 3) If the bacteriolytic property of the serum is too strong so that one cannot recognize an opsonic action even if serum dilutions are used, one can observe an action after inactivation of the serum (through heating for 15 minutes at 60°C), because the cholera opsonins, like other immune-opsonins, are thermostable . . . ". [Trans.]

As shown by Amako with the aid of numerous graphs, there existed in individual cases, as a rule, a parallelism between the agglutinatory, bacteriolytic, and opsonic properties of the sera.

It is of interest and of some practical importance to add that according to the investigations of Eisele et al. (1948) already referred to above, opsonophagocytic tests with brucellae, made according to the method recommended in the handbook on brucellosis in animals and in man of Huddleson (1943), were found to give positive results in 16 out of 20 cholera-vaccinated individuals. Marked reactions (phagocytosis by 80%-100% of the cells) were noted in about two thirds of this group.

Allergic and skin tests

Shwartzman (1928) observed that if a preparatory intradermal injection of rabbits with a filtrate of *S. typhosa* was followed 20-48 hours later by

an intravenous administration of the same or a suitable heterologous bacterial filtrate, in the majority of the animals tested marked haemorrhagic lesions developed at the site of the preliminary injection, which were apt to undergo necrosis and ulceration.

As first established by Gratia & Linz (1931) and confirmed by Uyeda (1934) and Vassiliadis (1935c), this curious, though not, or at least not strictly, specific reaction, known under the name of the Shwartzman phenomenon, could also be produced by culture filtrates of *V. cholerae*.

Gratia & Linz (1931) found that this phenomenon could be produced not only in rabbits but also in guinea-pigs through a preliminary intradermal injection followed by a second injection into the jugular vein or into the heart. They also drew attention to the similarity of Shwartzman's phenomenon with a reaction described by Sanarelli (1924a), according to whose observations a preparatory intravenous injection of rabbits with living cholera vibrios, followed 24 hours later by a second intravenous administration of either the homologous or a heterologous culture filtrate produced haemorrhagic reactions in the intestines, occasionally massive intraperitoneal haemorrhage, congestion of the genital organs apt to lead to abortion in pregnant animals, and sometimes immediate death.

Gratia & Linz tried, therefore, to produce Sanarelli's reaction by the administration of cholera filtrates according to Shwartzman's technique. Results were not uniform, some animals showing reactions neither in the skin nor in the intestine, others a typical Shwartzman reaction at the site of the intradermal injection, and some finally, instead of this haemorrhagic intestinal reactions similar to those described by Sanarelli. One guinea-pig, which died 2 days after the second injection, though free from either skin or intestinal lesions, showed abundant blood clots in the peritoneal cavity and identical findings were made in another guinea-pig, which had been given a preliminary dose of 3 ml *V. cholerae* filtrate intraperitoneally and had died a few hours after it had received a second dose intravenously.

The conclusion reached by Gratia & Linz was that there existed between the phenomena of Sanarelli and of Shwartzman " a close relationship or probably even an identity ".

Vassiliades (1935c), exploring whether Shwartzman's phenomenon might be elicited with El Tor as well as with cholera vibrios, recorded the following results:

	Number of animals	*Dose for preparatory injection*	*Dose for intravenous injection*	*Results positive*	*negative*
Filtrate of	3 rabbits	0.25 ml	1 ml per kg body-weight	2	1
V. cholerae	4 guinea-pigs	0.11 ml	0.75-1.25 ml for 400-600 g	0	4
Filtrates of	6 rabbits	0.25 ml	,, ,,	0	6
V. El Tor	8 guinea-pigs	0.10 ml		0	8

It will be noted that (*a*) administration of *V. cholerae* filtrates produced Shwartzman's phenomenon in rabbits, but—in contrast to the findings of Gratia & Linz—not in guinea-pigs; and (*b*) on the contrary, filtrates of two different El Tor strains failed to elicit this phenomenon. It would be of interest to establish with the aid of a larger material whether this difference between cholera and El Tor vibrios holds generally true.

Continuing investigations in this field, Raynal et al. (1940) were able to produce local skin reactions in guinea-pigs, which had been immunized one month previously through intraperitoneal injection of live cholera

vibrios, through intradermal administration of cholera antigens obtained with the aid of trichloracetic acid extraction. Normal guinea-pigs, receiving such antigens by the intradermal route, failed to react.

Kovacs (1932) reported that intracutaneous administration of the toxin of El Tor vibrios produced marked local reactions, consisting of infiltrations and hyperaemia or, if higher doses were used, even of necrosis, in the skin of rabbits, guinea-pigs and, as he showed on himself, also in the human skin. The appearance of these reactions could be inhibited by the simultaneous administration of sufficiently large doses of antitoxic sera and the reactions remained absent in guinea-pigs which had been immunized with the toxoid of the *V. kadiköj*, unless toxin amounts ten times exceeding the dosis necroticans were used.

Kovacs maintained that such intracutaneous tests could be used for a differentiation of El Tor and related vibrios from *V. cholerae*, because administration of 0.1 ml of the centrifugate of 6-day-old cholera broth cultures produced no skin reactions in a guinea-pig, whereas such reactions could be elicited with the centrifugates of El Tor and Kadiköj vibrios.

In contrast to these findings, Yu et al. (1932) obtained positive skin reactions with toxic solutions prepared by (*a*) growing typical non-haemolytic cholera vibrios, the virulence of which had been enhanced by repeated guinea-pig passages, in buffered glucose-free broth; (*b*) centrifuging the culture fluid and filtering the supernatant through N Berkefeld-candles. Reporting on their findings, Yu et al. stated that

(*a*) three normal rabbits, intradermally injected with 0.1 ml of such a filtrate, showed skin reactions of considerable size;

(*b*) tests on 6 rabbits which had been immunized either with the toxic filtrate or with cholera vaccine, gave negative results;

(*c*) tests on human volunteers, who received 0.1 ml of the toxic filtrate intradermally into one forearm and, as a control, 0.1 ml of such a filtrate heated for 2 hours at 100°C, gave the following results:

	Positive *	*Negative*	*Total*
Vaccinated	1	27	28
Not vaccinated	60	3	63
Total	61	30	91

* Positive reactions appeared within 6-12 hours, reaching a maximum between 20 and 24 hours and fading in 48 hours.

Yu et al. suggested that their method might prove of value in testing individual susceptibility to cholera and also for checking the length of the immunity produced by cholera vaccination. They noted in this connexion that the single vaccinated individual who gave a positive skin test had received cholera vaccine about three years before he had been examined.

The usefulness of skin tests for assessing the value of cholera vaccinations was also upheld by Brounst & Maroun (1949) who obtained positive reactions in 5 out of 10 thrice-vaccinated individuals tested by intradermal

injection of 0.1-ml doses of the vaccine. Appearing after 48 hours, these reactions consisted of local congestion and oedema, sometimes accompanied by the appearance of a central necrotic zone or by a nodular infiltration.

Large-scale use of skin tests was made by Sabry (1950) during and after the 1947 cholera outbreak in Egypt. The antigen for these tests was prepared by (*a*) simultaneously growing three cholera strains in broth; (*b*) killing the organisms by exposure to 52°C for one hour; and (*c*) centrifugation, the lower more concentrated part of the centrifugate being used in a dilution of 1:300. Frankly positive results obtained with this antigen through intradermal injection consisted in the appearance of an oedematous papule surrounded by an erythematous zone, the reaction becoming fully developed after 24 hours and then disappearing within 48 hours. In the case of mild reactions, no, or only an ill-defined, erythema became manifest round the papules.

Tests made in a cholera hospital on 13 patients, 9 convalescents, and 7 carriers produced only mild reactions, a result ascribed by Sabry to (*a*) a supposed state of allergy in these individuals, and (*b*) massive doses of sulfaguanidine which they had received. Sabry stated in the latter connexion that in the course of his further work he had succeeded in rendering the skin test negative by administering to a group of originally positive individuals 6 g of sulfaguanidine daily for 9 days.

Further results recorded by Sabry may thus be tabulated:

	Reactions		
Groups tested	*positive*	*mild*	*negative*
5 stool positive cholera carriers	5	—	—
37 vibrio-positive carriers	20	—	17
265 non-cholera patients	9	20	236
186 members of the hospital staff	32	51	103

Note. 31 individuals giving originally a negative skin test, remained negative when re-tested 7-30 days after they had received one dose of cholera vaccine.

Commenting upon further observations, Sabry stated that

" the percentage of the positive cases in the more recent experiments decreased among the domestic hospital staff as well as among ordinary cases, indicating that at least some of the carriers are on their way to recovery. As a decisive proof of the validity of this observation, 1001 cases were inoculated (i.e. skin-tested) during the period from 27-4-1948 to 6-7-1948 without the occurrence of one single positive reaction."

Sabry emphasized, therefore, the significance of the skin tests, maintaining that " the persistently positive cases represent the dangerous carriers responsible for the propagation of epidemics, who should be detected and kept under strict control ". However, apart from the fact that most workers do not share Sabry's belief in a dangerous role played by carriers in the spread of cholera, it has to be kept in mind that in a majority of his

observations he seems to have been unable to correlate positive skin tests with findings of *V. cholerae* in the stools of the individuals concerned.

Natural Immunity

Resistance and natural immunity

As shown by ample experiences, and well illustrated by the classical observation of Macnamara (1876) that out of 19 persons drinking water from a vessel which had been accidentally polluted with fresh cholera excreta, only five actually contracted the infection, the ingestion of materials containing *V. cholerae* is by no means invariably followed by clinical manifestations of the infection. General agreement exists, however, that such a non-appearance of the disease is due, if not solely so, in the first line to an unspecific resistance to the infection instead of being the result of a specific natural immunity.

Various factors contribute to the unspecific resistance against cholera infection. As has been noted in the third of these studies, some evidence has been adduced to show that the saliva of healthy persons exerts an anti-bacterial action on *V. cholerae* and might thus form a first line of defence against not too massive infection. Be this as it may, it is certain that the acidity normally prevailing in the stomach forms a potent barrier against the entry of cholera vibrios into the intestines where owing to the presence of an alkaline reaction conditions are favourable for a multiplication of the organisms. There can be no doubt, however, that even in the intestines unspecific defence mechanisms against cholera infection exist. The competition of the normally present bacterial flora is apt to exert an influence in this respect. Moreover, it is likely that a normal condition of the intestinal mucosa is capable of preventing an entrenchment of the invaders. Whether this defence mechanism is vested in the normally present mucous coating of the mucosa, as maintained for instance by Harvey (1929), or dependent upon the intactness of the epithelium itself (Romano, 1912), is difficult to decide. Probably both these factors play a role to a varying extent.

There can be no doubt, however, that the protection afforded to individuals in full health through the above-described means of an unspecific resistance to cholera is relative in degree. Kolle & Schürmann (1912), discussing this problem, pointed out with much reason that (*a*) like other acidophobe bacteria, cholera vibrios enclosed in copious amounts of food are apt to escape the action of the acid gastric juice, and (*b*) infected fluids, particularly cold drinks, are apt rapidly to pass the stomach, particularly the empty stomach. Experimental observations supporting the latter contention have been noted already in the third of these studies. Kolle & Schürmann insisted also that even in normal persons the acidity of the gastric juice was subject to variation and could be low at times.

While thus even healthy persons are by no means invariably proof against an entry of *V. cholerae* into their system, ample observations have shown that individuals with a permanently low acidity of their gastric juice are particularly apt to fall victim to cholera infection. Thus it is a well established fact that the disease is particularly rampant among individuals suffering from chronic gastritis due to the habitual abuse of alcoholic drinks. Sticker (1912) quoted in this connexion the observations made by Adams (1849) during an 1848-9 outbreak at Glasgow, according to which cholera killed 91 out of 100 drunkards as against 19 out of 100 abstemious persons. That also temporary gastro-intestinal disturbances favour cholera infection has been confirmed by observations on a greatly increased frequency of cholera admissions on the days immediately following Sundays or holidays, recorded during various outbreaks. Sticker, drawing attention to these records, also noted that according to several of the early observers the use of emetics and also that of even small doses of laxatives seemed to promote cholera infection.

The concept suggested by these observations that, besides the normally prevailing acidity of the gastric juice, a normally present unspecific resistance of the mucous surface of the intestines prevents cholera infection, is supported by laboratory experiences. As will be fully discussed in the sixth of these studies, several workers have succeeded in producing syndromes similar to, perhaps even identical with, human cholera in experimental animals ordinarily not amenable to oral infection with *V. cholerae*—in part by creating conditions analogous to those found to promote the appearance of cholera in man. Thus Pottevin & Violle (1913) recorded success in this direction by administering saline purgatives to monkeys before oral cholera infection, and Cantacuzène & Marie (1914) noted the appearance of a syndrome corresponding to that of human cholera in guinea-pigs, the resistance of whose intestine had been lowered through administration of podophyllin. Two guinea-pigs which had been dosed with this drug contracted the disease by mere contact with cholera-infected animals.

Most authorities are sceptical, and many frankly deny, that in addition to the well-documented presence of unspecific defence mechanisms a specific natural immunity against cholera infection exists. As far as it is permissible to adduce in this respect the evidence supplied by tests with the sera of normal, non-vaccinated subjects, it speaks against the presence of such a specific immunity, immune bodies being either altogether absent or demonstrable only at negligibly low titres.

Ample observations made in parenterally infected animals, though not directly applicable to the problem presently under review, are of interest in so far as they showed up a fundamental difference between an unspecific resistance and a specific immunity to cholera infection. It was found that previous administration of heterologous bacteria, such as *Chromobacterium*

prodigiosum, *Proteus* and *Ps. pyocyanea* could protect guinea-pigs against intraperitoneal injection of lethal doses of *V. cholerae*. However, as shown by classical investigations of Pfeiffer & Issaeff (1894), the protection afforded in this manner was distinct from the specific immunity produced by the *V. cholerae* by its early appearance and rapid disappearance as well as by the failure of the non-specific organisms to produce cholera-immune sera.

Naturally acquired immunity

While it was maintained by some early observers that persons who had survived a cholera attack had become permanently immune against this infection, Koch (1884) upheld that this acquired immunity

" does not seem to persist for a long time because there is a sufficiency of examples to show that an individual who had been affected during one epidemic fell ill with cholera a second time during another outbreak; but one hears but rarely that somebody had been attacked twice during the same cholera epidemic ". [Trans.]

However, Sticker (1912), emphasizing that several workers had observed cholera attacks in individuals who had recovered from the disease but some weeks previously (" Spätrecidive "), denied the development of a specific immunity against this infection and postulated that the rarity of second attacks was due merely to extrinsic causes, particularly the infrequency and short duration of the epidemics. Harvey (1929), discussing this problem, also adopted a cautious attitude, stating that

" the probabilities against an individual being in a position to contract a second attack of cholera must be great. This does not apply merely to his being in contact with cases of cholera, but to the likelihood of any individual's contracting infection even after the ingestion of the cholera vibrio."

Nevertheless, Harvey felt convinced that cholera attacks produce a naturally acquired immunity, but qualified this statement by adding that " but little information exists as to the longer or shorter duration of that immunity ".

As far as one can judge from the scanty evidence available on this point, it appears that cholera attacks, though not rendering the individuals concerned permanently immune, protect them against the infection for several years. This view was advocated for instance by Salimbeni (1915), who stated that

" the cured cholera patients are without any doubt immune against cholera for a longer or shorter time, because, though one knows of comparatively quite rare instances of individuals who had the disease two or even three times some years apart, as far as I know, no cases have been recorded of persons attacked by well characterised cholera during one and the same epidemic ". [Trans.]

Similarly it was recently stated by Maxcy (1951) that

" an attack of cholera does not necessarily confer protection against a subsequent attack. Nevertheless, second attacks within a period of a few years are uncommon."

A most interesting and important question arising in this connexion is whether, as considered probable by Harvey, the inhabitants of cholera-endemic areas, by suffering from slight and unnoticed attacks, become immune against the infection. It would be highly desirable to study this problem through large-scale investigations made in truly endemic areas with the aid of the now-available immunological and experimental methods.

Dealing with the information then available on the presence of immune bodies in the sera of cholera patients and convalescents, particularly the experiences of Svenson (1909) referred to earlier in this study, Hetsch made in 1912 the following statement :

" The experience that the agglutinin and bacteriolysin content of the blood in man and animals is considerably higher after cholera vaccination than after spontaneous cholera attacks and that nevertheless even after the slightest spontaneous attack the immunity is most considerably higher than after vaccination, justifies the assumption that recovery from cholera produces a local immunity of the intestine, which is not produced to such a degree in animal experiments and through vaccination." [Trans.]

Plausible though this assumption is, it appears that Hetsch, again dealing with the problems of cholera immunology in 1928, laid no more stress upon a local immunity as contrasted to a systemic immunity against the infection. Be this as it may, it is certain that persons who have recovered from cholera can remain immune against the infection, even though no immune bodies are demonstrable in their sera.

Active Induced Immunity

Introductory remarks

The first attempt to confer protection against cholera through a method of active immunization was made by Ferrán (1885) during the epidemic rampant in Spain during 1884. Noting that guinea-pigs which had survived an injection of living cholera vibrios cultivated from faeces in broth, were resistant to administration of further doses lethal to untreated animals, he applied this method to man. Ferrán made for this purpose initial injections of 8 drops of a broth culture of *V. cholerae*, to which bile had been added, and administered at intervals of 6-8 days two further doses of 0.5 ml each.

As summarized by Kolle (1896b) and by Voges (1896), it was soon shown by several workers that Ferrán worked with impure cultures containing only a minority of cholera vibrios besides numerous contaminating organisms. It is not surprising, therefore, that his method of vaccination not only gave no satisfactory results, but often produced severe, according to some observers occasionally even fatal, reactions. Modern writers are nevertheless unanimous in stating that Ferrán, though acting rather injudiciously, deserves credit for having first demonstrated the possibility of an actively induced immunity against cholera.

It was the great merit of Gamaleia (1888) to have first shown that it was possible to protect guinea-pigs against lethal doses of *V. cholerae* not only with the aid of living cultures, the virulence of which had been reduced, but also with growths *killed* by heating at 120°C. Curiously enough, the great practical importance of the latter observation was at first overlooked, Haffkine (1892b) recommending once more a method of cholera vaccination based upon the use of living organisms.

Following the scheme successfully used by Pasteur for rabies prevention, Haffkine used two cholera vaccines of different strength, administering first a " weak virus " obtained through cultivation of cholera vibrios under continuous aeration at 39°C, and five days later a " virus fixe ", consisting of organisms the virulence of which had been exalted through repeated intraperitoneal passage directly from guinea-pig to guinea-pig. As summarized by Hetsch (1912), Haffkine used originally a suspension of a tenth part of a slant, prepared with boiled water, as a dose for adults, and gave to children 1/20th and to infants 1/100th part of a slant, but afterwards repeatedly changed these dosages, using for instance 1/12th and 1/8th of slants of the weak and the exalted virus respectively (Voges, 1896). Later on, in order to reduce the reactions and so to win the good will of the population, Haffkine often used lesser doses (1/20th of a slant).

The difficulties of using Haffkine's method of cholera vaccination on a large scale were tremendous. As he admitted himself (see Kolle, 1896a), it was a particularly heavy task to keep sufficient amounts of " virus fixe " available through continuous animal passages. In large-scale practice it also proved often impossible to administer second doses, so that only one third of the 40,000 persons inoculated up to 1895 in India according to Haffkine's method received these. Nevertheless, as will be discussed in the last of these studies when dealing with the problems of cholera control, some of the records published by him leave no room for doubt that his method was apt " to protect man against natural cholera infection " (Kolle 1896b).

