STUDIES ON VIBRIO CHOLERAE

by

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PREFACE

Cholera is one of the most important health problems of many areas of Asia. Despite extensive research, workers are still confronted with a number of unsolved problems that stand in the way of control and ultimate eradication of the disease. The immediate problems presented by cholera at this time are related to its treatment and its practical control.

The clinical and biochemical abnormalities that occur in the cholera patient can be rapidly and consistently corrected by intravenous administration of fluids approximately equal in volume and electrolyte concentration to the fluids lost via the gastrointestinal route. Although treatment by rehydration is good, specific treatment must wait until we know more regarding the mechanism of pathogenicity in cholera. Alternatively, it may be that phage therapy still is worthy of consideration but this is doubtful.

The control of cholera depends on several factors.

(i) Improvement of sanitation, provision of safe water, satisfactory disposal of excreta and health education of the public to improve personal hygiene are essentials.

(ii) Further investigation of the epidemiology of cholera and cholera like diseases will assit control. This demands more research into cholera phages and genetic mechanisms so that epidemics can be traced and understood.

(iii) Finally, active immunization may achieve control of cholera, but we meanwhile require more knowledge of mechanisms

of pathogenicity so that good protective antigens may be prepared.

The writer has therefore investigated the 2 obviously important aspects of

(A) The toxins of cholera

(B) The phage, and genetics related to cholera.

These studies are reported in Sections I and II of this thesis respectively.

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SECTION I.

Introduction.

Historical outline.

The history of the world incidence of cholera has been described by Pollitzer and Swarup (Pollitzer, 1959). It would appear from many observations on record that cholera was confined to the East and almost exclusively to the Indian and Burmese sub-continent until the second decade of the last century. For several centuries cholera had been spreading from India into China along routes followed by caravans of tea and silk traders. Although importation of cholera took place from India to the neighbouring countries in the East (Simmons, 1879), no long-distance spread of the disease occurred until the early 19th century. A severe epidemic broke out in Bengal in 1817 and spread to China in the same year; the infection was carried to Burma, Indonesia and Thailand in 1819; to Malaya and the Philippines in 1820 and to Japan in 1822. Arabia was affected in 1821 through a British expeditionary force sent from India, and within a few months the disease spread to the southern Mediterranean coast, Zanzibar and East Africa. The first westward spread was recorded in 1821 when cholera originating from India was carried far and wide in 6 pandemics during 1817-1923 (Haeser, 1882; Sticker, 1912; Pollitzer, 1959). The introduction of steam power in navigation greatly increased maritime commerce and sea travel between India and the West. This contributed to the rapid and extensive spread of cholera.

Europe was affected in 1829 by the second pandemic of 1826-37. England and Scotland were affected in 1831. Four more pandemics scourged Europe in quick succession from 1852-1923.

Cholera was considered to be a more acute form of plague by many Londoners and the panic of the people has been vividly described by Snow (1855, p.153): "Many houses were closed altogether, owing to the death of the proprietors; and in a great number of instances the tradesmen who remained had sent away their families, so that in six days from the commencement of the outbreak the most afflicted streets were deserted by more than three-quarters of their inhabitants".

The scientific investigation of cholera began soon after its appearance in Europe and attempts were made to classify the disease according to the ideas of the two schools of epidemiologists - the "Contagionists" and the "Localists". The former held that cholera was a highly contagious disease and they insisted upon rigid measures of isolation of the patients, elaborate methods of disinfection and quarantine. On the contrary, the localists believed that the manifestations of cholera and other infectious diseases were dependent upon local "miasmatic" factors and considered that all of the above control measures advocated by the "contagionists" were unnecessary. Although microscopy revealed the causative microbes, their connection with epidemic disease was not established satisfactorily. Even in 1839 Schönlein ascribed contagion to microbes originating from derangements of humours (Bulloch, 1938). The "contagionists" were at a loss to account for the spread of cholera nor could they employ effective quarantine measures without having sufficient

knowledge of the spread of disease through food, water or animate carriers. de Jonnes (1832), in a series of memoirs published between 1821-23, declared that cholera was contagious. The idea of transmission of cholera by water gained firm support as a result of the labours of Snow (1855).