However, while admitting the value of Haffkine's method, Kolle (1896b) maintained that not only difficulties met with in manufacture but also considerations of a principal nature spoke against the use of live cholera vaccines. Believing that the toxins of *V. cholerae* were instrumental in conferring immunity, he considered it unnecessary to use strains of a particularly high virulence for vaccination, because according to the observations of Dungern (1895) virulent cholera cultures were no more toxic than avirulent ones. It has to be noted in this connexion that, since according to the now-accepted views it is the antigenic structure and not the toxicity of the strains used for vaccine manufacture which is of decisive importance, the above contention of Kolle is no more fully acceptable, the use of virulent strains being advantageous at least in so far as these are bound to be smooth and therefore antigenically suitable. However, one

must fully agree with Kolle's contention that the use of live instead of killed cholera vaccines offers no advantage in so far as, according to Haffkine's own observations, the organisms contained in his " virus fixe exalted " succumbed soon after injection. The action of live vaccines depended, therefore, not upon a survival of the organisms but upon the liberation of immunologically active substances contained in their bodies, as Kolle believed, of the toxins.

Far more important than these considerations were the results of comparative determinations of the bactericidal properties of the sera obtained from persons who had been vaccinated by Kolle (1896a, 1897) either three times according to Haffkine's method or once only with killed cholera vaccines, i.e., with suspensions of cholera cultures exposed (*a*) to heating at 56°C for one hour; or (*b*) to the action of chloroform vapours. Kolle established in this manner that the bactericidal titres of the sera derived from the individuals once injected with killed cholera vibrios were as high or even higher than those of the persons thrice injected with Haffkine vaccine. This held true of the determinations made 10 days after the completion of vaccination as well as of those which could be made one year or even longer afterwards. Kolle (1896a) summarized, therefore, that

" since an equal effect is obtained, it is better to use *sterile* cholera vaccines than vaccines containing *living* vibrios, because the manufacture of the latter (production of the cultures) is difficult as well as dangerous. Further, the pain produced by the injections deters many from submitting to a second inoculation. We know through my investigation that *one* injection of a somewhat larger dose . . . produces the same effect as multiple inoculations." [Trans.]

It is somewhat surprising to find that in spite of these observations of Kolle a few subsequent observers again advocated the parenteral use of live cholera vaccines. Thus Nicolle et al. (1912) used living virulent cholera vibrios for intravenous[m] injections.

As summarized by Hetsch (1928), these workers prepared their vaccines by subjecting suspensions of 20-hour-old agar cultures to centrifugation followed by repeated washing and final re-suspension of the sediment, one drop of the fluid thus obtained being adjusted to contain 4 million of *V. cholerae*. This amount, diluted in 50 ml of normal saline, was used as the initial dose for adults and this was followed 10-15 days later by administration of a dose six times stronger.

Nicolle et al. stated that the 36 persons thus vaccinated showed no serious reactions, particularly no diarrhoea. The application of the vaccine led to the abundant formation of antibodies in the sera of these individuals and three of them remained healthy when afterwards given virulent cholera vibrios per os.

[m] The method of *intravenous* administration of cholera or mixed vaccines was also recommended by Quarelli (1917). He found that this method of vaccination, while causing but mild reactions, led to a more rapid and persistent production of antibodies than the subcutaneous method.

Castellani (1913) stated that he had prepared a live attenuated cholera vaccine for subcutaneous injection by heating 48-hour-old cultures for one hour at 48°C or 45°C. However, these vaccines produced much more severe local and general reactions than killed cholera vaccine and a further disadvantage was that the attenuated vaccines had to be used soon after preparation, because, even if heating at only 45°C had been resorted to, the organisms died within two months. One may truly say, therefore, that there is nothing to recommend the use of Castellani's live vaccine in actual practice. The method of Nicolle et al. seems to be capable of producing a solid immunity, but its large-scale use would be fraught with great difficulties and even some danger. It is not surprising, therefore, that —apart from some attempts to practice oral vaccination against cholera— parenteral administration of killed vaccines or, to a lesser extent, of extracts prepared from *V. cholerae* in various ways, has been adopted as the standard practice for large-scale vaccination campaigns. The various methods used for this purpose, and also the problem of oral vaccination, will now be dealt with seriatim.

Agar-grown killed vaccines

Agar-grown killed vaccines have been continuously used for the purposes of cholera control since their recommendation by Kolle in 1896, first on a large scale during the 1902 cholera epidemic in Japan (Murata, 1904; Takano et al., 1926).

While it would be redundant to enter into a detailed description of the technique of preparing agar-grown vaccines, which is set forth in the textbooks on bacteriology and laboratory methods, it is of importance to discuss the following special problems of their manufacture, standardization, administration, and storage.

(1) *Choice of strains.* Dealing with the problem of selecting strains suitable for the manufacture of cholera vaccines, Hetsch (1912) noted that some of the early workers had insisted upon the necessity of choosing highly virulent strains, while others reached a contrary opinion, pointing out that part of the avirulent strains had equally good antigenic properties. Agreeing with the latter view, Hetsch emphasized that the immunizing properties of cholera strains did not run parallel with their virulence and that it was essential, therefore, to select those strains which showed a marked antibody formation in preliminary experiments. However, as will be stated below, some workers have recently again laid stress upon a high virulence of the strains used for the preparation of cholera vaccines. It is certain that the use of freshly isolated (and, therefore, presumably virulent) strains is of great importance in so far as these are unlikely to have undergone a loss in antigenic properties through roughening.

Though the phenomena of dissociation were still unknown at the time, it can be gathered from the summary of Hetsch (1928) that most workers engaged in the manufacture of cholera vaccines during the first World War insisted upon the use of freshly isolated strains. It was usually recommended to select several of these for the preparation of polyvalent vaccines.

Schwarz (1919), one of the workers quoted by Hetsch, aptly distinguished between vaccines manufactured from strains isolated locally during a cholera outbreak (" epidemieeigene Impfstoffe ") and those prepared from strains of a heterologous origin (" epidemiefremde Impfstoffe ")—either polyvalent vaccines or vaccines made from single selected strains. The criteria for choosing such specially suitable strains, established by tests with individually prepared vaccines, were according to Babes (1914) (*a*) absence of a severe reaction after vaccination, and (*b*) marked immunizing properties, as shown through tests in guinea-pigs injected simultaneously with dilutions of the sera of persons who had been given doses of the vaccines in question, and lethal doses of *V. cholerae*.

In the experience of Schwarz it was of importance to use vaccines prepared locally during the epidemic to be dealt with. He noted in this connexion that individuals who contracted infection even though they had been injected with such vaccines, had slight attacks or merely became carriers of *V. cholerae*, whereas usually severe forms of the disease were observed in persons who fell ill with cholera though they had received injections of heterologous vaccines.

The necessity of using fully smooth cholera strains for vaccine manufacture was stressed by Steward (1933). He noted that in actual practice during the off-seasons eight subcultures were made from a recently isolated strain, which were kept in the refrigerator[n] and successively used to prepare vaccines. During the epidemic seasons freshly isolated smooth strains were used for this purpose and were frequently replaced by strains of the same character. Thus, as Stewart stressed, the old method of preparing cholera vaccines from stock strains had been given up. Many of the subcultures made from the latter showed roughness.

Dealing again with the problem of selecting cholera strains for vaccine manufacture, Taylor et al. (1936) stated that:

" The maximum degree of protection in animals against infection with strains of the prevailing serological type is obtained by the use of vaccines prepared from strains which show both ' H ' and ' O ' agglutination with a serum of the Japanese ' original ' type and which also show the chemical structure (Linton's groups I and II) characteristic of the majority of agglutinable vibrio strains isolated from cases of cholera in India. Agglutinable strains from carriers and agglutinable variants produced from strains of origin other than cholera cases give a lower degree of protection."

[n] The statement made in the text of Stewart's article that these subcultures were kept in the *incubator* is obviously due to an error in translation, because he noted that, in contrast to cholera cultures kept in the refrigerator, those left at room temperature or in the incubator had a great tendency to become rough.

Quoting recommendations made by the Cholera Advisory Committee of the Indian Research Fund Association, Taylor (1941) stated more specifically that:

" The strains ordinarily used in manufacture of (cholera) vaccine should show the following characters:

(a) Typical smooth translucent colony appearance.

(b) Stable in salt solution.

(c) Serological characters of O group I (Gardner and Venkatraman) sub-type Inaba and should agglutinate to titre with a serum prepared against the dried O Inaba antigen issued from the Standards Laboratory, Oxford.

(d) Producing acid from mannose and saccharose but not from arabinose.

(e) Non-haemolytic."

Taylor added that in the opinion of the Cholera Advisory Committee there was no evidence to show whether the use of multiple strains for cholera vaccine manufacture was necessary or not. However, since in certain areas strains of the Ogawa subtype had been isolated from a considerable number of cholera cases during epidemics, it was in the opinion of the Committee " for consideration whether strains for the Ogawa subtype should be incorporated in the vaccine and this is the practice in the Madras Presidency ".

Yu (1938, 1942) laid emphasis upon selecting fully virulent strains for the manufacture of cholera vaccines.

He recorded in this connexion the following results obtained when (*a*) injecting groups of mice at intervals of 3 days with 2 doses of heat-killed cholera vaccines (1,000 and 2,000 million respectively), which had been prepared individually from 6 strains varying in virulence, and (*b*) challenging the animals 20 days afterwards with 3 MLD of one of these strains:

Vaccine	*Virulence of organisms*	*Number of mice tested*	*Survived*
(a)	++++	40	40
(b)	++++	40	38
(c)	+++	40	31
(d)	+	47	18
(e)	−	48	18
(f)	−	20	8

Yu added that stock cholera cultures were not suitable for vaccine manufacture because they were not perfectly smooth. They could be rendered negative to Millon tests by repeated mouse-passages but these led to only a slight rise of the virulence of the strains.

During the following years considerable attention was paid to the question of whether a cross-protection existed between the Inaba and Ogawa sub-types of *V. cholerae* and whether, consequently, monovalent cholera vaccines or vaccines consisting of a mixture of equal parts of vaccines prepared from Inaba and Ogawa strains respectively should be issued.

Ranta & Dolman (1944) recorded in this connexion the following observations:

" . . . Each of 50 mice was inoculated with the prescribed two doses of vaccine prepared from a single Inaba-type strain. A fortnight after the final dose, half of this vaccinated group was challenged with 10 m.l.d. of the Inaba-type strain, and half received 10 m.l.d. of an Ogawa-type strain. A similar number of mice, inoculated with 2 doses of Ogawa-type vaccine were divided into two groups, which were challenged with 10 m.l.d. of Inaba- and Ogawa-type vibrios respectively. All vaccinated mice survived."

Though in the opinion of Ranta & Dolman these findings did not necessarily imply that the type-specific O antigens played a part in mouse-protection, they pointed nevertheless to the existence of a cross protection between the two subtypes of *V. cholerae*.

This postulation was fully supported by Burrows, Mather, Elliott & Havens (1947) who concluded from large-scale active and passive immunization experiments that there existed " complete cross protection between vibrio types ".

For a further study of this problem, Ahuja & Singh (1948) injected guinea-pigs subcutaneously with two doses of cholera vaccines prepared respectively from strains of the two sub-types or with a mixed Inaba-Ogawa vaccine and challenged the animals 10 days after the second injection intraperitoneally with mucinized suspensions of live cholera vibrios of either the Inaba or the Ogawa subtype. Tabulated, the results of these tests were as follows:

Vaccine used	*Number of guinea-pigs immunized*	*Type of challenge strain*	*Survivals up to 96 hours*	
			Number	(%)
Inaba	15	Ogawa	14	93.0
Inaba	15	Inaba	14	93.0
Ogawa	14	Ogawa	14	100.0
Ogawa	15	Inaba	13	89.0
Inaba+Ogawa	15	Ogawa	15	100.0
Inaba+Ogawa	15	Inaba	15	100.0

Note : None of the 30 non-vaccinated controls challenged with either the Inaba or Ogawa type vibrios survived.

Though considering that the results obtained with the mixed vaccine were not significantly different from those obtained with the two monovalent vaccines, Ahuja & Singh concluded that

" On the basis of these findings the use of both sub-types of *V. cholerae* for the preparation of prophylactic cholera vaccine would be more satisfactory than the use of either an Inaba or an Ogawa sub-type alone ".

Dealing not only with the problem presently under review but with the selection of strains for the purpose of cholera vaccine manufacture in general, Pandit (1948) made the following important statement:

" In view of the evidence . . . regarding the prevalence of subtypes of vibrios in India, the vaccines used in the country are prepared from both the types of vibrios, particularly

those isolated from fatal cases of cholera. It is customary in most laboratories to replace the strains used by new ones as they are isolated. Pending further information on the question of virulence of vibrios, this procedure was considered to be the most suitable for adoption in the manufacture of cholera vaccines. However, it would seem that with the development of the technique for the measurement of antigenicity of vibrio strains, it should be possible to select such strains only for vaccine production as show a sufficient high degree of antigenic potency. Recently Ranta & Dolman (1944) and subsequently Burrows and his collaborators (1947, p. 157) obtained evidence to show that practically complete cross protection exists between the two sub-types of cholera vibrios. However, recent observations by Venkatraman in the King Institute tend to show that this may not always be the case, particularly if minimal quantities of antigens are used for protection."

As will be discussed below, the conclusion tentatively reached by Venkatraman was vigorously supported by Sokhey & Habbu (1950b), who denied that a cross protection existed between the Inaba and Ogawa subtypes.

Under these circumstances it seems indicated for the present to use both Inaba and Ogawa strains for the manufacture of cholera vaccines destined for wide distribution. Though, as shown by the observations of Burrows, Mather, Elliott & Havens (1947) and of Sokhey & Habbu (1950b) the virulence of cholera cultures may be preserved for prolonged periods through freeze-drying (lyophilization), it is certainly best to replace the strains used for vaccine preparation by recently isolated ones whenever possible.

(2) *Cultivation methods.* While generally speaking the high quality agar media available for diagnostic work are also utilized for the manufacture of cholera vaccines, some workers recommended for the sake of economy cheaper media, for instance one prepared with 3% yeast instead with meat or meat extracts and peptone (Fischer et al., 1915). However, Ungermann (1917), giving a systematic description of the methods of cholera and typhoid vaccine manufacture in the Berlin Gesundheitsamt, warned against the use of cheap substitutes, particularly prefabricated meat extracts which, because possibly made from meat of doubtful freshness, were apt to contain products of protein decomposition and thus to cause untoward reactions in the vaccinated. Since, moreover, media made with fresh beef gave a more abundant growth than those prepared with horsemeat, the former alone were used for vaccine manufacture in the Gesundheitsamt. However, as shown by the excellent quality of the cholera vaccine made in the Kasauli Institute in India, in countries where the use of beef for media preparation is out of question, mutton digests are apt to prove equally advantageous.

While, in general, Roux bottles or similar containers or, as recommended by Ungermann, large covered glass dishes (diameter 21 cm) are used in vaccine manufacture, Fischer et al. (1915) advocated the use of tin dishes. They admitted, however, that these became rusty after they had been used several times.

Ungermann claimed that more abundant yields were obtained when, instead of suspensions made directly from agar subcultures, *ad hoc* subcultures made from these in broth (" Bouillonvorkulturen ") were used for seeding the flasks or dishes. He adduced as explanation that in agar cultures of *V. cholerae* even after a growth for only 24 hours a part of the organisms was no more viable, whereas in broth the overwhelming majority of the vibrios was still in the stage of multiplication at that time.

As generally agreed, an incubation at 37°C for 20-24 hours is suitable for the mass production of *V. cholerae* in the course of vaccine manufacture.

(3) *Killing methods.* The first and until more recently most amply used method for killing the vibrios in the course of cholera vaccine manufacture was to expose the organisms to heat. As noted above, Gamaleia (1888) resorted in his pioneer work to a temperature of 120°C. Fairbrother (1928) established that the substance of *V. cholerae* which on inoculation gave rise to protection in animals was heat-stable, withstanding an exposure to 100°C for one hour. Uyeda (1922) claimed that a " koktoantigen," i.e., the supernatant obtained through centrifugation of *V. cholerae* suspensions which had been boiled for 15-30 minutes, was the best cholera vaccine.

Though it is not possible to share this view, it is noteworthy that according to the observations of Taylor (1936), Burrows, Mather, Elliott & Havens (1947) and Singer (1948b) prolonged boiling exerted no untoward influence on the immunogenic power of cholera antigens.

Notwithstanding these observations, the general tendency has been to use for heat-killing the organisms in actual cholera vaccine manufacture even lower temperatures than that of 56°C recommended by Kolle in 1896. Haffkine (1913), who in about 1911 began to use a killed cholera vaccine, resorted for its preparation to an exposure of the vibrio suspensions to only 50°C for a few minutes, but followed this procedure by the addition of 0.5% phenol. Generally speaking, however, temperatures of 53°C to 54°C have been applied for the period of one hour. If carefully implemented, this method gives fully reliable results, particularly if, according to the generally adopted practice, phenol to a final concentration of 0.5% is added as soon as initial samples have been withdrawn to test the sterility of the brews.

As summarized by Hetsch (1928) and Harvey (1929), methods to effect sterilization of agar-grown vaccines through the addition of antiseptics instead of through the application of heat have been recommended by several workers, the substances used being—besides 0.5% phenol—chloroform, ether, formol, glycerol, hydrochloric acid, and quinine. Singer et al. (1948b) found an alcohol-killed vaccine as satisfactory as those killed by heat or other methods.

Tentative use of 0.5% phenol was made already early in his work by Haffkine with the idea of abating the virulence of the cholera strains used for the preparation of his " weak virus ". However, as noted by Hetsch (1912), it was soon shown by some other workers that this chemical exerted a sterilizing instead of an attenuating effect. Large-scale advantage of the method of sterilizing cholera vaccines with phenol alone was afterwards taken by many workers, e.g., those in Japan (see Takano et al., 1926), and this procedure is still widely used. As quoted by Taylor (1941), the Cholera Advisory Committee of the Indian Research Fund Association recommended in this respect that :

" The vibrios should be killed by the addition of 1 per cent phenol to the suspension without the application of heat. The phenol should be reduced to 0.5% in the vaccine finally issued."

With the exception of formol which, as will be noted below, has been used to a considerable extent for the sterilization not only of agar-grown but also of other types of cholera vaccines, the other chemicals enumerated above have not been used routinely in the manufacture of such vaccines.

(4) *Standardization methods.* Introducing the method of cholera vaccination with agar-grown vaccines, Kolle (1897) recommended that these ought to contain one " normal " loop or 2 milligrams of fresh culture mass per millilitre. To ensure this standard or that of 2 loops (4 mg) per ml required in Germany at the time of the first World War (see Ditthorn & Loewenthal, 1915; Fischer et al., 1915), some workers merely computed the number of loops by determining the surface area of the media used for cultivation, assuming that one agar slant yielded 10 loops of culture mass, a Petri dish of a diameter of 8.8 cm, 66 loops, etc. (Soltmann, 1915). However, this rather crude method of standardization was criticized by other workers, for instance by Ungermann (1917) who pointed out that (*a*) successively prepared media were apt to give different yields, and (*b*) more important still, the abundance of growth depended not merely upon the surface area of the media, but also upon their mass and differed, therefore, according to the thickness of the agar layers. For these reasons the above-described method of standardization has been given up in favour of determinations of the bacterial contents of vaccines with the aid of gravimetric or counting procedures, or by opacity tests.