Dr. John Snow, anaesthetist to the University College Hospital, London, made his appearance at a critical moment in the evolution of the germ theory of cholera when the contagionists were unable to explain the occurrence of isolated cases in which there was no proof of contact. They had to modify or change their views by supposing that the atmosphere became a medium for the conveyance of the poison. Snow built his epidemiological concept on the understanding of the pathological process of the disease. He acquired experience of cholera by working in a mining village that was afflicted by the disease in 1832 (University College Hospital Magazine, 1954, p. 136).

The first edition of Snow's work "On the mode of communication of Cholera" was published as a pamphlet in 1849 (Snow, 1849) and it contained isolated examples suggestive of water-borne infection. His conclusive proof of the role of water in the wide and rapid dissemination of cholera was given in the second enlarged edition published in 1855 (Snow, 1855). Snow's arguments regarding the spread of cholera were attacked by various sections of his profession (see MacKintosh, 1955). Baly and Gull (1854) denied the role of water in the spread of cholera. The air of the cholera wards was examined microscopically and chemically and no

significant abnormality was found by Ehrenberg (cited by Hirsch, 1881) and Thomson (1850) before Snow published his work. The dejecta of cholera patients were examined by Boehm in 1838 and Hildebrand in 1838-39 (Hirsch, loc. cit.). The former found some microscopical spherical bodies and the latter tried to produce symptoms resembling cholera in experimental animals by feeding cholera stool. Brittan (1849) found some "annular bodies" in cholera stool and in the air of a cholera ward while he was working in the Bristol epidemic. Some peculiar granular bodies were observed by Williams (1849) in cholera evacuations and Budd (1849) described microscopic bodies in the drinking water of cholera-infected places. From these observations, the fungoid theory of cholera came into being for a short while but lost its ground by the declaration of Busk (1849), the President of the Microscopical Society of London, who observed that most of those supposed cholera fungi were nothing more than a species of Uredo or Smut.

The presence of very minute animalcules that produced sudden and rapid movements in recently voided rice-water stool of cholera patients was reported by Pouchet (1849). Hassall (reviewed by Pollitzer, 1959) observed myriads of vibriones in samples of rice water; many of them formed threads and the others aggregated into dot-like masses under the microscope. Hirsch (loc. cit.) credited Boubée for his observations about the influence of geological and physical characters of the soil on the spread of disease. He explained that the diffusion of the disease was not due to the geological character of the soil but due to its aqueous saturation.

This "Bodentheorie" was further developed by Pettenkofer (cited by Lewis, 1870). He suggested that a special factor passed by cholera patients and healthy contacts required for its development a special kind of porous soil containing organic matter, salt and free interchange of air and water. The poison might lie dormant in soil that was too dry or too wet only to revive when conditions were favourable. Subsoil water level fluctuations also had some relation to the aetiology of the disease and this was studied extensively in India by Lewis and Cunningham (1878) who confirmed the views of Pettenkofer.

As the pandemic of 1881 swept into Cairo in June, 1882, and into Alexandria in July, the Consulting Committee on Public Hygiene in Paris was requested by Pasteur to send a scientific mission to Alexandria. "Since the last epidemic of 1865", he wrote, "Science has made great progress on the subject of transmissable diseases . . . . Now the present knowledge demands that attention should be drawn to the possible existence within the blood, or within some organ, of a micro-organism whose nature and properties would account in all possibility for all the peculiarities of cholera, both as to the morbid symptoms and the mode of its spread." (Vallery-Radot, 1906, p. 378). Several Commissions were sent to Egypt. The French Commission under Roux and the German Commission under Koch were working in Alexandria by the end of the year. The then Secretary of State for India did not comply with the request of Macnamara to be sent to Egypt (Macnamara, 1892, p. 29). Koch observed and isolated vibrios from dejecta of patients and intestinal contents of victims