General agreement has been reached that the method of weighing in the course of vaccine manufacture the *moist* culture masses does not yield exact results, mainly because their weight depends upon a large and variable water content rather than upon their bacterial content. As shown by careful observations such as those of Brown (1914, 1919) and of Ungermann (1917), determinations of the *dry* weight of the bacterial masses give fully reliable results, but this method is not only rather tedious but of limited value in so far as, whether rightly or wrongly, standard values of bacterial

vaccines are usually given not in terms of weight but in numbers of the organisms per ml, which have to be determined with the aid of counting methods.

As far as the preparation of cholera vaccines is concerned, only two of the latter methods have been used on a large scale, namely, that introduced by Wright in 1902 and haemacytometer counts recommended about the same time (see summary by Soltmann, 1915). As is generally known, the principle of the former method is to mix equal volumes of the bacterial suspensions to be tested and of fresh human blood in a capillary tube, to prepare stained smears from this mixture and to compare the number of bacteria present with that of the blood corpuscles. Since it may be taken that 5 million of the latter are present per ml, this standard value can be used easily to compute the number of organisms per millilitre.

Though the present writer for one cannot agree with the assertion sometimes made that Wright's method gives inconsistent results, it is generally admitted that the number of organisms elicited with its aid is lower than that actually contained in the bacterial suspensions under test. There can be little doubt, therefore, that haemacytometer counts, which give exact values, are preferable for vaccine standardization but unfortunately, simple as the implementation of this method seems at first glance, it is fraught with considerable technical difficulties and thus reliable only in the hands of experienced workers. It is under these circumstances of great importance that, as shown by ample experiences, results approaching or even equalling in exactness those obtained by dry weight determinations or properly made counts may be obtained with the aid of opacity tests.

No doubt it would be simplest to carry out the latter by comparing the opacity of the bacterial suspensions under examination with that of previously prepared vaccines. However, though used by some workers, in the experience of most observers this method is as unreliable as it is expedient, because bacterial vaccines, quite particularly cholera vaccines, are apt to undergo a process of autolysis, thus changing in aspect and density. It is necessary, therefore, to carry out the opacity tests with the aid of standard suspensions of a non-bacterial nature, preferably those of a stable character which can be used on successive occasions.

Various substances have been suggested or actually used for this purpose. Ungermann (1917) noted in this respect that emulsions of lecithin in water, though formerly recommended for opacity tests, possessed in higher concentrations a colour of their own which interfered with their suitability for the standardization of cholera vaccines. He recommended, therefore, the use of alcohol dilutions of a 10% solution of dry Canada balsam in benzol. A suspension corresponding in opacity to that of cholera vaccines of the officially prescribed strength could be obtained by mixing 0.6 ml of the balsam-benzol solution (which was stable) with 9.4 ml of absolute alcohol. Results with this method were so satisfactory that it alone was used in the

Berlin Gesundheitsamt for the standardization of cholera and typhoid vaccines.

As shown by some other workers, particularly through the exhaustive studies of Brown summarized in his publication in 1919, reliable results in the standardization of bacterial vaccines could be obtained through opacity tests made with the aid of standard barium sulfate suspensions. According to Brown, these quite stable suspensions could be prepared as follows :

" A strong solution of barium chloride is made and to this is added an excess of sulphuric acid. The mixture is then boiled and the precipitate is poured on to a filter paper and is washed with tap water until the filtrate is neutral to litmus paper.

" The barium sulphate is then dried and thoroughly roasted. When cool a portion is accurately weighed and placed in a perfectly clean mortar. The powder is finely ground and the requisite amount of 1 per cent aqueous solution of sodium citrate is gradually added. From this 1 per cent suspension of barium sulphate an 8-fold dilution is made and similarly the other dilutions, the sodium citrate being used throughout as the diluent fluid."

A set of 10 tubes was thus prepared, the first containing the above-mentioned 8-fold dilution, the second 9 volumes of the same dilution and 1 volume of citrate solution or, in comparison to the first tube, 90% barium sulfate and so on, the last tube containing 1 volume of the original barium sulfate suspension and 9 volumes of sodium citrate solution.

Brown furnished the following figures showing the relationship of opacity to the weight of dried bacterial substance, expressed in milligrams per ml of *V. cholerae* suspensions :

Percentage of $BaSO_4$ suspensions [a]	*Serial number of opacity tube*	*Weight of vibrios (mg per ml)*	*Numerical equivalent of vibrios (millions)* [b]
100.0	10	2.22	10,926
90.0	9	2.00	9,833
80.0	8	1.80	8,741
70.0	7	1.55	7,648
60.0	6	1.33	6,556
50.0	5	1.11	5,463
40.0	4	0.90	4,370
30.0	3	0.67	3,278
20.0	2	0.44	2,185
10.0	1	0.22	1,093

Remarks. [a] As defined in text

[b] As established with haemacytometer tests by Cunningham & Timothy (1924)

As stated by Gardner (1931), bacterial vaccines may be roughly standardized as follows :

" A barium sulphate suspension is made by mixing equal parts of M/100 H_2SO_4 and M/100 $BaCl_2$. This is shaken and distributed into a series of 6 by 5/8 in. test tubes covering the maximum variation in diameter of the stock in use. They are then sealed. The measured bacterial suspension is diluted in a test tube until it is equal in opacity

to the standard in a tube of the same diameter. The bulk is diluted to the same extent or retained as a known multiple of the standard. Equal opacity is judged by viewing a small luminous flame through the tubes. The opacity will be equal in each if the image is obscured in each at the same distance from the flame."

Joetten (1917), dissatisfied with the exactness of all the above-mentioned methods, tested cholera and typhoid vaccines with the aid of complement-fixation tests, using the vaccines as antigens and a bacteriolytic cholera-immune serum as amboceptor. Though he found that vaccines which appeared to be of uniform value gave identical results in such tests, the implementation of this method in the course of mass vaccine production would be fraught with considerable difficulties. The same holds true of the important proposal of Gallut (1949c) to standardize cholera vaccines by determining the weight of the O antigen extracted with the aid of trichloracetic acid. Gallut maintained in this connexion that only strains with an adequate yield of O antigen, equalling at least 5% of the total dry weight of the organisms, should be used for cholera vaccine manufacture.

(5) *Dosages.* Kolle (1897), finding that a single administration of his vaccine, standardized to contain 2 mg of culture-mass per ml, produced a satisfactory antibody response in a group of volunteers, maintained that vaccination with such single doses was sufficient for the purposes of cholera control. As noted before, actual use of this method was first made during the 1902 epidemic in Japan. Reporting on this work, Murata (1904) stated that the dosage recommended by Kolle, though giving good results in general, was not invariably sufficient, a number of the vaccinated contracting the infection. He soon resorted, therefore, to single administrations of 1-ml doses of a vaccine of double strength (4 mg of culture-mass per ml) and found that none of the persons thus protected contracted cholera.

As has been noted above, a standard of 4 mg culture-mass per ml of cholera vaccine was also made obligatory in Germany, but this vaccine was administered to the armed forces during the first World War in *two* doses, usually of 0.5 ml and 1 ml, the second injection being given after an interval of 5-7 days. According to Takano et al. (1926), a 2-dose system of cholera vaccination was also soon adopted in Japan where, however, 1 ml and 2 ml respectively of a heat-killed and phenolized vaccine containing only 2 mg of the vibrios per ml were administered.

While the principle that, in order to confer a satisfactory degree of protection against cholera, a 2-dose system of vaccination should be adopted, has been generally accepted, in the experience of many field workers it is often impossible to act accordingly in large-scale vaccination campaigns. This situation has been well described by Russell (1935) who stated that

" The Cholera Commission of the ' Office international ' has recently reiterated its view that ' while vaccination by a single injection is of some value and can be employed in circumstances in which it is the only practicable method, vaccination by two injections

should remain the method of choice.' In countries like India, however, where the number of inoculations to be done urgently, may run into many thousands, it is usually impossible to give more than a single inoculation and the common practice is to give a single dose of 1 cc."

It is clear that in order to obtain the best possible results with the system of single-dose vaccination, which had to be adopted for large-scale campaigns not only in India but also in other countries, for instance in China, it is essential to use a high-standard vaccine so as to avoid the administration of too low dosages—a practice unfortunately not rarely resorted to—or to obviate the considerable drawbacks of injecting amounts in excess of 1 ml. This desideratum has been satisfactorily fulfilled in India, where a standard of 8,000 million of *V. cholerae* per ml has been adopted. In some other countries, however, cholera vaccines of lesser strength were used, e.g., in China often one of only 2,000 million of organisms per ml, administered as a rule in amounts of 1 ml. It was only after Dzen & Yu (1936) had demonstrated the inadequacy of this method through large-scale guinea-pig experiments, that better counsel prevailed, and Robertson & Pollitzer (1939), working under the auspices of the League of Nations, were able to introduce a vaccine with a vibrio content of 6,000 million per ml—a standard afterwards widely adopted in China. However, while single-dose administration of this vaccine gave satisfactory results, the present writer wishes to emphasize once more that this system of cholera vaccination was adopted merely out of necessity and that it ought to be replaced whenever possible by the administration of two vaccine doses.

(6) *Keeping qualities.* As confirmed by numerous observations, for instance by the special studies of Ungermann (1917), killed vaccines, quite particularly cholera vaccines, undergo a soon-commencing and progressive process of disintegration of the bacterial cells.

As maintained by Lange (1922), this process, though often designated as *autolysis*, is really one of *cytolysis* and thus fundamentally different from autolysis in the strict sense, due in Lange's opinion to a decomposition of the bacterial protein through the action of ferments, which had not been destroyed during the process of killing the vaccines by moderate heat nor by the subsequent addition of 0.5% phenol and were thus able to produce a progressive and fargoing reduction of the antigenic properties of the vaccines. Since it was inadvisable to destroy these ferments by the application of higher temperatures, Lange recommended that formol ought to be used to kill the organisms and to destroy the ferments at the same time. He evidently assumed that autolysis in the strict sense frequently took place in cholera vaccines killed by other methods, but this is not supported by the experiences of other workers quoted below.

In contrast to the contentions of Lange, Dold (1925) maintained that less " autolysis " took place in heat-killed vaccines than in those sterilized by other methods, both because a kind of protein coagulation took place at temperatures of about 60°C and because such temperatures damaged the autolytic ferments.

A further study of this problem was made by Gohar & Makkawi (1948). Finding that some of the numerous brands of vaccine used for combating the 1947 cholera epidemic in Egypt after a few weeks' storage no more conformed to the prescribed standard of 8,000 million per ml, these two workers compared the lysability of cholera vaccines killed and preserved in different ways, making for several days daily counts with a photoelectric colorimeter. It was thus established

" that heat-killed vaccines were by far the most stable, apparently because of the adequate coagulation of protein by the heat. Formalin-killed vaccines were also stable but they keep best when preserved with merthiolate instead of phenol. The phenol-killed and preserved vaccines were the least stable."

It is generally held that the cytolysis progressively taking place in killed cholera vaccines does not lead to an accompanying loss of their antigenic properties. Several workers maintained on the contrary that the " opening-up " of the bacterial bodies enhanced the efficacy of the vaccines. Be this as it may, it is certain that the rapidly commencing process of cytolysis soon leads to changes in the aspect and density of the vaccines, thus rendering them unfit for standardization by opacity tests. Raju (1930), who made a special study of this subject, found that in the course of four weeks the average opacity standards of 29 samples of phenol-killed cholera vaccines kept at room temperature (90°F) (32°C), and of 18 such samples kept in cold storage became lowered as follows :

Time of testing (days after preparation)	*Average opacity in millions per ml of samples kept at room temperature (90°F) (32°C)*	*in cold storage (32°F) (0°C)*
0 (freshly made)	49,000	49,000
1	29,000	41,000
2	25,000	39,000
3	19,000	38,000
4	18,000	38,000
6	16,000	37,000
14	15,000	36,000
21	13,000	33,000
28	12,000	32,000

These observations fully justify the plea of Raju that opacity tests ought to be made on the day of preparation of the vaccines and not a few days afterwards, as it was the practice in some laboratories. Raju added that, as shown by comparative tests, exposure to light exerted no influence on the alterations in opacity, but it altered the colour of the samples, giving them a brown tinge.

Though the ample observations made in regard to the keeping qualities of cholera vaccines at the time of the first World War in Europe are not always fully comparable in view of differences in the methods of preparation and of testing, it can be gathered that, in the opinion of a majority of the workers there, storage for periods of one year or even longer did

not cause a loss in potency of the vaccines. Nevertheless, Hetsch (1928), commenting upon these observations, considered it as unadvisable to utilize products older than six months to one year. He maintained, on the other hand, that cholera vaccines which had been stored for 3-5 weeks produced less marked reactions in man than those used immediately after manufacture.

In order to establish whether the formerly adopted policy in India to discountenance the use of cholera vaccines stored for periods longer than six months was justified, Maitra & Ahuja (1932) injected rabbits with a vaccine which had been stored for the period of one year either at 37°C or in a refrigerator at 4°C and determined the agglutinin titres in the sera of these animals. An analysis of the results showed that

" a temperature of 37°C does not cause any appreciable deterioration in the agglutinogenic power of the vaccine. Agglutinin titre of freshly prepared vaccine—administered within a week of its preparation—is about the same as that of one year vaccine stored under above experimental conditions."

Though storage in the refrigerator was found to be more efficient than that in the incubator, it appeared unnecessary, therefore, to take special precautions for the preservation of cholera vaccines for periods of at least one year under temperatures not exceeding 37°C. Under these circumstances, the possibility of an extension of the storage period for such vaccines up to 12 months after their manufacture seemed to deserve serious consideration.

Evidence not only confirming but even amplifying these experiences was obtained by Taylor et al. (1936) through a series of protection tests in guinea-pigs with a phenol-killed cholera vaccine, made either soon after manufacture or after storage for various periods. It was found that

" vaccines stored up to two years in the plains of India and exposed to hot weather temperature of 111°F. [44°C] or over give protection practically equal to freshly made vaccine".

Hence, as far as the knowledge gained through these experiments could be applied to human vaccination, a considerable extension of the six-months' storage period for cholera vaccines appeared to be permissible even if no cold storage facilities were available.

It is of interest to add that, in order to prolong the storage periods, a few workers such as Chiba (1922), Gutfeld (1922), and Pasricha et al. (1941) recommended the use of cholera vaccines which had been exsiccated. However, in view of the remarkably good keeping qualities of the usual vaccines, there seems to be no urgent call for the manufacture of such dry cholera vaccines.

Whole fluid culture (direct) vaccines

Though Brieger & Wassermann demonstrated in 1892 already that administration of broth cultures, which had been killed by heating for

15 minutes at 65°C, rendered guinea-pigs immune to infection with virulent cholera vibrios, it was but recently that direct cholera vaccines, prepared from whole fluid cultures, were recommended or actually used for human vaccination.

Jennings & Linton (1944) obtained by (*a*) cultivating *V. cholerae* under continuous aeration with a mixture of air and 20% v/v of CO_2 in a glucose-containing casein-digest medium, and (*b*) sterilizing the growth after an incubation for 24 hours through addition of phenylmercuric nitrate or acetate at the rate of 1 g per litre-batch of brew, a vaccine with a turbidity of 5 to 10 thousand parts per million of silica and a nitrogen content of about 0.05 per ml. They stated that this product, injected subcutaneously in 0.1 and 0.2 ml doses into human volunteers, caused no objectionable reactions, but that the serum of these individuals was as efficacious in mouse-protection tests as the sera of persons inoculated with a cholera vaccine of the usual type. It has to be noted, however, that this claim was not confirmed by Sokhey & Habbu (1950a), according to whose comparative tests the vaccine of Jennings & Linton possessed only 1/20th of the protective power of the casein-hydrolysate vaccine described below.

As set forth by Sokhey & Habbu (1950a), the shortage of agar supplies in India during the second World War necessitated a search for a liquid medium suitable for the manufacture of a direct cholera vaccine.

A casein hydrolysate medium, prepared according to detailed specifications of Sokhey et al. (1950) was chosen for this purpose, in which highly virulent cholera vibrios of the Ogawa and Inaba subtypes respectively were cultivated at 37°C for 3 days. 4 ml of 10% formol were then added per flask of 500 ml to make a strength of 0.08% and the flasks were again incubated at 37°C for three days. Then, after samples for testing sterility had been withdrawn, 15 ml of 0.05% phenylmercuric nitrate were added per flask as a preservative. The two monovalent vaccines thus prepared were then mixed in equal quantities and put into ampoules.

Sokhey & Habbu considered it as indispensable to prepare in this manner a divalent vaccine because in their experience (see also Sokhey & Habbu, 1950b) there was little cross protection between the Inaba and Ogawa subtypes.

The casein-hydrolysate vaccine was standardized by a method of biological assay (Sokhey & Habbu, 1950b), to which further reference will be made later in this study. The two workers noted in this connexion that, as shown by haemacytometer counts, their vaccine contained not more than 3,000 million organisms per ml. Though admitting that this count perhaps did not represent the total bacterial content, because vibriolysis was likely to have taken place during the prolonged period of manufacture, Sokhey & Habbu drew attention to the possibility that metabolites of *V. cholerae* might play a role in immunizing mice.

Making comparative tests with their new vaccine on the one hand, with Jennings and Linton's direct cholera vaccine and three samples of

agar-grown cholera vaccines manufactured in India on the other hand, Sokhey & Habbu noted that the casein-hydrolysate vaccine had a considerably higher mouse-protective power than any of the other products examined. They ascribed the low protective power of the agar-grown vaccines to a lessened virulence of the strains used for their manufacture, but claimed that

" even when virulent strains were used for the preparation of agar-grown vaccines, these were found to have 1/6th to 1/9th of the protective power of casein hydrolysate vaccines made from the same strains ".

Though the casein-hydrolysate vaccine has been amply used for the purpose of cholera control in India since 1945 with apparently good success, so far no large-scale statistics have become available to assess its value in the field as compared to that of agar-grown vaccines. Comparative tests on mice, made in the King Institute, Madras, with (*a*) the agar-grown vaccine produced in that institute, and (*b*) casein-hydrolysate vaccine, failed, according to Pandit (1948), to show a significant difference in the antigenicity of the two products, the animals immunized with either " withstanding approximately 100 times more of the challenge culture than the unprotected mice ".

Wahba (1952) compared the efficacy of (1) an agar-grown and formol-killed vaccine containing 8,000 million of cholera vibrios per ml, and (2) a fluid vaccine prepared by growing *V. cholerae* in a casamino-acid medium, which, being also formol-killed, had a titre of 5,000-6,000 organisms per ml. Though mouse-protection tests showed no superiority of the fluid vaccine, Wahba stressed the simplicity of its manufacture.

Comparative tests similar in scope to those described above were made by Ranta & McCreery (1953) with (1) the agar-grown and phenol-killed cholera vaccine prepared according to Ranta & Dolman (1943) with a titre of 8,000 million vibrios, and (2) a vaccine produced by cultivating the organisms in the chemically defined fluid tyrosine-asparagin-glycin medium of Ranta & McLeod (1950), diluting the growth to contain 8,000 million vibrios per ml and adding phenol to a final concentration of 0.5% for the purpose of sterilization. The direct vaccine (2) protected mice as well as the agar-grown product but was found to be less stable because it underwent rapid autolysis. Ranta & McCreery suggested, however, that it might be possible to overcome this drawback by the judicious use of formol.

It is of interest to add that Felsenfeld et al. (1950) recommended the sterilization of broth-grown cholera vaccines through the action of antibiotics. Neomycin was found to be most suitable for this purpose.

Vaccination with supernatants

Making comparative tests in animals, Fairbrother (1928) found that the supernatant fluid of 24-hour-old broth cultures of *V. cholerae* obtained

through centrifugation possessed but feeble immunizing properties, which were probably due to an incomplete removal of the organisms.

This experience was confirmed through further observations in India, Russell (1935) stating in this connexion that a

" study of the immunizing value of cholera vaccines prepared (*a*) from bacterial deposit, (*b*) from the supernatant fluid, and (*c*) from a mixture of deposit and supernatant fluid has revealed that vaccines prepared from supernatant fluid are not only very toxic but possess little protective value. Those prepared from the bacterial deposit are highly protective though slightly less so than vaccines prepared from the whole emulsion."

Vaccination with culture filtrates

As summarized by Hetsch (1912), some early workers, first apparently Vincenzi (1892), demonstrated through animal experiments that sterile filtrates of older cholera cultures in broth possessed immunizing properties.

A further noteworthy attempt to produce a cholera vaccine with the aid of candle filtration was made by Strong (1903, 1904) who kept for this purpose heat-killed suspensions of *V. cholerae* in saline for 3-5 days at 37°C before filtering them. It was not possible to gather sufficient experience as to the efficacy of this vaccine in the field. As shown by tests in laboratory animals and in man, the product gave rise to agglutinins and bactericidins, but exerted only a slight antitoxic action.

Analogous laboratory experiences were made by Bertarelli (1905) with a vaccine prepared according to a method similar to that of Strong.

Besredka & Golovanoff (1923) produced a cholera " antivirus " by (*a*) candle-filtering cholera cultures which had been incubated for 8-10 days; (*b*) re-seeding the filtrates with the homologous organisms and again incubating for about 8 days at 37°C; and (*c*) filtering once more.

When guinea-pigs which had received 1-2 ml of the final filtrate intracutaneously, subcutaneously, intraperitoneally, or intravenously, were challenged 24 hours later with 1/10th of a 24-hour-old virulent agar-slant culture of *V. cholerae*, the majority of the animals survived. Controls which received in place of the cholera antivirus plain broth or staphylococcus filtrates, succumbed rapidly.

The antivirus was found to be heat-stable; in fact heating for 20 minutes at 100°C seemed to enhance its activity. The protection conferred by it, which in the case of intravenous administration was sometimes manifest already after 12 hours or even after 6 hours, remained complete for 15 days, but then disappeared in the course of the following week.

In a subsequent paper, Golovanoff (1924a) reported on comparative tests with the sera of rabbits which had been injected with antivirus preparations and with heat-killed cholera vibrios respectively. It was found that, though endowed with immunizing properties, the antivirus preparations did not produce agglutinins at titres higher than 1:100.

In order to show that the action exerted by the cholera antivirus was of a specific character, Golovanoff (1924b) injected groups of guinea-pigs with filtrates prepared from *V. cholerae* and heterogenous organisms respectively, and afterwards challenged all of these animals with lethal doses of cholera vibrios. The filtrates of some of the heterogenous cultures (*Ps. pyocyanea, Proteus vulgaris,* and *Chromobacterium prodigiosum*) were found to confer an unspecific protection to animals challenged with *V. cholerae* after 24 hours, but to none of those challenged 4 days after administration of the heterogenous filtrates. All guinea-pigs injected with cholera antivirus survived, regardless of whether they had been challenged after one or four days.

Important immunological studies on cholera filtrates were undertaken by Singer et al. (1948a), who used for this purpose either Seitz-filtered cultures of *V. cholerae* or obtained material from agar cultures by cutting the media, extracting the juice by pressure through several layers of cloth, and then centrifuging at high speed before resorting to filtration.

The technique used by these workers to study the activity of the filtrates was as follows:

" Guinea-pigs of approximately 300 g weight are bled, the abdomen is opened and the ileo-coecal junction is located. The ileum is excised, freed of mesentery and put into a dish with Tyrode solution. The contents of the ileum are then removed by fitting a syringe into one end of the intestine and rinsing with Tyrode solution. A glass rod of suitable size is passed through the ileum and one end of the intestine is tied to the rod. With a gentle stripping motion the intestine is inverted over the glass rod so that the epithelium faces outwards. The ileum is washed in three changes of Tyrode solution, placed on several layers of thick filter paper which have been soaked with Tyrode solution and cut into pieces of approximately 3 mm in length.

" One half ml portions of the solutions to be tested are placed in test tubes and one piece of ileum is added to each. Tyrode solution is used as diluting fluid. After incubation for one hour in the waterbath the result is read.

" When the reaction is positive the liquid surrounding the intestine becomes turbid and floccules are suspended in it consisting of epithelial cells and mucus which detach themselves from the piece of ileum when the tube is shaken. When the reaction is negative the liquid remains perfectly clear."

Exhaustive studies showed that the " filtrate factor " responsible for the above-described reactions was most regularly produced by cultivation of *V. cholerae* in beef-extract broth containing 1% agar, only irregularly in digest and peptone-water media, not at all in synthetic media. It was absent from the saline washings of 24-hour-old agar cultures and the autolysates of cholera vibrios.

The filtrate factor was found to be rather heat-labile, being almost completely destroyed by exposure to a temperature of 50°C for 30 minutes. An untoward influence was also exerted by an acid reaction, storage in the incubator for one week, or addition of 0.3% formol, but not by addition of 0.5% phenol.

Discussing the significance of these findings, Singer et al. pointed out that the filtrate factor bore in its physical characteristics a close resemblance to a bacterial toxin. That according to Burnet and his co-workers (see the third of these studies), the filtrate factor—called mucinase by them—was a mucin-splitting enzyme, was in the opinion of Singer et al. not " incompatible with its nature as a toxin as it has become increasingly probable that bacterial toxins have some of the properties of true enzymes ". Nevertheless the fact that, as shown by tests with sera raised in rabbits with the aid of H + O and O cholera antigens on the one hand, and with filtrates on the other hand, a close antigenic relationship existed between the filtrate factor and the O antigen of *V. cholerae*, spoke in the opinion of Singer et al. against the former being identical with the cholera toxin. For, as they put it,

" All true bacterial toxins which have been described so far are antigenically specific and entirely different from the somatic antigens of the bacteria by which they are produced ".

The conclusion reached by these workers was, therefore, that antigenically the filtrate factor was very similar to, if not identical with, the somatic antigen of *V. cholerae*.

Considering the practical importance of their findings, Singer et al. stated that:

" The discovery of F.F. (i.e. the filtrate factor) will not alter the accepted methods of cholera vaccination at present, as the antibodies produced by the somatic antigen in cholera vaccines protect the guinea-pig ileum against the effect of cholera mucinase. Preliminary experiments have shown that the sera of human subjects who have been vaccinated with cholera vaccine exert a neutralizing effect similar to the effect of immune rabbit sera."

As stated by Singer et al. in a second publication (1948b), they had been able to confirm through further experiments that the sera of vaccinated human subjects as well as cholera-immune sera raised in rabbits were able to protect the guinea-pig ileum against the effect of cholera filtrates. In both instances the protecting antibodies could be removed by absorbing the sera with boiled suspensions of *V. cholerae*. The antibodies still demonstrable after absorption, being presumably of an anti-flagellar character, were unable to neutralize the factor responsible for the action of the filtrates.

It deserves attention that Singh & Ahuja (1953), to whose observations on the epithelium-desquamating enzyme of vibrios reference has been made already in the third of these studies, found that strips of intestines freshly dissected from cholera-vaccinated guinea-pigs were not protected against the desquamating effect of homologous or heterologous vibrio filtrates. These two workers militated, therefore, against the claim of Singer et al. that cholera vaccination should confer appreciable protection against the toxic effect of *V. cholerae* on the epithelium of the small intestine.

An important study of comparative characteristics of variously prepared cholera vaccines with regard to the preservation of mucinase in an antigenic form was recently made by Jensen (1953). For this purpose different vaccines as well as mucinase-containing solutions were used for the immunization of rabbits in order to determine the amounts of antimucinase which might be developed. It was found that immunization with cholera culture filtrates containing mucinase in an active form, gave rise not only to antimucinase at a relatively high titre, but also to agglutinins. Administration of filtrates which had been inactivated by heating for 30 minutes at 56°C, while giving rise to the latter antibodies, did not stimulate antimucinase production. Antimucinase was produced only to a low degree in rabbits which had been immunized with washed viable cholera vibrios or with heat-, phenol- or formol-killed vaccines.

Since these findings indicated that antimucinase production depended upon the presence of active mucinase in the materials used for immunization, a study of the stability of mucinase under varying conditions was made. Jensen established in this respect that:

" In a series with varied temperature-time combinations a loss of at least 75% of the activity was obtained at each of the following points: 45 C, 2 hours; 37 C, 4 hours; 21 C, 48 hours, 4 C, 2 weeks; −20 C, 50% loss in 8 weeks.

" Similar losses within 30 minutes were observed upon the addition of formalin to 0.3% and of phenol to 0.5%. With merthiolate added to a final concentration of 0.01% the losses were no greater than those recorded for preparations without preservative.

" Lyophilization gave preparations which appeared to be quite stable during an 8-week period of testing. The lyophilized material in sealed ampoules withstood the stress of 100°C for 1 hour without loss of activity upon rehydration."

Thus merthiolate seemed a suitable bacteriostatic agent in vaccine manufacture and lyophilization appeared to be useful for the stabilization of the products. Further, though under experimental conditions immunization with the filtrate factor gave rise to satisfactory agglutinin titres, it was in Jensen's opinion desirable nevertheless to use for human vaccination a killed vaccine to which mucinase had been added. To obtain such a vaccine,

" a suspension of washed *V. cholerae* containing 8×10^9 viable bacteria per ml was mixed with an equal volume of filtrate factor. Merthiolate was added to the mixture to a final concentration of 0.01%. This final mixture was then lyophilized, the ampoules being finally filled with dry nitrogen and glass-sealed."

Jensen stated that the mucinase titre of this vaccine was 1:1,600 and that rehydrated lyophilized material gave the same titre. Since there was no detectable loss when the latter material was heated for 1 hour at 100°C, it could be anticipated that this new vaccine would keep well when stored for long periods under lower temperatures. While giving good antibody production in rabbits, it appeared to be non-toxic for these animals and for mice even upon intracerebral administration.

Vaccines prepared from autolysates

As will be gathered from the foregoing section, some workers, particularly Strong, though ultimately resorting to filtration when manufacturing cholera vaccines, depended in the first line upon procedures promoting what is usually called an autolysis of the vibrios. Attention has now to be drawn to some further attempts to utilize autolysates of *V. cholerae* as vaccines.

Gohar (1934) resorted for this purpose to suspensions of cholera vibrios which had been kept in the laboratory for several weeks until all organisms had died and most of them had become lysed. Used directly for the immunization of guinea-pigs, this autolyzed vaccine appeared to be more effective than the vaccines killed by exposure to a temperature of 60°C.

Violle (1950) succeeded in producing with the aid of suprasonic vibration a lysate from cholera vibrios which possessed moderate antigenic properties. He fully realized, however, that this method would not be suitable for the purposes of practical vaccine manufacture.

Vaccines prepared by extraction methods

Attempts to use extracts prepared in various ways from the organisms for the purposes of cholera vaccination have been made by numerous workers. While, as described below, most of them resorted to chemical procedures, a few implemented mechanical methods, such as expressing the " plasmatic juice " of previously ground-up vibrios (Hahn, 1897) or breaking up the bodies of the organisms at the temperature of liquid air (Macfadyen, 1906). However, Hahn's vaccine, though found to confer a long-lasting immunity to experimental animals, was apparently never used for human vaccination, while the antigen prepared by Macfadyen served only for serum manufacture.

To prepare antigens suitable for cholera immunization by chemical methods, some workers, first apparently Klebs (1892), resorted to alcohol extraction. Gohar (1934) used distilled water to extract cholera vibrios which had been dried in vacuo. To judge from guinea-pig experiments, addition of such extracts to heat-killed cholera vaccines enhanced the protective power of the latter.

Gohar & Isa (1948) stated that they had prepared a soluble extract from cholera vibrios

" by adding to a suspension containing 8,000 million organisms per ml an equal quantity of normal sodium hydroxide and incubating at 37°C. for a few hours until the organisms are dissolved and the suspension becomes clear. This is subsequently neutralized with HCl until it is just alkaline (pH about 7.5)."

Fifteen out of 25 rats, which had been twice injected with this extract at a week's interval, survived intraperitoneal challenge with LD_{50} doses of living cholera vibrios.

The possibility of using the nucleoproteids of *V. cholerae* referred to earlier in this study for the purposes of vaccination was experimentally explored by several workers, first by Heller (1905), Schmitz (1906), and Blell (1906).

Though in the opinion of Heller and of Blell nucleoproteids were suitable for human cholera vaccination, Hetsch (1912) stressed on the contrary that, in view of their high toxicity, the mediocre titre of the antibodies produced by them, and the technical difficulties of properly manufacturing them, administration of the usual vaccines was by far preferable. It appears in fact that vaccination with nucleoproteids has never been used for the purpose of protecting man against cholera infection.

Toxoids

The use of toxoids prepared from 10-day-old El Tor cultures by addition of 0.5% formol and storage for eight days was recommended for human vaccination against cholera by Kraus & Kovacs (1928), because in their experience (*a*) subcutaneous injection of these products into rabbits and guinea-pigs protected these animals not only against one or several lethal doses of the El Tor exotoxin but also against intraperitoneal administration of living El Tor or cholera vibrios, leading as well to agglutinin production in the sera of these animals, and (*b*) the reactions produced by these toxoids in man were not marked.

Felsenfeld & Young (1945) experimenting on rabbits, guinea-pigs, and mice with differently prepared cholera vaccines, obtained the best results by using formol- or phenol-killed cholera vibrios of the Inaba sub-type combined with formolized filtrates of Inaba or El Tor vibrios. They preferred the latter organisms for preparing such toxoids because these were less toxic and stimulated the production of antibodies against the haemodigestive and necrotoxic action of *V. cholerae* to a higher level. The sera of human volunteers tested with this toxoid-vaccine, which was used in combination with a dysentery vaccine, were found capable of protecting mice against cholera infection.

In order to obtain a cholera toxoid, Gohar & Isa (1948) treated the soluble extract they had obtained from suspensions of *V. cholerae* through the addition of sodium hydroxide (see page 1053) with 0.7% formol and incubated the mixture for 20 days at 37°C. While the lethal dose of the original soluble extract for rats was 3.5 ml subcutaneously, the formol-treated product was almost atoxic and rendered animals immunized with it resistant to large toxin doses. Used experimentally for the vaccination of rats which were afterwards challenged with LD_{50} doses of *V. cholerae*, mixtures of vaccine and toxoid gave better results (84% survival) than toxoid alone (56% survival), the toxic extract or killed vaccines.

Following up preliminary trials by Gohar & Isa, Gohar (1948) experimented with an alum-precipitated cholera endotoxoid prepared by

(*a*) cultivation of *V. cholerae* for 2-3 days in broth filled into flat flasks, which were placed horizontally to provide a maximum of aeration; (*b*) addition of 0.6% formol followed by further incubation at 37°C for a week; (*c*) precipitation with 0.5% alum; and (*d*) collection of the precipitate and re-suspension to the required density in phenolized normal saline.

Results obtained by single administration of this vaccine in mice, which were afterwards challenged with LD_{100} doses of living cholera vibrios, were almost as good as those produced by two administrations of a heat-killed vaccine (40% as against 45% survival).

Tested on a small scale in man, the alum-precipitated vaccine produced, when injected intradermally, indurated masses which persisted for a long time and were, in Gohar's opinion, thus capable of exerting an antigenic stimulus lasting for several days. Administration of full doses subcutaneously produced somewhat severe reactions. These observations speak against the practicability of this method of cholera vaccination, recommendable though it might be on theoretical grounds.

Sensitized vaccine

The use of sensitized cholera vaccines has been recommended by Japanese workers, first apparently in 1916 by Takano and by Yabe (see Shiga et al.. 1918; Takano et al., 1926). The method used by Takano for the preparation of such vaccines has been summarized by Takano et al. as follows:

" A 20-hours agar culture 1 gm. is suspended in 2 c.c. of a cholera immune horse serum (bactericidal titre of at least 0.0001 c.c.) diluted four times with the salt solution; the mixture is then incubated for 2 hours during which it is shaken from time to time. Then the suspension is centrifuged at very high speed and the organism is washed twice with salt solution. The organism thus sensitized is suspended in physiological salt solution containing 0.5% of carbolic acid. Of such a suspension 1 c.c. contains 2 mgm. of the organism. The cholera vaccine thus prepared is placed in an incubator overnight . . . An essential part of this process is to prevent the death of the cholera vibrio until the sensitisation is complete. If dead vibrios were to be used, the antigen would be largely set free in the medium and lost in the process of washing, so that the antigenic power of the vaccine would be greatly reduced."

In contrast to these recommendations, a few workers, particularly Besredka (1922) and Masaki (1922a) advocated the use of living sensitized cholera vaccines, but these—though found satisfactory in the laboratory—seem not to have been utilized for human vaccination. Sensitized vaccines prepared according to Takano's method have been used on a fairly large scale in Japan with satisfying results, it being claimed in particular that in contrast to the usual type of cholera vaccines the sensitized products caused but mild reactions, yet produced a quickly setting-in immunity which reached

a considerable degree even after single doses only had been administered. Since, however, the difficulties of preparing sensitized vaccines on a scale sufficient for mass campaigns are enormous it is not surprising to find that in spite of their undeniable advantages the use of such products has been given up entirely.

Cutaneous vaccination

Making parallel observations on guinea-pigs which had been injected intraperitoneally at eight days' interval with two doses of a heat-killed cholera vaccine and on a second group of animals which had the same amounts of vaccine (0.3 and 0.6 ml) rubbed into their freshly-shaven skin, Ciuca & Balteanu (1924a) found that the animals of both groups resisted intraperitoneal challenge with a lethal dose of *V. cholerae* made 12 days after the second vaccine administration. However, it was found that only the intraperitoneally vaccinated guinea-pigs and not those protected by the percutaneous route showed agglutinins, bacteriolysins, and complement-fixing antibodies in their sera. Further testing the response to intracutaneous injection of a suspension of live cholera vibrios in 0.2-ml amounts, Ciuca & Balteanu (1924b) noted (*a*) a marked skin reaction in normal guinea-pigs; (*b*) a less marked reaction in animals protected by two intraperitoneally administered doses of cholera vaccine and tested 12 days after the second injection; and (*c*) practically no skin reaction in the animals which had been vaccinated by the percutaneous route. Ciuca & Balteanu claimed on account of these findings that percutaneous cholera vaccination produced a local cellular immunity.

Interesting as this postulation is, it has to be noted that according to all workers who subsequently devoted attention to this point (see Neuhaus & Prausnitz, 1924, for example), intracutaneous administration of cholera vaccines did lead to the appearance of antibodies in the sera of the vaccinated animals. Panja & Das (1947) as well as Singer et al. (1948b), who made corresponding observations in man, found that antibody formation was apt to take place in the intradermally vaccinated persons to a more marked degree than was the case in subcutaneously vaccinated individuals.