at autopsy in 1883. As the outbreak of the cholera epidemic in Egypt came to an end, he was deputed by the German Government to continue his work at the Medical College, Calcutta, where he confirmed his Egyptian findings in 1884 (Koch, 1884). He was also the inventor of the nutrient gelatin plate; vibrios produce characteristic colonies on this medium because of their ability to liquefy gelatin rapidly. Koch reported his discovery before Virchow, the Czar of German medical science at that time and the chairman of the Imperial Board of Health. He hailed Robert Koch saying "I thought it very probable that the bacillus was, indeed, the causis morbi; but from what I have heard today, my conceptions on the subject have arrived at a much greater degree of certainty." (British Medical Journal, 1884).

Pettenkofer, as described by Dubos (1951), admitted that the comma bacillus was the specific cause of cholera yet he maintained that certain local, seasonal and individual conditions had to be satisfied before it could produce the disease. Pursuing this view he swallowed during 1892, at the age of 74, recent isolates of virulent cholera vibrios, after neutralising his stomach contents with a draught of sodium carbonate solution: a procedure recommended by Koch as the most favourable for infection. His pupils also joined this self-experimentation. He escaped with mild diarrhoea and among his pupils Emmerich nearly died of cholera; Metchnikoff and others remained well. This was regarded as a proof of their theory that certain individual conditions were involved.

Many other forms of vibrios were isolated from diverse sources

by various workers (for reference see Mackie, 1929) and new tests were devised to differentiate the specific germ of cholera from other vibrios. The first step towards specific identification of the cholera vibrio was made by Pfeiffer who developed the well-known Pfeiffer's reaction (Pfeiffer, 1894). The specificity of the agglutination reaction (Grüber and Durham, 1896, cited by Pollitzer and Burrows, 1955) remained suspect until the exhaustive cross-absorption tests by Gardner and Venkatraman (1935). Cholera and cholera-like vibrios having biochemical similarities and possessing a common H-antigen were classified by these workers into Group A. Vibrios having a different H-antigen and biochemically distinct from Group A were assigned to group B. Group A was further subdivided into O-subgroups I-IV on the basis of different O-antigens. V. cholerae and most of the El Tor vibrios (q.v.) fell under O-subgroup I of Gardner and Venkatraman. Thus, according to Gardner and Venkatraman, the true cholera vibrio is a non-haemolytic organism containing the specific O-antigen of subgroup I; excepting its lack of haemolysin production, it is indistinguishable from El Tor vibrios containing the same O-antigen. In further studies of the antigenic structure of the cholera vibrio the existence of subtypes, generally referred to as the Inaba, Ogawa and Hikojima types, was recognized. Nobechi (1933) postulated the antigenic formulae for these as AX, BCX and ABX.

The greatest confusion in the serological identification of the cholera vibrio rose from the isolation of 6 strains of vibrios by Gotschlich (1905, 1906), at the El Tor quarantine station, from

dead bodies of returned Mecca pilgrims. Though these victims showed no sign of choleraic disease during life, the vibrios found in their intestine were identical with true cholera vibrios according to biochemical and serological reactions. Kraus (cited by Kolle and Hetsch) observed that these strains were strongly haemolytic. This observation of Kraus added to the evidence of the absence of any further cases at El Tor and the history of the absence of typical symptoms of cholera in the patients from whom these organisms were isolated. The isolation of a similar vibrio from stools of healthy persons and from persons suffering from non-choleraic disease (Omar, 1938; Marrs, 1940) provided a basis for the conclusion that the El Tor vibrio was not identical with V. cholerae. Observations as to whether the haemolytic property could be exploited as a criterion for the differentation between El Tor and true cholera vibrios were made. Thus Doorenbos (1936) showed that V. cholerae could also be haemolytic depending on the composition of the medium, the species of the red cells used and the age of the culture. De, Bhattacharya and Roy Choudhury (1954) demonstrated that V. cholerae lysed human red cells only in the presence of a soluble salt of calcium while the El Tor vibrio lysed red cells in the absence of calcium and failed to lyse them in the presence of excess of calcium. The following table shows some of the tests recommended by various authors for differentiating V. cholerae from V. eltor.