Commenting upon their experiences when vaccinating 11 persons intradermally with 0.1 ml and 0.2 ml of the standard Kasauli vaccine and 10 individuals (controls) subcutaneously with 0.5 ml and 1.0 ml doses of the same vaccine, Panja & Das lauded the great economy in material effected by the former method and also stated that the reactions produced by intracutaneous vaccination were negligible. They admitted, however, that in mass campaigns it was considerably less expedient to use the last mentioned in place of the subcutaneous method.

Singer et al. (1948b) noted the appearance of small abscesses at the site of the intracutaneous injection in some of the persons vaccinated three

times with 0.2-ml doses. Since, however, such abscesses became manifest only at the third injection, they were probably the result of an allergic reaction and might be avoided by administering only two doses at 5-7 days' interval. Be this as it might, no doubt one must share the misgivings of Panja & Das regarding the extrinsic difficulties of using the cutaneous method of cholera vaccination in mass campaigns.

Vaccination by the nasal route

Sanarelli (1924b) found that insufflation of a powder consisting of toluene-killed cholera vibrios, boric and lactic acid into the nasal cavity of rabbits led to an often quite considerable formation of agglutinins in the sera of the animals and rendered them immune against cholera infection by the intravenous route.

Oral vaccination

While Brieger et al. (1892) reported that they had rendered guinea-pigs immune against oral infection with *V. cholerae* by intraperitoneal administration of heat-killed vaccines, Klemperer (1892) claimed that it was also possible to protect the animals against oral infection by the introduction of small doses of living cholera cultures into their stomach. Similarily, Cantacuzène (1894) reported that repeated introduction of cholera vibrios into the stomach of guinea-pigs rendered the animals fully resistant to intraperitoneal infection with *V. cholerae*, provided that they were challenged not earlier than 18 days after the last intragastric inoculation. However, Sobernheim (1893), though finding that intragastric immunization with rather large doses of cholera vibrios conferred some degree of protection against subsequent intraperitoneal infection, was unable to protect guinea-pigs against oral infection either by the intragastric or any other method of cholera vaccination.

Pfeiffer & Wassermann (1893), who infected a considerable number of immunized guinea-pigs per os with recently isolated cholera vibrios found similarly that

" the percentage of immunized guinea-pigs which survive this mode of infection is not appreciably higher than in the control guinea-pigs. The mode of immunization seems to exert no influence in this respect. We found no difference when we immunized the guinea-pigs with living or killed cultures, subcutaneously or intraperitoneally, when we challenged a few days after immunization or waited for weeks."

Sawtschenko & Sabolotny (Zabolotny) reported in 1893 upon observations they had made when orally administering numerous doses of an agar-grown and heat-killed cholera vaccine during about a month to themselves and during about two weeks to a student. The total amount of

vaccine taken by Sawtschenko was 180 ml, equalling 1.4 g of cholera vibrios weighed in the dry state, while Zabolotny ingested 110 ml (dry weight of vibrios about 0.84 g) and the student 135 ml (dry weight of vibrios about 1 g). The sera of Sawtschenko and the student, when administered intraperitoneally to guinea-pigs in doses of 0.1-1 ml, 25 days after immunization had been completed, protected the animals against challenge with two lethal doses of *V. cholerae* made three days afterwards.

After they had partaken of further vaccine doses, bringing the total dry weight of cholera vibrios administered orally to about 1.7 g and 2.3 g respectively, Sawtschenko & Zabolotny ingested after previous alkalinization of their stomach content 0.1 ml of a 24-hour-old virulent cholera broth culture. Though it was possible to demonstrate the presence of the organisms in the stools of Zabolotny up to three days after infection and in the faeces of Sawtschenko on the second day, neither of them showed any clinical signs of the disease.

Remarkable though these experiences are, it has to be pointed out that (*a*) even the attempts to produce cholera artificially through oral infection of non-vaccinated persons were by no means always crowned with success, and (*b*) in view of the prolonged course of oral vaccination with enormous doses, the results of the two Russian workers did not furnish any proof for the practicability of this mode of cholera immunization.

In a further report made in 1894 Zabolotny stated that he had been able to protect sisels (*Spermophilus guttatus*) against intragastric as well as against intraperitoneal cholera infection through several times repeated administrations of live attenuated or heat-killed *V. cholerae* cultures per os, results which were afterwards confirmed by Korobkova (1922). Subcutaneous or intraperitoneal immunization of sisels with killed cholera vibrios on the contrary failed to protect the animals against intragastric infection.

In analogy with the last-mentioned observation, Metchnikoff (1894) stressed that he found it impossible to protect young unweaned rabbits by parenteral administration of either living or killed cholera vibrios against oral infection with *V. cholerae*, which produced in such animals a process apparently identical with that observed in human victims of the disease. These negative results were confirmed in 1911 by Choukevitch. As summarized by Hetsch (1912), analogously disappointing results were also obtained by some other workers with dogs and cats.

The possibility of conferring immunity to cholera through intragastric administration of Galeotti's (1912) nucleoproteid was explored by de Bonis & Natale (1913). They found that only two out of 11 guinea-pigs which had been given with the aid of a stomach tube 1-3 doses of 0.005-0.02 g of cholera nucleoproteid dissolved in 0.5% sodium bicarbonate solution, survived this treatment. They were able to establish, however, that (*a*) the sera of 9 of these animals contained agglutinins (maximal titre 1:1,000),

and (*b*) the two survivors resisted infection with 0.25 ml of a cholera broth culture, while a control given the same dose died in 48 hours.

The problem of oral cholera vaccination received far more attention than in the past after Besredka, in a series of articles published in 1918 and 1919 in the *Annales de l'Institut Pasteur* had reported upon successes obtained in the experimental prevention of dysentery, typhoid and paratyphoid by the combined administration of ox-bile and vaccinating doses of the organisms in question by the oral route. Masaki (1922b) investigating whether these observations were applicable in the case of cholera, thus summarized the findings he had made in this respect:

" (a) Both rabbits and guinea-pigs are completely refractory to the ingestion of cholera vibrios in any dose.

(b) Oral administration of bile alters the intestinal wall of rabbits, facilitates the entry of the cholera endotoxin and its passage into the system: as a consequence agglutinins appear in the animals which had ingested either living or killed vibrios after bile sensitization.

(c) Ingestion of either living or killed vibrios does engender protective antibodies in sensitized as well as in non-sensitized rabbits.

(d) Only bile-sensitized rabbits react to the ingestion of living vibrios: very high doses (two agar cultures in Roux bottles) kill the animals in one to two weeks; median doses (one culture) render the animals ill for some days; doses less than half a Roux bottle finally cause no harm.

(e) Only bile-sensitized animals which have shown illness after the ingestion of living vibrios, become vaccinated against intravenous administration of a surely lethal dose of vibrios." [Trans.]

Masaki added that the immunity engendered in this manner was in all probability a local (intestinal) one. He laid, in this connexion, stress upon the fact that, though ingestion of bile followed by that of living cholera vibrios led to the appearance of agglutinins in the sera of the rabbits, these antibodies, instead of augmenting, decreased and finally disappeared, apparently because administration of the initial vibrio doses had led to a " vaccination " of the intestinal wall which thus became impermeable to the organisms or their products.

The validity of Masaki's conclusions was supported by some laboratory observations, e.g. those of Glotoff (1923) and of Horowitz-Wlassowa & Pirojnikova (1926), but some other workers, such as Sdrosdowsky (Sdrodowski) (1924) and Klüchin & Vigodtschikoff (1925) took a definite stand against the method of oral cholera vaccination. Engelhardt & Ray (1927) concluded from an exhaustive study that it was possible to immunize bile-sensitized rabbits by oral administration of very large doses of *living* cholera vibrios against intravenous infection. The agglutinin titre in the sera of these animals rose but slightly and then decreased. In the rabbits which were orally given *killed* cholera vibrios after bile administration, no immunity resulted but the agglutinin titre of their sera rose constantly during immunization and the following two weeks.

In view of these discrepant results it is not surprising to find that the opinions held by the different observers in regard to the question of whether cholera immunization per os led to a local or a general immunity, were rather divided. Some, for instance Horowitz-Wlassowa & Pirojnikova, were in favour of the former view, but others, Sdrosdowsky for example, denied the existence of a separate enteric immunity of histogenous origin —an opinion also vigorously expressed by Hetsch (1928).

On account of the observations he had made in the past with Sawtschenko, Zabolotny (1922) advocated the large-scale use of oral vaccination for coping with the cholera situation in Russia. As summarized in the *Tropical Diseases Bulletin* (1923), he

" recommends the use of vaccines prepared from thick suspensions of organisms killed by heat, carbolic acid or alcohol (20-40 per cent.), from 3 to 5 doses of 2-10 c.c. every other day. Each dose contains from 10 to 100 milliard vibrios, or from 0.01 to 0.1 gm. of dried organisms. Vaccines were also prepared in the form of tablets with sugar or cocoa, each containing 0.1 gm. of dried organisms."

Zabolotny stated that, as shown by preliminary experiences, in persons who had been immunized against cholera in this manner, the agglutinin titre rose to 1:400 and the bactericidal titre to 1:60. His article does not indicate whether large-scale advantage of oral cholera vaccination was taken in Russia.

Among the studies made during the years following Zabolotny's publication in regard to the appearance of antibodies in the sera of individuals orally vaccinated against cholera, the following deserve mention:

Korobkova & Zénine (1923) tested the sera of 49 out of 348 persons who had been immunized by oral administration on each of 3 subsequent days of one tablet respectively containing 50 milliards (US billions) of heat-killed cholera vibrios, 115 of these individuals also receiving on each occasion a bile tablet. In the sera of 19 individuals, which were examined 17 days after immunization, an agglutinin titre of 1:100 (the maximum tested) was found to be present invariably, regardless of whether or not bile tablets had been given.

In 30 persons, whose sera were tested 5 weeks after immunization, agglutinins were but rarely demonstrable, but bacteriolysins at titres ranging from 1:10 to 1:25 were found to be present with one exception, both in the group receiving vaccine only and in that receiving vaccine and bile tablets. In Korobkova's opinion it was therefore uncertain whether bile administration had to be combined with the oral administration of cholera vaccines. The same doubt was also expressed by Peverelli (1924).

Gluchow et al. (1923), testing the sera of 73 individuals vaccinated orally, noted the appearance of agglutinins and bacteriolysins which persisted for 9 months, but decreased in the majority 4 months after vaccination to half the titre. A repetition of oral vaccination did not lead to an increased antibody titre, but such an increase was noted after the subcutaneous administration of booster doses. In the opinion of the above mentioned workers these observations supported the view that oral vaccination created a barrier against the passage of vibrios or their products through the intestinal mucosa, which could be circumvented through parenteral revaccination. This view was opposed by Stepanoff-Grigorieff & Iljina (1924), in whose opinion the appearance of agglutinins in practically all persons orally vaccinated and that of bacteriolysins in part of these individuals manifested the development of a general immunity.

Far more important than the experiences recorded above were large-scale trials of the method of oral cholera vaccination in India,[o] made simultaneously with mass-vaccinations by the subcutaneous route.

The procedure adopted in these campaigns for oral vaccination was to administer on each of three consecutive mornings, before food had been taken, first a bile tablet and 15 minutes later a commercially prepared bilivaccin tablet with a bacterial content of about 70 milliards (billions) of dried cholera vibrios. Parenteral vaccination consisted of the subcutaneous injection of either one or two doses of a standard cholera vaccine with a vibrio content of 8,000 million.

Reporting on the first of these trials, Russell (1928) submitted the following figures:

		Cholera		*Percentage*	*Percentage mortality*
		attacks	*deaths*	*attacked*	*among attacked*
A. *Bilivaccin*					
Number given 3 doses of bilivaccin	4,982	18	4	0.36	22.2
Number not treated (controls)	11,004	222	93	2.02	41.9
B. *Cholera vaccine*					
Number of persons given one dose (0.5 ml)	17,160	59*	25*	0.34	37.3
Number of persons given two doses (1.5 ml)	8,485	31	2	0.37	6.5
Number of persons not treated (controls)	25,645	489	184	1.67	37.6

* Attacks and deaths occurring within three days after vaccination excluded.

While these figures, besides illustrating the value of the usual method of cholera vaccination even with a single 0.5-ml dose, demonstrate also the efficacy of oral vaccination, it has to be noted that in the experience of the field staff

" the bilivaccin sometimes produced acute diarrhoea of such a severe type that the persons affected refused to take further doses, and, in certain cases indeed, the medical officers were accused of inducing cholera. Fortunately, no untoward incident occurred, as those affected quickly recovered."

Submitting the gross figures quoted above and the subsidiary statistics to a painstaking analysis, Russell reached the conclusion that

" [*a*] the immunity developed five days after a single dose of anti-cholera vaccine is nearly as high as that conferred three days after a full course of oral bilivaccin;

" [*b*] . . . it may be inferred that a high degree of immunity is conferred by both the subcutaneous anti-cholera vaccine and the oral bilivaccin, but that the former is, in the long run, superior to the latter. In view of the fact that, with ordinary precautions, the risk of injury from inoculation is inappreciable and that even transitory discomfort is uncommon, the case in favour of anti-cholera vaccine as a practical and cheap preventive measure is complete."

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[o] As reported by Sarramon (1930) a comparative study of oral and parenteral cholera vaccination was also made in Indochina. It showed attack rates of 0.36% in the 4,982 persons who had received bilivaccin and of 0.37% in 8,485 individuals vaccinated parenterally as against attack rates of 2.02% and 1.67% respectively in the two control groups of 11,004 and 29,254 persons.

As stated by Russell (1935), during 1932 another large field-experiment with bilivaccin and with anti-cholera vaccine was carried out in endemic-cholera areas of Madras Presidency (now Madras State), several thousand persons being protected by the former method and an additional 6,000 persons by the latter. The conclusions reached after statistical analysis were that

" (1) both the full three-dose course of bilivaccine and the 1 c.c. dose of anti-cholera vaccine conferred a considerable degree of protection against cholera, and

" (2) the incidence of cholera amongst the unprotected was 8.5 times higher than among those protected by bilivaccine (3 doses) and 5.5 times higher than amongst those protected by anti-cholera vaccine."

In Russell's opinion these experiences seemed to confirm those made in the first field-study. He added:

" The question of the substitution of bilivaccine for anti-cholera vaccine for the protection of Haj pilgrims was recently referred to the ' Office International ' for an expression of opinion, but that body has declared that while vaccination *per os* probably produces a certain immunity, this is much inferior to that obtained by subcutaneous inoculation. Moreover, the difficulty of exercising a strict control appeared to the ' Office International ' to be sufficient reason for rejecting the suggestion."

There can be no doubt that the almost insurmountable difficulties of properly using oral cholera vaccination under the conditions ordinarily prevailing during mass campaigns as well as the unpleasant and sometimes even serious reactions apt to follow bile administration strongly speak in favour of parenteral immunization. It was probably for these reasons as well as on account of the difficulty and costliness of preparing oral vaccines that, after having received much attention for some time, the method of cholera vaccination per os has been given up entirely.

Mixed vaccines

Castellani, who seems to have been the first worker to draw attention to the possibility of using mixtures of vaccines manufactured individually from different bacterial species for simultaneous immunization against the respective infections, suggested in 1913 that combined administrations might be made of vaccines prepared from live attenuated cholera vibrios and dysentery bacilli. He and Mendelson (1915) followed this proposal by recommending the use of a tetravaccine obtained by mixing vaccines prepared separately from typhoid, paratyphoid A and B bacilli, and from cholera vibrios, the finished product containing per ml 500 million of the first mentioned organisms, 250 million each of the two paratyphoid strains, and 1,000 million of *V. cholerae*. It is of interest that 0.5% phenol alone was used to sterilize these vaccines, a storage of the phenolized suspensions at 10°-20°C for a few hours being found sufficient for this purpose. Dealing

exhaustively with the use of various combined vaccines, Castellani (1916) recommended inter alia a mixed cholera and plague vaccine and a " penta-vaccine " for simultaneous immunization not only against these two infections but also against typhoid and paratyphoid A and B.

As summarized by Hetsch (1928), numerous European workers recommended and to some extent practised combined vaccinations against typhoid and cholera at the time of the first World War, while in the Philippines Manalang (1925) made ample use of single-dose administration of a tetra-vaccine prepared according to Castellani's method, which contained per ml 4,000 million cholera vibrios, 2,000 million typhoid bacilli, and 1,000 million each of paratyphoid bacilli A and B.

More recently, Gefen (1945) recommended a polyvalent vaccine prepared with the aid of extraction methods for simultaneous vaccination against cholera, typhoid, paratyphoid, dysentery, and tetanus. Similarly, Ranta & Dolman (1943) recorded favourable results of laboratory tests (production of agglutinins in rabbits) with a combined vaccine containing 4,000 million of cholera vibrios, 700 million of typhoid bacilli, 225 million respectively of paratyphoid bacilli A and B per ml of tetanus toxoid. Felsenfeld & Young (1945), to whose method of manufacturing a toxoid-vaccine against cholera reference has been made above, tested a mixture of this and a similarly prepared dysentery vaccine on a group of volunteers and obtained satisfactory results when using the sera of these individuals for serological and mouse-protection tests.

While in some instances the combined vaccines were issued ready-made by the manufacturing laboratories, some workers mixed the individual vaccines they proposed to administer in combination immediately before use. Schwarz (1919) was not satisfied with either of these procedures, fearing on the one hand that storage of combined vaccines might lead to a deterioration of their immunizing power and, on the other side, that an instantaneous mixture of individual vaccines might lead to contaminations. He advocated, therefore, mixing the vaccines destined for combined administration two days before their use, so that the phenol or other antiseptic contained in them could cope with contaminating organisms.

Judging from experiences gained through laboratory tests, mainly those with the sera of persons to whom the combined vaccines had been administered, the various workers using such products were unanimous in asserting their efficacy. Indeed, some observers, e.g., Manalang (1925), maintained that the combination of different vaccines exerted a stimulating influence on the immunizing power of the individual components. It was also generally upheld that the reactions caused by the administration of mixed vaccines were not more marked than those following the separate use of the single components of these products. However, fairly ample experience of combined cholera and typhoid-paratyphoid vaccination in China has convinced the present writer that the latter claim holds true only to a limited

extent: while the reactions produced by these mixed vaccines were not more marked than those caused by the administration of typhoid-paratyphoid vaccines alone, they were much stormier than those resulting from the sole use of cholera vaccines. Hence, while the absence of marked reactions was instrumental in overcoming the prejudice against sole cholera vaccination, the use of the combined vaccines invariably led to complaints seriously hampering the campaigns. In the considered opinion of the present writer it is not advisable, therefore, to use mixed vaccines in the course of general anti-cholera campaigns, the less so because as a rule these have been prepared with sub-standard amounts of cholera vibrios—a drawback which becomes particularly serious when only single vaccine doses can be administered. One must admit, however, that under special conditions, particularly when applying vaccination methods for the protection of armed forces, the advisability of using combined vaccines of an adequate standard deserves consideration.

Negative phase

As in the case of other infectious diseases, so also in that of cholera it has been asserted by some workers that vaccination is followed by a negative phase during which the susceptibility of the immunized to the infection is temporarily increased. As can be gathered from a study of the literature, particularly the statements of Pfeiffer & Friedberger (1908b), Aaser (1910), Bessau & Paetsch (1912), Papamarku (1917), Schwartz (1919), and Hetsch (1928), these claims were mainly based on the one hand on observations of changes in the titre of immune bodies in the sera of the vaccinated and on the other hand on experiences regarding the incidence of cholera in recently immunized individuals.

The claims made by some observers that a negative phase was created through a drop in the antibody content of the sera of the vaccinated deserve no credence, not only because such a lowering of the antibody titres has not been confirmed by other workers but also because the laboratory experiences discussed below clearly show that cholera vaccination is not followed by a phase of temporarily increased susceptibility to the infection.

Exhaustive studies made in this respect by Pfeiffer & Friedberger (1908b) showed that experimental animals which had been immunized with specific vaccines, instead of becoming increasingly susceptible to cholera during the period following immediately, showed on the contrary at once a resistance—probably at first an unspecific resistance—to the infection. Bessau & Paetsch (1912), continuing these studies, were also unable to demonstrate the presence of a negative phase through animal experiments performed " under conditions which had to be considered very favourable in comparison to human immunization ". Attention has been drawn already (see page 989) to further observations by Papamarku (1917) who

showed that the drop in bactericidal power observable in the sera of guinea-pigs after cholera re-vaccination was as a rule not accompanied by a loss of resistance to challenge infection.

When it is considered that (*a*) as generally agreed, a period of at least about three days has to elapse before the immunity engendered by active cholera immunization begins to become manifest, and (*b*) during outbreaks it is inevitable that some persons, because they are incubating the disease at the time of vaccination, fall ill before being protected, it is easy to understand why, as soon as Haffkine's method of vaccination began to be practised, its many adversaries clamoured that this procedure, instead of preventing, caused or at least facilitated the appearance of cholera. Haffkine took a determined stand against the idea of a negative phase, stating for instance in a speech given in 1899 before the Royal Society, London, in reference to some of his early statistics that:

> " Inoculation has again acted, so to say, immediately; or as we have adopted to generally formulate the result, has acted within the time necessary for the subsidence of the general reactionary symptoms produced by the inoculation."

In spite of these and other reassurances, the idea of a negative phase following cholera vaccination in man continued to be ventilated from time to time, for instance as late as in 1950 by Dani. However, a large majority of the cholera workers considered the appearance of the disease in quite recently vaccinated persons merely as a *post hoc* and not as a *propter hoc* phenomenon. Thus Simpson (1915), referring to careful observations made in this respect in India, denied that vaccination against cholera led to a negative phase in man and his opinion has been endorsed by many workers dealing with this infection in Central Europe during the first World War. Petrovich (1915) even stated that he had used cholera vaccine with good success for the treatment of a quite considerable number of patients who had been attacked by the disease. It was also pointed out with much reason by several workers (see Hetsch, 1928) that the non-appearance of clinical signs of the disease in specifically vaccinated carriers of *V. cholerae* strongly spoke against the appearance of a negative phase. More important still, a large-scale statistical study by Adiseshan et al. (1947), to which full attention will be paid in the tenth of these studies, failed to show that the administration of a standard cholera vaccine in single doses led to a significantly increased incidence of the disease among the inoculated.

The evidence adduced above suffices to show that one should not hesitate in emergencies to make ample use of cholera vaccination even during epidemics. At the same time it is clear, however, that both in order to benefit as many persons as possible and to avoid alarming the people by the occurrence of cases in recently vaccinated individuals, every possible effort should be made to administer the vaccinations before onset of the cholera seasons.

Duration of immunity

While, as discussed above, general agreement exists that active cholera immunization does not confer protection against the infection during the days immediately following administration of the vaccine and it is also usually held that a period of about a week has to elapse before a substantial immunity becomes established, opinions regarding the duration of the immunity vary. Haffkine, summarizing the experiences with his vaccines up to 1906, maintained in this connexion that

" their effect becomes rapidly accentuated during the first few days and lasts, when moderate doses are used, for about 14 months, after which time it begins to decrease markedly and probably to disappear ".

Though some workers stated that cholera vaccines manufactured according to Kolle's method conferred immunity for a year, others maintained that the period of protection afforded by killed cholera vaccines lasted only for 7-9 months, or merely for 6 months or even less. Adiseshan et al. (1947), summarizing the observations they were able to make in the course of their above-mentioned statistical study, stated in this connexion that:

" In the villages which had second outbreaks within the first six months after the first outbreak, the incidence of cholera in persons who had been inoculated at the time of the first outbreak is definitely lower than in the uninoculated. These differences are statistically significant. The protection afforded by anticholera inoculation continues, therefore, for at least six months.

" Although the figures available are too small to warrant a definite conclusion, the virtual absence of cholera among inoculated persons in villages which were re-infected between six and twelve months after the first outbreak suggests that immunity may last for as long as twelve months."

It follows from these observations that in localities were cholera becomes, or is apt to become epidemic perennially, at least yearly re-vaccinations are indicated. Hetsch (1928), summarizing the experience acquired regarding the duration of the immunity after cholera vaccination during the first World War, was of the opinion that as long as a danger of infection continued to exist, 0.5-ml booster doses of cholera vaccines ought to be administered at half-yearly intervals—a procedure found satisfactory in the German and Austrian armies.

Evaluating tests

It is essential to state that the tests used to assay the immunogenic value of bacterial vaccines in general, and of cholera vaccine in particular, form only part of the examinations necessary to ascertain the suitability of these products. It is of prime importance to determine on the one hand that the finished products are of proper standard and to ascertain on the

other that they are free from aerobic or anaerobic contamination and, in the case of killed vaccines, also that the method of sterilization used has been effective. Unless it is incumbent upon a laboratory to assay commercially produced vaccines, the implementation of the tests just mentioned forms a part of the routine followed for vaccine manufacture.

A further preliminary step of great importance is to ascertain that the cholera vaccines issued produce no undue reaction on account of either an exalted toxicity or of too high a content in phenol or other antiseptics. Pasricha et al. (1938) recommended that the absence of an unduly high toxicity be proved by the survival of guinea-pigs given 5-ml doses of the products under test intraperitoneally, and that the absence of an excess of antiseptics be demonstrated by showing that adult mice which had been subcutaneously injected with 0.5-ml doses of the vaccines examined, remained free from serious symptoms during the period of one week.

The important methods available in practice for assessing the immunizing properties of cholera vaccines may thus be classified :

(1) direct agglutination tests with the vaccines as antigens and standard cholera-immune sera;

(2) serological tests (including Pfeiffer tests) to ascertain the presence of antibodies in the sera of actively immunized animals or vaccinated human subjects;

(3) active immunization experiments in laboratory animals;

(4) protection tests with the sera of vaccinated animals or human subjects.

The importance of these various methods will now be dealt with seriatim.

(1) *Direct agglutination tests.* Summarizing the experiences of Pasricha et al. (1938, 1941) when examining numerous cholera vaccines of different origin, Taylor (1941) stated that these workers took advantage of three categories of tests, namely, (*a*) direct agglutination of the finished vaccines with pure O sera of the Inaba and Ogawa sub-types; (*b*) agglutinogenic tests in rabbits; and (*e*) protection tests in guinea-pigs. Taylor emphasized that :

" The results of these tests were found to run parallel to each other and when a vaccine did not agglutinate to satisfactory titre with the O sera no protection against an infecting dose of *V. cholerae* was obtained."

Endorsing on account of these experiences the great value of direct agglutination tests, Taylor concluded, therefore, that " if a vaccine is sterile and shows satisfactory agglutination it can be considered satisfactory".

(2) *Serological tests.* As has been stated before, agglutination tests with the sera of immunized animals and more still with those of vaccinated human beings have been made by numerous workers but have yielded

rather discrepant results. There can be no doubt that, besides showing up differences in the character of the various vaccines examined, these discrepant results were due to a large extent to extrinsic causes, particularly (*a*) differences in the size and number of the vaccine doses administered, and (*b*) differences in the technique implemented by the various workers, especially the use of less suitable killed antigens in place of live cholera vibrios. At the same time it must be admitted, however, that even if the tests are performed in an adequate manner with the sera of suitably vaccinated individuals, agglutinins, if at all demonstrable, appear at different and not rarely rather insignificant titres and persist for different periods, not necessarily coinciding in length with those during which immunity is supposed to last.

While in the opinion of some workers these inconstancies sufficed to render agglutination tests with the sera of vaccinated subjects of little value for the assay of cholera vaccines, other observers made a still far more serious objection, namely, that the results of such tests merely demonstrated the antigenicity instead of the immunizing power of the vaccines examined. Since, however, in the experience of many workers a considerable parallelism existed between the results of agglutination tests and those obtained with active immunization of test animals, one should not be rash in denying the value of the former, far more expedient, method. It also deserves great attention that, as indicated by the studies of Burrows, Mather, Elliott & Havens (1947), possibly together with other antibodies O agglutinins do play a role in the protection against cholera infection.

It is generally acknowledged that the demonstration of bacteriolysins in the sera of specifically vaccinated experimental animals is indicative of a state of immunity against parenteral infection with *V. cholerae*. Since, however, a fundamental difference exists between the morbid process produced in this manner under experimental conditions and the disease spontaneously developing in man after the ingestion of cholera-contaminated materials, it is difficult to decide whether or to what extent the presence of bacteriolysins in the sera of cholera-vaccinated human subjects testifies to the existence of an immunity against natural infection with *V. cholerae*. Even some of the workers who were agreed that cholera vaccination is apt to protect man against such an infection, ascribed little importance to the presence of bacteriolysins in the sera of the vaccinated. For instance, Papamarku (1917) maintained in this connexion that these bodies may be absent or present at low titres only in the sera at times when the individuals in question are supposedly still protected against cholera infection by the previous vaccination.

However, while one must admit that the presence of bacteriolysins in the sera of cholera-vaccinated individuals furnishes no direct proof for the existence of an immunity against the infection, bacteriocidal tests with such sera are of value in so far as a considerable degree of parallelism has

been found to exist between the results they yield and those of active and passive immunization tests. The technical difficulties attendant upon the bactericidal tests and their consequent tediousness render them less practicable in routine work than the above-evaluated agglutination tests. While, therefore, the latter seem to be preferable, it has to be kept in mind that no close parallelism has been found to exist between the results yielded respectively by these two serological methods. It also deserves attention that in the experience of Ahuja & Singh (1948) the outcome of bactericidal tests alone compared favourably with that of passive mouse-protection tests.

Complement-fixation tests with the sera of the vaccinated, while presenting considerable technical difficulties, seem to possess no superior value in comparison with the two serological methods discussed above.

(3) *Active immunization tests.* The method of testing cholera vaccines by actively immunizing experimental animals with the products under examination and then challenging the animals thus protected with doses of *V. cholerae* found to be lethal for controls, which already guided Ferrán and Haffkine, has been continuously used by subsequent workers.

While at first exclusive use of guinea-pigs was made for this purpose, other species of experimental animals have been preferred by most of the recent workers. As noted before (see pages 1053 and 1054), Gohar & Isa (1948), in order to compare the efficacy of various cholera vaccines, resorted to active immunization tests with rats. White mice seem first to have been used for this purpose by Fennel (1919) to assess the efficacy of a cholera lipovaccine he had manufactured. However, large-scale use of these animals for the assay of cholera vaccines became possible only when Griffitts (1942) showed that suitably small, yet highly virulent test doses for the intraperitoneal challenge of mice could be obtained by suspending the cholera vibrios chosen for this purpose in 5% mucin instead of in normal saline. It is of historical interest to note in this connexion the previous observation of Cantacuzène & Marie (1919a) that otherwise sublethal doses of *V. cholerae*, to which extracts from the small intestine or caecum of guinea-pigs had been added in small quantities, proved fatal if intraperitoneally administered to animals of this species.

Taking advantage of Griffitts' findings, the National Health Institute at Washington recommended tentatively in 1942 a mouse-protection test for the assay of cholera vaccines. As summarized by Ranta & Dolman (1943), this method

" involves vaccinating each of a group of at least 30 white mice, about five weeks old and weighing 8-10 gm., with a single intraperitoneal dose of the test vaccine, equivalent to about 400 million vibrios . . . An equal number of similar mice is set aside at the outset for control purposes. Fourteen days later one-half of the mice in both the vaccinated and non-vaccinated groups are given intraperitoneally approximately 500,000 live vibrios of a virulent Inaba-strain suspended in mucin, while the remainder receive similar doses of a virulent Ogawa type strain. The requirement is that at least 50 per cent of the mice

in each vaccinated group should survive for 72 hours, while at least 75% of the non-vaccinated mice should die of cholera septicaemia within 72 hours."

Ranta & Dolman (1943) confirmed both the advantage of using suspensions of *V. cholerae* in 5% mucin for the challenge of immunized mice and the value of mouse-protection tests for the assay of cholera vaccines. They recommended in 1944 a modification of such tests, based upon the use of two spaced doses of the vaccines under examination and requiring survival of 100% of groups of not less than 15 mice challenged with 5 MLD of mucinized vibrios, and of at least 80% of batches of such animals challenged with 10 MLD. They considered it unnecessary to challenge the animals with both Inaba and Ogawa strains, as had been recommended by the National Institute of Health, because in their experience there existed a cross protection between these two sub-types.

The problems involved in the mouse-protection tests were exhaustively studied by Burrows, Mather, Elliott & Havens (1947) who reached the conclusion that

" A standard dose or fold increase method of titration of protective antibody was found to be impractical, but protective titer expressed as the ratio of the LD_{50} dose for immune mice to that for control mice was reproducible within reasonable limits, and the results were comparable provided that the virulence of challenge strains was substantially the same. The variability was such that 100 fold differences in titer were regarded as significant, 10 to 100 fold suggestive, and 10 fold or less as not significant. "

In a further study on the biological assay of cholera vaccines, Sokhey & Habbu (1950b) pointed out that the mouse-protection test suggested by Ranta & Dolman (1944) was not sufficiently exact because it used too large vaccine doses for the immunization of the animals. It was for this reason that Ranta & Dolman postulated the existence of a cross protection between the Inaba and Ogawa subtypes which Sokhey & Habbu were unable to confirm.

The principle of a new method for the biological assay of cholera vaccines introduced by the two last-mentioned workers, which gave reproducible results within narrow limits, was to determine " the dose of vaccine required to protect 50% of the immunized animals against a challenge dose constant both in numbers and virulence and producing 100% mortality among the controls ".

As noted before, Sokhey & Habbu preserved suitably virulent strains for challenging their animals by freeze-drying. After regeneration the selected strain was grown for three hours in nutrient broth and one part of a 10^{-3} dilution of this was added to four parts of a 5% mucin suspension. 0.5 ml of this mixture, which was used for intraperitoneal injection of the test animals, contained about 100,000 organisms and represented 100 times the minimum lethal dose.

Though, as will be discussed below, some modern observers are inclined to place more reliance upon passive protection tests, there can be no doubt

that properly conducted active immunization tests yield fully reliable results as far as the degree of immunity conferred by parenteral administration of the vaccines in question against parenteral infection with test doses of *V. cholerae* is concerned. It is clear, however, that the results of such tests, even if most favourable, furnish no direct answer to the question of to what extent parenteral administration of the vaccines concerned is apt to protect man against oral cholera infection.

In order to obtain such final proof, it would be necessary to demonstrate that the vaccines in question, if subcutaneously administered, are capable of protecting the vaccinated animals or human subjects against oral cholera infection. As has been discussed before (see pages 1057-1058), experiments made in this respect by some earlier workers not only failed to furnish such final proof in a convincing manner, but gave as a rule frankly negative results. A few analogous trials made in man proved likewise disappointing. Metchnikoff (1911), summarizing the results of such attempts, stated that

" in our 1893 memoir we referred to observations made in three persons, two of whom had been vaccinated by Haffkine, whereas the third served as control. All three showed the same symptoms of benign cholera which one observes in the majority of cholera infections in the laboratory. Ferrán himself as well as some of the individuals vaccinated by him suffered after ingestion of cholera vibrios from diarrhoea like the non-vaccinated. His co-worker Pauli had choleraic diarrhoea even though he had received 13 vaccine injections. Zlatogoroff (1904) . . ., though vaccinated four times with killed and living cholera vibrios, suffered after ingestion of a cholera culture from diarrhoea and was obliged to take calomel after he had a third fluid stool." [Trans.]

It might be argued that the cholera attacks in the above-mentioned vaccinated subjects were invariably slight, but one must fully agree with Metchnikoff that the attempts to induce cholera artificially in man gave rather inconstant results in non-vaccinated as well as in immunized individuals, producing often only slight symptoms, if any at all.

However, in marked contrast to the above-mentioned failures or uncertainties, recently Burrows & Ware (1953) obtained impressive results when immunizing guinea-pigs intraperitoneally with three doses of cholera O vaccine and afterwards challenging the animals by the intragastric route. As summarized by the two workers:

" Active immunization with cholera O vaccine results in a 14-fold increase in the ID_{50} dose (median infective dose) at the height of the immune response, 4 days after a course of vaccine, which declines to 8.7-fold at 14 days and 1.9-fold at 28 days."

These results as well as previous observations of Burrows and co-workers on the appearance of antibodies in the faeces of actively cholera-immunized guinea-pigs and human volunteers, which will be discussed later, seem to endorse the value of active immunization-tests for an assay of cholera vaccines.

(4) *Passive protection tests.* A passive mouse-protection test for assaying the results of cholera vaccination has been recommended by Griffitts (1944). As summarized by Burrows, Mather, Elliott & Havens (1947), this worker, when reporting the results of titration of protective antibodies in the sera of immunized human volunteers,

" expressed the titer in two ways, the number of LD_{50} doses protected against by 0.1 ml of serum, and the amount of serum required to protect 50% of the mice receiving various doses of vibrios. By the first method, normal serum showed a titer of less than 3,000, and the immune serum of 100,000 to 200,000. By the second, 0.1 ml of normal serum did not protect against 590,000 vibrios, and of pooled immune serum, 0.068 ml protected 50% of mice receiving 59 million vibrios, 0.01 ml 50% of those receiving 5.9 million, and 0.0014 ml those receiving 590,000 vibrios."

Burrows et al. (1947b), using the sera of 25 cholera-immunized volunteers for passive mouse-protection tests, formed a most unfavourable opinion on the value of this method. However, Ahuja & Singh (1948), using guinea-pigs for passive as well as for active protection tests, reached the conclusion that the former represented " the most sensitive method available in the present state of our knowledge for demonstrating differences in the immunizing value of vibrio strains".

As has been noted before, Ahuja & Singh considered bactericidal tests as fairly trustworthy because they gave a response approximately parallel to the results of passive protection tests. They were, on the contrary, not favourably impressed by the utility of agglutination tests with the sera of vaccinated subjects for an assessment of the value of cholera immunization.

Mechanism of active cholera immunity

As pointed out by Pfeiffer & Wassermann (1893) in a classical study on the mechanism (" Wesen ") of the active immunity against cholera, the fact that immunized guinea-pigs resisted challenge with larger amounts of living cholera vibrios than non-immune animals, could be interpreted by assuming that " immunization might have conferred either *antitoxic* or *bactericidal* properties ".