Table 1.

Differentiation of Cholera and El Tor vibrios.

Author	Test	Result obtained with	
		V. cholerae	V. eltor
Greig (1914)	Haemolysis of goat cells.	-	+
Taylor, Pandit and Read (1937).	VP reaction by Barritt's method.	-	+
Moor (1938)	Lysis of sheep blood in agar plates under anaerobic conditions.	-	+
Tanamal (1948)	Soda serum test: (agglutination with antiserum diluted in 0.2 per cent. Na_2CO_3. $2H_2O$).	-	+
	Soda-sublimate test: vibrio suspension + a few drops of 0.5 per cent. $NaHCO_3$ + equal volume of 0.5 per cent. $HgCl_2$, shows clumping within 15 min.	+	-
Mukerjee (1961)	Lysis by cholera phage IV.	+	-
Finkelstein and Mukerjee (1963)	Agglutination of fowl red cells by agar culture in saline.	-	+
Gan and Tjia (1963)	Inhibition by (i) 0.9 per cent. trypsin broth (pH7) and (ii) polymyxin B disk (50 units).	+ +	- -

The monograph of Roger (1921), the report of Taylor (1941) and the reviews of Mackie (1929) and Greig (1929) are largely based on the experiences of these authors with cholera in India and Egypt. The classification of the vibrios by Heiberg was published from Copenhagen in 1934, but the disappearance of cholera from the West has partly shifted the weight of research on the problem to Asia and to workers stationed there. Thus the United States Naval and Army Research Unit (NAMRU-2) in Taiwan, Formosa, is engaged in studying the altered physiology of cholera patients and infected animals. The SEATO laboratory at Bangkok in Thailand has made interesting contributions on the aetiology of cholera, and the Pakistan-SEATO Cholera Research Laboratory at Dacca has been conducting field and laboratory studies on the pathogenesis and immunology of the disease. More accurate data on various aspects of natural and experimental cholera have been provided by investigators working in these places. In spite of their valuable work, the basic problems have not yet been solved. Confusion still exists regarding the types of vibrios capable of causing cholera (Lindenbaum et al., 1965); the factors determining host susceptibility (Rosenberg et al., 1965); the pathogenesis of the disease (Phillips, 1965); the optimal regime for replacement and antibiotic therapies (Carpenter et al., 1965); and the efficiency of various vaccines (Oseasohn, Benenson and Fahimuddin, 1965).

The Pathogenesis of Cholera.

Cholera is an acute infectious disease of sudden onset usually

coming in epidemic form and characterized by copious watery diarrhoea, vomiting, muscular cramps and extreme collapse. The classical disease is caused by a specific bacterium - V. cholerae. The organisms multiply freely in the lumen of the small intestine of the affected person and they are present in large numbers in the intestinal contents and dejecta. They do not penetrate deeply into the intestinal mucosa and there is practically no invasion of the blood stream but the gall bladder may be infected.

The mechanisms by which the cholera vibrio causes enormous losses of fluid and electrolyte from the plasma by way of the gut have not yet been clearly defined. Pathologists in the past have been almost equally divided between those who thought that the vibrio induced a denudation of the gastrointestinal tract and those who believed that there was no denudation. Those who supported the former theory believed that, as a result of denudation, there was an outpuring of a transudate which comprised the cholera stool. Cohnheim (1890) rejected the idea of a "true transudate" fluid passing from the blood vessels of the intestinal mucosa to the lumen of the gut. He argued against calling the cholera evacuations a transudate because of the very low albumen content and absence of red blood cells in the rice water stool. He maintained that the presence of amylase in the cholera stool was indicative of hypersecretion from the glands of the small intestine. He also failed to find epithelial cells in the rice water stool. At autopsy some of the intestinal villi were seen to be denuded of epithelium to a variable depth; some retained their epithelium. The denudation that he observed was considered

to be a post-mortem change and the possibility of leakage of a "true transudate" through intact epithelium during life was denied.

Peroral Crosby capsule biopsies of the bowel epithelium were obtained by Gangarosa et al.(1960) and Fresh et al. (1964) from patients with acute and severe cholera. These specimens showed intact mucosa with mild oedema, congestion, and cellular infiltration. The biopsies were from jejunum because the lower part of the ileum was more difficult to reach. This was also confirmed from radiographs and histological sections. Gangarosa et al. (1960) aspirated intestinal contents from the biopsy sites. On culture the samples did not yield V. cholerae although the patient continued to excrete vibrios. These investigations did not conclusively exclude the possibility of ulceration in the distal part of the ileum which Dutt, Mondal and De (1964) could demonstrate in materials fixed immediately after death by injecting Zenker's solution into the abdomen of the victim. The low protein content of the bacteria-free cholera stool, however, is evidence against significant ulceration of the ileum in the acute phase of the disease. Watten et al. (1959) demonstrated that when I^{131} -tagged polyvinyl-pyrrolidone was administered intravenously it does not appear in the cholera stool in abnormal amounts. This is further evidence that there is no mucosal sloughing with denudation of the villi and an outpuring of fluid from the denuded surface.

It has been found from studies on the pathophysiology of cholera (Phillips, 1963) that the fluid loss is isotonic and there is excess loss of bicarbonate and potassium. Movement of large

volumes of electrolyte solutions from plasma to gut lumen and from gut lumen to plasma in normal dogs has been studied by Visscher et al. (1944a, b) and Berger et al.(1959) with the use of isotopes. If this study could be directly transposed, in a man weighing 50 kg. a total of 40-80 litres of fluid would pass from his plasma into the gut lumen and back again from gut lumen to plasma in 24 hr making the net fluid flux zero. Visscher has also shown that the flux of the fluid from gut lumen to plasma is done by an active transport of sodium ions by intestinal mucosal cells, the so-called "sodium pump", and the mechanism could be inhibited by different poisonous metabolites.

Huber and Phillips (1960) applied the sodium pump concept to explain the depression of transport of water and sodium ions across frog skin treated with cholera stool filtrates, and to explain the diarrhoea in cholera. They observed the presence of a sodium pump inhibitor in the stools of patients suffering from classical Asiatic cholera and from disease associated with the El Tor vibrio but not in the stools of normal healthy individuals. The inhibitor was found to be thermolabile. De and Chatterje (1953) found that the fluid that collected in the ligated loop of the small intestine of rabbits after inoculation with a loopful of culture of V. cholerae was rich in protein and contained Evans blue when this dye was previously injected into the ear vein. De, Sarkar and Tribedi (1951) found protein-rich fluid in the peritoneal cavity of rabbits after intraperitoneal inoculation of a suspension of V. cholerae killed by heating at 56°C for 15 min. It is not unusual to find a variable number of

red blood cells in the cholera stool so that it may appear mucosanguinous or like meat infusion (Liebermeister, cited by Pollitzer, 1957). The presence of variable numbers of red cells in the cholera stool also suggests that there is altered permeability of the intestinal capillaries in cholera. These observations suggest that there is a toxic effect of V. cholerae on the permeability of the intestinal capillaries leading to leakage of plasma protein into the lumen of the gut, but the mechanism remains unexplained.

It is convenient, at this point, to review the exoenzymes and exoproteins that may be involved in mechanisms of pathogenicity of the cholera vibrio and to consider the part that endotoxic reactions may play in producing the symptoms and signs of the disease.

Enzymes of Vibrio cholerae.

Burnet and Stone (1947a) described an enzyme in cultures of V. cholerae that produced desquamation of the intestinal mucosa of guinea pigs. They referred to this as mucinase and showed that it was distinct from the receptor-destroying enzyme that is also produced by the cholera vibrio. Similar results were reported by Burnet (1948) who showed that the mucinase dissolved the mucin coating of the intestinal epithelium and led to desquamation of the epithelial cells.