Exhaustively investigating which of these two factors was at work, Pfeiffer & Wassermann found that guinea-pigs, regardless of whether they had been immunized with live or killed vibrios, by the subcutaneous or the intraperitoneal route, were practically as susceptible to intraperitoneal challenge with killed cholera vibrios as non-immune animals. It was clear, therefore, that the immunized animals had not acquired a resistance against the cholera toxin (" Giftfestigkeit "). On the other hand, it could be shown that living cholera vibrios injected into the peritoneal cavity of immunized guinea-pigs perished there far more rapidly than was the case in normal animals. Even if immunized guinea-pigs succumbed to challenge infection, because injected with overwhelming doses of *V. cholerae*, their peritoneal

cavity was as a rule sterile. The conclusion reached by Pfeiffer & Wassermann on account of these experiments as well as of passive immunization tests, which will be discussed later, was that

" it was erroneous to consider cholera immunity as a resistance against the toxin ['Giftfestigung'], as has been invariably done in the previous publications. In active as well as in passive immunization there develop exclusively bactericidal properties ". [Trans.]

An identical conclusion was reached independently by Sobernheim (1893) who stated that

" substances must have been produced in the blood of [cholera-] immunized animals which impede the development of living bacteria and, therefore, the production of the lethal amount of toxin, but which do not interfere with the deleterious action of the already formed toxin. Consequently, the animals are immune in the strict sense, but not resistant to the toxin ['giftfest'] ! ". [Trans.]

As can be gathered from a study of the literature, particularly from a valuable summary by Hetsch (1912), the views of Pfeiffer & Wassermann and of Sobernheim, though soon adopted as the creed of the official German school, were not shared by some other workers, who explained the mechanism of active cholera immunity in different ways.

Most noteworthy in this connexion are the views of Metschnikoff (1895) and some other French observers (see Hetsch, 1912), according to whom not a humoral immunity depending upon the action of bactericidal substances but the phagocytic activity of the leucocytes took the decisive part in the destruction of the cholera vibrios in the bodies of immunized animals. However, Pfeiffer (1894b), quoting experimental observations according to which vibriolysis took place in the peritoneal cavity of cholera-immunized guinea-pigs without any marked participation of leucocytes, maintained that phagocytosis, instead of being of prime importance in the process of cholera immunity, was an accompanying phenomenon (" Begleiterscheinung ") of a secondary character—an opinion which appears to have been shared by most subsequent workers.

Another noteworthy objection to the views of Pfeiffer and colleagues was made by Gruber (1896), in whose opinion agglutination of the causative organisms was of primary importance in the process of cholera immunity, the agglomerated vibrios then becoming amenable to the action of protective substances present in the bodies not only of immunized but also of normal animals.

However, as summarized by Sobernheim (1897) and by Hetsch (1912), Gruber's theory was not in accord with the observations of several other workers. Pfeiffer & Kolle (1896) stressed in this connexion that the phenomenon of agglutination observable in vitro represented merely a passing stage, after which the vibrios became again capable of multiplication. More important still, they as well as other workers showed that cholera-immune sera, including the sera of convalescents, even though they had

become devoid of agglutinating properties for various reasons, could still exert a bacteriolytic action in the animal body. It also deserved great attention that, as demonstrated for instance by Kolle (1901), different methods of immunization led to differences in the antibody content of the resulting sera, agglutinins appearing rapidly and to a high titre in the sera of intravenously immunized animals in particular, whereas subcutaneous or intraperitoneal administration of *V. cholerae* led to the production of prevalently bacteriolytic sera. Considering these and analogous experiences, Hetsch (1912) concluded that

" the specific bacteriolysins of R. Pfeiffer and the agglutinins of Gruber-Durham are different substances occurring side by side in the cholera-immune serum. The agglutinins can be considered as the result of a reaction of the organism to the infection and to some extent also as indicators of an immunity. The typical bacteriolysis, however, ... is produced solely through the bactericidal substances of R. Pfeiffer." [Trans.]

However, while feeling convinced of the paramount importance of the bacteriolysins in the protection of immunized guinea-pigs against infection with *V. cholerae*, Pfeiffer & Wassermann (1893) warned against using these " theoretically interesting facts " for an explanation of the immunity against human cholera, because as they stressed, the latter was

" absolutely different from the mixed process of infection and intoxication which is produced in guinea-pigs through intraperitoneal injection of cholera bacteria ". [Trans.]

In order to study in which organs of the animal body the cholera-immune bodies were formed, Pfeiffer & Marx (1898) subcutaneously injected strong young rabbits with agar-slant doses of heat-killed cholera vibrios and then determined the bacteriolytic titres of their sera and leucocytes as well as of extracts of their organs, prepared by (*a*) triturating weighed quantities with the aid of glass powder; (*b*) mixing the triturates with measured amounts of broth; and (*c*) removing after one day's storage in the refrigerator the glass particles by centrifugation.

While obtaining no evidence that the leucocytes served " as the matrix or even as the vehicles of the cholera immune bodies ", Pfeiffer & Marx found, as summarized by Hetsch (1912),

" when examining after different intervals the extracts of the various organs for their antibody content that, hand in hand with a rapid increase of the immunity, in certain organs a considerably higher quantity of bacteriolysins was demonstrable than in the circulating blood. This held true in the first line of the spleen and the bonemarrow, next of the lymph nodes and the lungs. Unexpectedly it was further found that in the majority of the experiments the spleen contained already during the second day after the vaccination clearly demonstrable amounts of cholera immune bodies, even when hardly any traces of such were recognizable in the blood serum." [Trans.]

In the opinion of Pfeiffer & Marx, the abundance (" Plus ") of the immune bodies found in the spleen, bonemarrow, and the lymph nodes indicated that a rapid production of these substances took place there,

which was in excess of their secretion into the blood stream. Apparently this excess of immune bodies in the above-mentioned organs gradually decreased and was no more manifest when immunity had become maximal. While these findings referred in the first place to the bacteriolysins, Pfeiffer & Marx obtained some evidence to show that the agglutinins behaved in an identical manner.

On account of these and related observations Pfeiffer & Marx felt convinced that the production of cholera-immune bodies took place in the blood-forming (" blutbereitenden ") organs, the spleen, the bonemarrow, and the lymph nodes.

This conclusion of Pfeiffer & Marx, because in accord with the now generally accepted concept that the reticulo-endothelial cells, found in organs like the spleen, the bonemarrow, the lymph nodes, and the liver, are the site of production of the immune bodies, continues to be considered as valid. However, a considerable debate arose over the question of whether a production of cholera-immune bodies took place in the intestine.

Attention has first to be drawn in the latter connexion to observations made by Cantacuzène (1894), Cantacuzène & Marie (1919a, 1919b), and Inouyé (1928).

Cantacuzène (1894) noted that cholera vibrios which had been introduced by the intragastric route into subcutaneously or intraperitoneally vaccinated guinea-pigs, disappeared from the small intestine after 3 hours, whereas they persisted there abundantly for 24 hours in non-vaccinated animals. He postulated, therefore, that a " bactericidal milieu " existed in the small intestine of cholera-vaccinated guinea-pigs.

As already alluded to (page 1069), Cantacuzène & Marie (1919a) found that extracts prepared from the small intestine of guinea-pigs by mincing, drying in vacuo, suspension in normal saline, storage in the refrigerator for 24-48 hours, centrifugation, filtration through paper and inactivation by exposure to 56°C for ½ hour, if added in quantities of 0.5 ml or 1 ml to a non-lethal dose of cholera vibrios, rendered the latter rapidly fatal for intraperitoneally infected guinea-pigs. This " activating " property was manifested not only by extracts obtained from the intestines of normal guinea-pigs, but to an even more marked degree by those derived from cholera-vaccinated animals. However, the extracts obtained from the latter category of animals were found to protect guinea-pigs against intraperitoneal injection with lethal cholera doses if administered subcutaneously 6 hours before infection.

Supplementing these observations by complement fixation tests with intestinal extracts prepared in the manner described above, Cantacuzène & Marie (1919b) found that:

(*a*) The complement-fixing properties of extracts from the intestines of normal guinea-pigs were variable but as a rule not marked.

(*b*) On the contrary, the extracts obtained from the small intestines of guinea-pigs which had either received 24 hours previously a lethal dose of *V. cholerae* intragastrically, or had been injected intraperitoneally 24 or 72 hours previously with heat-killed cholera vibrios, showed most marked complement-fixing properties. The extract from the caecum of these animals gave much feebler reactions, their blood sera quite feeble reactions or even none at all.

(*c*) The extracts from the small intestines of solidly cholera-vaccinated guinea-pigs as well as their sera showed most marked complement-fixing properties.

Inouyé (1928), working with extracts prepared like those of Cantacuzène & Marie, but usually without resorting to desiccation, found that

(*a*) guinea-pigs which were intraperitoneally injected with intestinal extracts from animals vaccinated subcutaneously, resisted an immediately following challenge with lethal doses of *V. cholerae*, whereas guinea-pigs previously injected intraperitoneally with intestinal extracts from non-vaccinated animals succumbed even to non-lethal cholera doses;

(*b*) such non-lethal doses proved also fatal to animals which were given intraperitoneally extracts prepared from the liver or kidneys or muscles of normal guinea-pigs, in a solitary experience also to an animal injected intraperitoneally with the intestinal extract or an orally vaccinated guinea-pig;

(*c*) on the contrary, extracts from the liver or the spleen of parenterally vaccinated guinea-pigs did not exert a sensitizing action, thus not causing the death of animals challenged with sublethal doses of *V. cholerae*.

Though in view of the method of experimentation chosen by Inouyé the value of his results appears to be limited, it is noteworthy that the extracts prepared from the intestines of parenterally vaccinated animals protected guinea-pigs against lethal cholera doses.

Cantacuzène (1920), discussing the findings made by him and Marie, felt convinced that the general immunity produced by parenteral cholera vaccination was accompanied, or rather preceded, by a rapidly appearing local immunity. Judging from ample and favourable experiences made in the Rumanian army during the Balkan wars and during the first World War, he considered parenteral cholera vaccination, which in his opinion conferred an antibacterial immunity, as an " absolutely rational " method.

As has been stated already, some of the advocates of oral cholera vaccination, such as Masaki (1922), maintained that this mode of immunization led to a local intestinal immunity, but one must fully agree with Hetsch (1928) that the evidence brought forward in this respect was by no means convincing, whereas not only other experiences made in the case of cholera (see Wassermann & Sommerfeld, 1915, for example) but also ample evidence adduced in the case of other bacterial infections spoke against this concept. In order to support his view, Hetsch quoted the following conclusion reached by Neufeld (1924):

" There exist markedly different degress of active immunity, but no different kinds, as if for instance through preliminary treatment with living cultures another kind of immunity would be produced as after the injection of killed cultures, or after oral administration of bacteria one different from that following subcutaneous administration or as if natural recovery from an (infectious) disease would necessarily [' grundsätzlich '] lead to a state of immunity different from that following artificial immunization. The new knowledge on the formation of antibodies supports the basic concepts of Ehrlich, even though his ideas have to be modified in a few respects [' in einzelnen Punkten '] ." [Trans.]

Attention has now to be paid to recent exhaustive studies by Burrows and his co-workers, which shed new light on the mechanism of active immunity against cholera.

Taking advantage of the new methods for the isolation and purification of the endotoxin of *V. cholerae* introduced by Burrows (1944), he and his co-workers studied first the immunological properties of this endotoxin (see Burrows, Mather, Wagner & McGann, 1944). The main results of this investigation were that:

(*a*) An immunological, haptene-like activity of cholera endotoxin prepared by preliminary extraction with alcohol and three subsequent precipitations with chilled acetone was indicated by skin reactions in immune rabbits as well as by specific precipitation and complement fixation produced by rabbit immune sera.

(*b*) In a series of rabbit immunization tests with differently prepared types of endotoxin most remarkable results were obtained with dialysates, which in the case of animals immunized in 5 or 6 doses with a total of 7.5-8.5 mg led to a protective titre of at least 100,000 as well as to a marked agglutinin response (titres 1:50,000 or more).

Thus, as measured by the agglutinin response, these dyalysates possessed antigenic properties markedly superior to those of whole vibrios. Similarly, mice could be actively immunized by three intraperitoneal inoculations of an alcohol-saline suspension of alcoholic toxin with doses far below those needed to afford the same degree of protection with heat-killed cholera vaccines.

(*c*) It was not possible, however, to demonstrate an in vitro neutralization of the activity of the endotoxin preparations by either antibacterial or anti-endotoxic sera or to immunize mice either actively or passively against the lethal effect of intraperitoneal administration of the purified cholera endotoxin.

Studying the permeability of the small intestine of rabbits and guinea-pigs in vitro, Burrows, Wagner & Mather (1944) found that addition of living vibrios or of crude or purified cholera endotoxin to the Ringer-Locke solutions used for these tests markedly accelerated the rate of flow through strips of normal intestine. If, however, in place of these, strips of intestine from immune animals were used,

" they were completely, or almost completely, resistant to the action of the toxin and in its presence showed little or no difference in permeability to fluids from normal intestine in the absence of toxin ".

As summarized by Burrows and his co-workers, these findings, which were consistent with and complementary to those recorded in the first paper by Burrows, Mather, Wagner & McGann (1944), indicated that

" active immunity to Asiatic cholera in the experimental animal, and presumably also in man, includes antitoxic as well as antibacterial immunity ".

It deserves attention, however, that according to further experiments recorded by Burrows (1953), the immunity resulting from active immunization with purified cholera endotoxin proved to be inferior to that produced by active immunization with O antigen.

Reporting upon observations made in guinea-pigs which had been infected with *V. cholerae* by the intragastric route following alkalinization and intraperitoneal administration of opium tincture, Burrows, Elliott & Havens (1947) stated that

(*a*) the infection thus produced was confined essentially to the lumen of the bowel with no consistent or significant spread into the tissues and organs, and could be

considered to be of a true nature in view of an enormous multiplication of the vibrios in the intestine of the animals;

(*b*) prior administration of a non-lethal dose of *V. cholerae* per os or intraperitoneal immunization with 2 mg of cholera O vaccine two weeks before challenge protected the animals against as much as three lethal doses and altered the pattern of vibrio excretion characteristic for non-immunized infected animals by bringing about a sharp reduction in the number of organisms, especially early in the infection, and usually leading to a lessened persistence of the infection;

(*c*) antibody activity, manifested by the appearance of agglutinins and protective antibodies, and shown to be due to the presence of immuno-globulin, was demonstrated in the faeces of immunized animals and also in the faeces of human volunteers who had been vaccinated against cholera;

(*d*) the antibody in the faeces, called *coproantibody* to distinguish it from that in the serum, though appearing early and reaching peak titres before antibodies became manifest in the serum, disappeared in contrast to the serum antibodies in 3-4 weeks.

The correlation found to exist between the pattern of vibrio excretion characteristic for cholera-immunized animals and the presence of copro-antibody led to the conclusion that " effective immunity to enteric infection is associated with pre-existing coproantibody ".

As shown by further studies of Burrows & Havens (1948), immune globulin, immunologically indistinguishable from immune-serum globulin, was excreted in the faeces and urine of actively or passively cholera-immunized guinea-pigs as well as of cholera-vaccinated human volunteers. It was established in this connexion that the barrier between the tissues and the lumen of the bowel was readily permeable to the immune globulin, which thus could pass in either direction.

Burrows & Havens confirmed that there was a lag between the appearance of peak agglutinin titres in the faeces and urine of immunized guinea-pigs and in their sera respectively. This lag was still more conspicuous in cholera-vaccinated human subjects, in whom the peak of the faecal agglutinin titre was reached about two weeks after the second inoculation, and that in the urine apparently a few days later, as compared to the occurrence of peak titres in the serum after 30 to 42 days. There was also a differential in the disappearance of the antibodies, the agglutinins demonstrable in the urine and faeces falling to insignificant levels in the case of immunized guinea-pigs after 3-4 weeks, in that of human subjects two to three months after vaccination, whereas—as far as could be ascertained—the antibodies persisted in the sera for considerably longer periods.

It could not be established definitely where the faecal antibodies were formed. Considering various possibilities, Burrows & Havens stated that:

" It is possible that fecal antibody is that which is formed locally. The assumption that, with the general antigenic stimulus of parenteral inoculation, the antibody-forming cells of the intestine form antibody to excess much more rapidly than those of, for example, the spleen, seems unlikely and is without foundation. The corollary assumption, that the association of falling fecal titer and rising serum titer may be explained by an earlier cessation of antibody formation by the local cells or a breakdown of the mechanism of

diffusion or secretion, is hardly tenable especially in view of the results of experiments reported here in which fecal titer was maintained by periodic reinoculation. Neither of these need be made, however, for it is possible that the observed independence of serum and fecal antibody titers is a concentration effect. Thus, the dilution of immune globulin liberated by antibody-forming cells during the early stages of immunization is relatively much greater in the body fluids than in the feces, but antibody accumulates in the tissues and not in the feces. Rising serum antibody would, then, represent a rate of accumulation of serum globulin while the titer of fecal antibody would reflect more directly its rate of diffusion from the antibody-forming cells."

The findings made in regard to the coproantibody were confirmed by some further investigations of Burrows and co-workers, particularly by the results of a study on the quantitative relationship between the faecal and serum antibodies made with the aid of complement-fixation tests by Koshland & Burrows (1950).

It would be rash to assert that the observations of Burrows and colleagues, the main features of which alone could receive attention within the scope of the present disquisition, have fully solved the problems of the mechanism of active cholera immunity. In fact, they have furnished reason to assume that, as Burrows, Mather, Elliott & Havens (1947) put it when commenting upon the mouse-protection test :

" It may well be that protection is a manifestation of more than one kind of antibody, possibly O agglutinin, bacteriolysin, immune opsonin and the like, as well as antibody to the vibrio endotoxin; at least present evidence does not justify the assumption that protective antibody is homogeneous. "

There can be no doubt, however, that the demonstration of a local response to parenteral cholera immunization in the intestine, manifested by the rapid appearance of coproantibody, goes a long way to support the assumption that this method of immunization is an effective means to protect man against natural infection with *V. cholerae.*

Passive Immunity

Observations which demonstrated the possibility of conferring a passive immunity against cholera seem to have been recorded first in 1892 by Gamaleia, Klemperer and Lazarus.

Gamaleia reported at a meeting of the Société de Biologie, Paris (October 29, 1892) that Ketscher in St. Petersburg had (*a*) found that the milk of cholera-immunized goats, if intraperitoneally administered to guinea-pigs in 5-ml doses, protected the latter animals against lethal doses of *V. cholerae* given intraperitoneally or intramuscularly, and (*b*) noted even the survival of guinea-pigs which had received intraperitoneal injections of such milk after they had been infected with cholera vibrios by the intramuscular or intraperitoneal route.

Klemperer (1892c), besides confirming that the milk of immunized goats was capable of protecting guinea-pigs against intraperitoneal cholera infection, also found the same to hold true of the serum of a human volunteer who had been injected with 5 ml of milk

from a cholera-immunized goat. Klemperer (1892b) further established that the serum of persons vaccinated with living or heat-killed cholera vibrios protected guinea-pigs against intraperitoneal injections of *V. cholerae*.

As already alluded to (page 986), Lazarus (1892) found that the serum of cholera convalescents, if administered in extremely small doses (minimum one decimilligram, 0.0001 g) intraperitoneally to guinea-pigs, protected the animals against intraperitoneal challenge with lethal doses of *V. cholerae* made a few hours afterwards. He established, on the other hand, that even enormous doses of cholera convalescent serum were incapable of saving previously cholera-infected guinea-pigs which already showed signs of illness. Lazarus maintained, therefore, with great reason that

> " in the case of animals which it was possible to save from death through treatment [with convalescent serum] following infection, we cannot speak of a successful cure but rather of an immunization administered during the incubation stage ".

As can be gathered from a study of the literature (see summaries by Hetsch, 1912, and Kraus, 1929), during the years following these initial findings the question of whether passive immunization against cholera conferred an antitoxic or merely a bactericidal immunity, became the subject of considerable debate. Though, as will be shown later, this controversy is now of historical interest rather than of actual importance, the following of the several claims made for the production of cholera-antitoxic sera (see in addition to the summaries just mentioned those of Hetsch, 1928, Harvey, 1929, and of Burrows, Mather, Wagner & McGann, 1944) deserve attention.

Ransom (1895, see also Ransom & Kitashima, 1898), immunized goats and horses with a soluble cholera toxin, which he claimed to have obtained by heating 5- to 10-day-old broth cultures of *V. cholerae* for a short time at 100°C, filtering through a Pukal filter and then resorting to concentration in vacuo and alcohol precipitation at 30°C. As admitted by Ransom himself (1898), his sera possessed feeble (" schwache ") antitoxic properties. Pfeiffer (1895) even maintained that the antitoxic effects of Ransom's serum were not higher than those of normal sera.

Metchnikoff et al. (1896) prepared cholera sera by immunizing goats and horses as well as guinea-pigs and rabbits with what they considered a soluble toxin of *V. cholerae*. This thermostable product was obtained from strains, the toxigenicity of which had been enhanced through passage in collodium sacs kept in the peritoneal cavity of guinea-pigs. The vibrios in question were afterwards grown in a medium containing besides 2% peptone, 2% gelatin, and 1% sodium chloride, also 10% of normal horse-serum for 4 days (when toxicity was found to be maximal) and then filtered.

The sera obtained with this toxic product through prolonged immunization of horses had marked bactericidal and agglutinating properties. At the same time they were endowed with what Salimbeni (1908) considered as a somewhat feeble antitoxic power, at least 1 ml being needed to protect a guinea-pig against 4 lethal toxin doses. Nevertheless, as summarized by Salimbeni (1908), " the serum proved to be very efficacious prophylactically and gave in curative tests very good results in intestinal cholera of young rabbits produced experimentally according to Metchnikoff's method ".

Salimbeni (1908), continuing the above-described work, modified the methods originally adopted by Metchnikoff et al. (1896) by (*a*) ceasing to use the preliminary passage of the strains in collodium sacs through guinea-pigs, which was found to be unnecessary when freshly isolated human cholera strains, never passed through animals and kept with rare transplantations on agar at room temperature, were available; (*b*) increasing the

content of horse serum in the medium used for toxin production to 25%; and (*c*) adopting in place of the subcutaneous the intravenous route of immunization. He noted in this connexion that, while 1.5 ml of a subcutaneously immunized horse had been needed to neutralize 4 lethal doses of cholera toxin, after continued intravenous immunization of the same horse 1/3 ml of its serum sufficed for this purpose.

It is noteworthy that prolonged intravenous immunization of a horse with living cholera vibrios (saline suspensions of agar cultures) also gave a serum which combined marked agglutinating and bactericidal properties with a quite marked power to neutralize the toxin. This was in contrast to the results of intraperitoneal immunization with living cholera vibrios attempted by Metchnikoff et al. (1896), the serum obtained in this manner from a horse, though endowed with marked agglutinating and bactericidal properties, showing less antitoxic power, since 1.5 ml were necessary to neutralize two lethal toxin doses.

As alluded to before (page 1053), Macfadyen resorted, in order to liberate the cholera endotoxin, to the method of fragmentating growths of *V. cholerae*, obtained through cultivation on agar for 18 hours, at the temperature of liquid air. The thermolablie endotoxic products obtained in this manner were capable of producing sera which possessed, besides agglutinating and bacteriolytic properties, anti-endotoxic power. For instance, the serum of an intravenously immunized goat was found to neutralize in guinea-pig tests 8 lethal endotoxin doses, whereas normal goat serum proved incapable of protecting these animals even against 2 lethal doses.

Brau & Denier (1906), in order to obtain toxic products for the manufacture of cholera-immune sera, (*a*) grew a strain of *V. cholerae* isolated in Saigon, which was found to coagulate milk rapidly but was non-haemolytic, in a medium consisting of 90% normal horse-serum and 10% defibrinated horse-blood, and (*b*) resorted after an incubation at 39°C for 7 days to filtration first through paper and then through Chamberland or Berkefeld filters.

Summarizing their experiences when using sera produced with the aid of this toxic filtrate and, for the sake of comparison, also a serum raised by Salimbeni against live cholera vibrios, Brau & Denier stated that:

> " (*a*) Subcutaneous injection of the toxin into goats, rabbits, guinea-pigs and horses produces with difficulty an active immunity. The serum thus obtained is feebly antitoxic;
>
> (*b*) Intravenous injection on the contrary immunizes the animals and leads to the appearance of very manifest antitoxic properties in their serum;
>
> (*c*) The animals which had been intravenously injected with living cultures, furnish a more active serum than those treated with the soluble toxins."

Brau & Denier maintained, therefore, with much reason that there seemed no need to " establish a difference between the cholera toxin contained in the bodies of the microbes and that obtained in the culture fluid ".

It is important to add that (*a*) the serum produced by Brau & Denier had also quite good agglutinating properties (titre 1: 5,000); (*b*) their toxin, the production of which they ascribed to a maceration of the vibrios in the course of cultivation, was found to be thermostable, a serum raised with a filtrate which had been heated for 20 minutes at 100°C, proving as efficacious as those prepared with unheated toxic filtrates, and (*c*) Brau & Denier established that neutralization of lethal doses of their toxin by gradated serum doses did not take place according to the law of multiple proportions, as is characteristic for true bacterial exotoxins.

Kraus, summarizing in 1909 the results obtained in the production of antitoxic cholera sera (see Kraus, 1907; Kraus & Russ, 1908), stated " that not only the cholera vibrio, but also the El Tor vibrios and many other vibrios have the property to produce toxins (' Gifte vom Charakter der als Toxine charakterisierten Gifte ') and that these can be

neutralized with specific antotixins ". Therefore, in order to produce antitoxic sera, Kraus and Kraus & Russ used not only true *V. cholerae* strains, but also El Tor strains as well as the *V. Nasik*, a haemolytic vibrio not reacted upon by cholera immune sera.

The sera raised with the toxins, i.e., the filtrates of 6- to 8-day-old broth cultures, of true cholera vibrios, though also producing bacteriolysis of El Tor vibrios, exerted an antitoxic action only against the *V. cholerae* toxins. The El Tor serum, while bacteriolytic not only for the El Tor but also for classical cholera vibrios, was found to be antitoxic not only in the case of these two organisms, but also of cholera-like vibrios. Serum obtained with *V. Nasik* toxins, while bacteriolytic for this organism only, proved antitoxic also for El Tor vibrios.

While finding guinea-pigs rather unsuitable for tests with these sera (see below), Kraus reported that:

" 1. Mice which are infected with cholera vibrios, are cured if one treats them either with antitoxic cholera serum or with serum obtained with the toxin of specific El Tor vibrios (i.e. El Tor vibrios in the strict sense). Sera obtained with *V. Nasik* toxins were inefficacious in this case.

" 2. Mice infected with specific El Tor vibrios are cured if treated with sera prepared with El Tor or Nasik toxins. Cholera serum produced in horses failed completely (but, as stated by Kraus in a footnote, a cholera serum produced by Pfeiffer in goats neutralized also El Tor toxin).

" 3. Mice infected with *V. Nasik* are cured, if they are treated with sera prepared with El Tor or Nasik toxin. Cholera serum exerts no curative action."

On account of these findings Kraus stressed that success in cholera treatment could be obtained only with those sera which contained antitoxins as well as bacteriolysins. It has to be noted, however, that the mice used in such tests could be saved only if treated with the appropriate sera not later than one hour after infection. Guinea-pigs could be saved but occasionally if given large serum doses intravenously half an hour after infection. Kraus warned, therefore, against concluding from favourable results obtained in one animal species upon the possible effect of the sera in other species and expressed at the same time the fear that man might react in respect to specific cholera treatment like the guinea-pig and not like the mouse.

Schurupow (1909), reporting upon tests with one of Kraus' cholera sera, which had been sent to Russia, stated that this serum possessed but minimal antitoxic properties and produced agglutination of cholera vibrios only at a titre of 1: 500.

In order to prepare a cholera serum of his own, Schurupow tried first to immunize horses with living cholera vibrios (route not stated). However, the serum of these animals was found to possess only marked agglutinating and bactericidal properties but no antitoxic power. Schurupow admitted, however, that these experiences were not in accord with those of some other workers, Kraus for instance having obtained through subcutaneous immunization of horses with living cholera vibrios a serum, 0.07-0.1 ml of which neutralized 1 ml of toxin.

In view of the disappointing results he had obtained with live cholera vibrios, Schurupow immunized horses with Chamberland-candle filtrates of *V. cholerae* cultures which had been treated with alkali. The resulting sera possessed agglutinating properties (titre 1: 10,000), but had according to Schurupow no bactericidal power. As far as can be gathered from the somewhat scanty data furnished by this worker, his sera showed quite considerable protective and even curative action against the endotoxin, but unless quite high doses were administered prophylactically, the guinea-pigs tested showed marked signs of intoxication before eventually recovering.

As will be gathered from the above summary, the various workers enumerated, though using different antigens for the immunization of their

animals, unanimously stated that they had obtained sera more or less endowed with antitoxic properties. The validity of these claims was, however, vigorously opposed by Pfeiffer and his co-workers.

Pfeiffer & Wassermann (1893), making passive immunization tests in guinea-pigs with cholera convalescent sera, found ample evidence of a bactericidal action of these sera, but none of a role of antitoxins, and concluded, therefore, that passive as well as active cholera immunity depended upon the presence of bacteriolysins. This contention was supported by Issaeff (1894) who found that the sera of cholera-immunized guinea-pigs as well as those of human convalescents, though endowed with marked protective and to some extent even with curative properties, possessed no antitoxic properties, the maximal cholera toxin doses tolerated by immunized guinea-pigs being not higher than those for control animals.

The findings of Issaeff were confirmed through exhaustive studies made by Pfeiffer (1895b) with the sera of goats which had been subcutaneously immunized for prolonged periods with increasing and finally with enormous doses of living cholera vibrios. Even the most potent of these sera were found to exert " no true antitoxic effect " either against the toxin of chloroform-killed cholera vibrios or against that contained in broth cultures of *V. cholerae* which had been sterilized with toluene after an incubation for 20 days.

It seemed at first glance in contrast to these observations that guinea-pigs which had been injected intraperitoneally with a mixture of the toxic substances and large amounts of the immune serum, tolerated toxin doses two to three times in excess of those lethal for the controls. Pfeiffer found, however, that almost as high a tolerance for increased toxin doses could be produced if, instead of immune serum, the serum of normal goats was used for such tests. He assumed, therefore, that under the conditions of these experiments the immune as well as the normal serum exerted merely an unspecific action by hampering and retarding the absorption of the cholera toxins.

A further interesting study of the problem presently under review was made by Pfeiffer & Friedberger (1908a) who used for this purpose El Tor sera put at their disposal by Kraus in comparison to a purely bactericidal cholera serum obtained through single intravenous injections of rabbits with minimal doses of *V. cholerae*. The El Tor toxin used to test these sera was obtained by (*a*) centrifuging the peritoneal exudate of guinea-pigs which had succumbed to intraperitoneal infection with *V. El Tor*, and (*b*) adding to the supernatant small amounts of the bactericidal cholera serum so as to lyse the organisms which had not become sedimented.

In confirmation of the experiences of Kraus and Kraus & Russ, Pfeiffer & Friedberger found that the acute effects of the thermolabile exotoxin produced by *V. El Tor* were neutralized by El Tor serum. They stated, however, that

" in the case of the true toxins and antitoxins we find an exactly quantitative relationship; the lethal dose is either neutralized or not. In the case of the mixtures of Tor exudates with Tor serum things are not so simple. Here one observes, even when manifold multipla of the protective minimal [serum] dose are used, a drop of the temperature [of the test guinea-pigs] down to 34°C, which may be accompanied by other signs of intoxication (most marked prostration) and which appears characteristically only 5-6 hours after injection of the mixture." [Trans.]

Pfeiffer & Friedberger concluded from these observations that the peritoneal exudates of El-Tor-infected guinea-pigs contained two kinds of toxins—a neutralizable exotoxin and a second component, which represented the endotoxin liberated in the peritoneal cavity of the animals through disintegration of the organisms, and which, as shown by a large series of carefully conducted experiments, was not neutralized by the El Tor sera. Pfeiffer & Friedberger further adduced evidence to show

" that in El Tor infection the antitoxic serum acted mainly through its bactericidal component, whereas the antitoxin contained in the serum according to our quantitatively conducted experiments was incapable of exerting a favourable influence on the course of the infection ". [Trans.]

Preventive and curative tests made with the El Tor sera in cholera-infected guinea-pigs convinced the two workers that

" the El Tor antitoxin is by no means a universal antitoxin as is postulated by Kraus & Russ. It fails completely as far as the endotoxins of the cholera vibrios and also as far as the toxic substances are concerned which form in the body of cholera-infected guinea-pigs." [Trans.]

Entertaining no doubt that in the case of cholera as well as in that of El Tor infection the immune sera exerted only a bactericidal effect, Pfeiffer & Friedberger concluded that " so far the investigations of Kraus had furnished no proof for the existence of true antitoxins against the toxin of *V. cholerae* ".

The views of Pfeiffer & Friedberger were fully shared by Raskin (1909) who, re-testing Schurupow's serum found that this product, while exerting no anti-endotoxic action, possessed a high bacteriolytic titre. In accordance with these observations Raskin came to the conclusion that the curative action of Schurupow's serum in guinea-pigs was not superior to that shown by purely bacteriolytic sera.

Pottevin (1913b) found that a classical strain of *V. cholerae*, which had been isolated in Constantinople, possessed only a thermostable endotoxin which was neutralized neither by its homologous serum, produced through prolonged subcutaneous immunization of a donkey, nor by the serum raised in an identical manner with one of the two El Tor strains examined at the same time. The latter two strains possessed also an exotoxin which (*a*) was thermolabile; (*b*) was endowed with haemolytic properties; (*c*) in contrast to the endotoxin of the cholera and El Tor vibrios was lethal for intravenously injected pigeons; and (*d*) also in contrast to this endotoxin

was neutralized within certain limits by either of the two above-mentioned donkey sera.

These interesting observations, besides confirming the views held by Pfeiffer in regard to the toxin of *V. cholerae*, also speak in favour of an identicity of the El Tor exotoxin with the " haemotoxin " (haemolysin) of these organisms. The continued presence of endotoxic properties after the action of the exotoxin had been inhibited by heating, as demonstrated by Pottevin, was probably responsible for the belief of some workers that the *V. El Tor* produced a soluble toxin distinct from its haemolysin.

Carrière & Tomarkin (1910) summarized the results of an exhaustive experimental study on the problem of cholera serotherapy, made in Kolle's laboratory at Berne, Switzerland, as follows :

" 1) Bacteriolytic or purely bactericidal cholera sera obtained through a few intravenous injections of cholera vibrios possess the least curative effect, even when their lytic and agglutinating titres are high.

" 2) The greatest curative effect in animal experiments is exerted by sera, which have been produced through prolonged immunization with cholera bacteria, particularly if a mixture of the sera of different species of animals, immunized . . . by various methods is used.

" 3) Such sera contain considerable quota of anti-endotoxins, but it is difficult to assess the anti-endotoxic properties in view of the comparatively low neutralizing power of the sera and the varying resistance of animals to the cholera toxins.

" 4) The law of multipla is not valid for the anti-endotoxins of the cholera serum.

" 5) The administration of comparatively large amounts of bactericidal serum is innocuous for animals with experimentally produced cholera peritonitis, provided that considerable amounts of anti-endotoxic substances are administered simultaneously.

" 6) Serum treatment of cholera patients is innocuous, if one uses instead of purely bactericidal sera such with some anti-endotoxin content. In constrast to the widely accepted opinion that owing to the action of the bacteriolysins serum administration leads to the massive liberation of endotoxins, our sera as well as those manufactured in Russia caused absolutely no harm even if given to cholera patients in very large doses.

" 7) To obtain therapeutic success, the administration of considerable amounts of serum is indispensable.

" 8) It is essential to use for therapeutic purposes exclusively those sera which have been obtained through as much as possible prolonged immunization of different animals and animal species by subcutaneous and intravenous administration of living and killed cholera vibrios as well as of endotoxins and extracts of the latter." [Trans.]

Commenting upon the results of their studies, made mainly with the sera of goats, horses, and rabbits, Carrière & Tomarkin found no reason to abandon the concept of the cardinal importance of the cholera endotoxins, but militated against the opinion often advocated that it was impossible to produce antitoxins against them. However, while claiming to have proved the fallacy of such beliefs, Carrière & Tomarkin admitted that the endotoxic substances at work

" probably do not represent the whole endotoxin of the bacterial cells in its original form and produce antibodies which do not completely correspond to the endogenous bacterial

toxins. The incomplete efficacy of the sera might be due, therefore, not to the fundamental impossibility to produce anti-endotoxic substances, but merely to the inadequacy of the methods available for immunization." [Trans.]

Kolle (1909a), in a preliminary communication on the work of Carrière & Tomarkin, expressed full agreement with the views of these two workers and maintained that

" prolonged immunization leads, perhaps more in some animal species than in others, to the formation not only of bacteriolysins and agglutinins but to some extent also of anti-endotoxins, further of complement-fixing substances and bacteriotropins. Moreover, this enumeration probably does exhaust the substances which come in question for the treatment of cholera. It would be wrong, theoretically to dismember an immune serum and to conclude from such theoretical considerations upon its efficacy in animal experiments and in patients." [Trans.]

However, while one must agree with Kolle that the mechanism of passive cholera immunity is of a more complex nature than is indicated by the rigid dicta of Pfeiffer and his school, and that anti-endotoxins probably play a role in it, one should not lose sight of the fact that all cholera sera produced thus far exhibited but feeble anti-endotoxic properties, if any at all. It is under these circumstances not surprising that, as will be discussed in the eighth of these studies, in spite of the optimistic opinions expressed by some of the early observers, the inefficacy of cholera serotherapy is now generally admitted. In fact, after rather unfavourable experiences had been made in this respect during the first World War (see Hetsch, 1928, and particularly Kraus, 1929), apart from a solitary attempt made by Ghosh (1935, 1936) the method of treating cholera patients with the aid of immune sera has not been used any more.

RÉSUMÉ

Passant en revue les problèmes relatifs à l'immunologie dans le choléra, l'auteur discute la question, encore controversée, de l'endotoxine cholérique, celle de la structure antigénique du vibrion et de la composition chimique des antigènes. Il examine ensuite les races sérologiques du vibrion, la sérologie du choléra, les réactions sérologiques des sujets vaccinés et des porteurs sains, la spécificité des réactions d'agglutination, la fixation du complément, les tests cutanés et l'allergie. Il développe la question de l'immunisation naturelle, de l'immunité induite, des divers types de vaccins, du mécanisme de l'immunisation active, et explique les difficultés rencontrées dans l'application de l'immunisation passive, aujourd'hui pratiquement abandonnée.

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