Narayanan, Devi and Menon (1953) and Gurkipal Singh and Ahuja (1953) confirmed the presence of mucolytic activity in cultures of cholera vibrios. This activity was also detected in

culture products of some El Tor and non-agglutinable water vibrios. The enzyme was capable of desquamating intestinal epithelium and of reducing the viscosity of ovomucin solutions. Lam and Mandle (1954) demonstrated that the vibrio mucinase desquamated intestinal mucosa and increased the permeability of isolated intestinal loops. According to Proter (1955), mucinases acting on the gut were produced in high concentration by non-agglutinating avirulent water vibrios and they existed in at least 2 serologically distinct forms.

De (1961) found mucinase in toxic culture filtrates of V. cholerae grown in 5 per cent. peptone water medium. The activity was detectable in dilutions of the filtrates up to 1 in 40. Non-toxic filtrates produced by growing the organism in other liquid media or in semi-solid agar were much less active; mucinase activity was demonstrable in these in dilutions up to 1 in 10 only. This observation gave De the impression that higher degrees of mucinase activity were related to enterotoxicity of filtrates, but he subsequently found that filtrates from the 5 per cent. peptone water medium inoculated with a rough strain possessed high mucinase activity without being necessarily enterotoxic. This indicates that mucinase and enterotoxin are not the same.

Felsenfeld (1944) detected the presence of lecithinase C in cultures of V. cholerae. De (1961) showed haemolytic activity in the bacteria-free filtrate obtained from saline washings of agar cultures of V. cholerae and he suggested that lecithinase C activity was present. He maintained that such an enzyme might

increase the permeability of intestinal capillaries by breaking down substrate in the gut wall and causing the outpouring of fluid into the lumen of the intestine. He further observed persistent haemolytic activity in filtrates from agar cultures although enterotoxic activity was only present occasionally. On the other hand, filtrates obtained from cultures grown in 5 per cent. peptone water were always enterotoxic but possessed no haemolytic property. He concluded from these observations that the enterotoxic property is not likely to be related to haemolytic or lecithinase C activity.

The presence of receptor-destroying enzyme (RDE) was demonstrated by Burnet and Stone (1947b) in filtrates obtained from cultures of V. cholerae in nutrient broth and in semi-solid agar medium. De (1961) found that some filtrates having a high RDE content had no effect on rabbit loop. This suggested that the enterotoxic activity was not related to the receptor-destroying enzyme of V. cholerae. The receptor-destroying enzyme has since been characterized as a neuraminidase.

Cholera toxins.

"The basis of all harmful effects of bacterial infection is quite certainly chemical; and only when the chemist has replaced the immunologist shall we be able to give an intellectually satisfying account of what happens when a particular parasite invades a particular host". (Topley and Wilson, 1936).

The harmful effects of parasitic microorganisms may be manifested by interference with host metabolism or with

detoxification mechanisms of the host, by production of mechanical lesions, or by inducing the formation of toxic or inflammatory substances from the tissues of the host. It has been known for many years that some bacteria injure tissues with what are called bacterial toxins which are classified as endotoxins or exotoxins according to whether they are found inside or outside the parent organisms. Exotoxins are found outside the parent cells and they are also called "true" or "soluble" toxins. Such a toxin is excreted during the active phase of growth of the organism and reaches its highest concentration when the growth of the organism is maximal, but there are instances of the highest production of the toxin being attained long after the maximal growth of the organisms has been reached. Some exotoxins can also be recovered from washed cells but more occurs extracellularly than intracellularly.

Despite a great deal of research on exotoxins, very little is known about the physiology of their production, their mode of action and the part they play in disease. The mode of action of only one classical bacterial toxin can be defined. This is the alpha toxin of Clostridium perfringens which was recognised as a phospholipid-splitting enzyme by Macfarlane and Knight (1941) more than 20 years ago. Recently the release of degradative enzymes by bacterial toxins from lysosomes may help in elucidating the consequences of the action of some toxins. Weissman, Keiser and Bernheimer (1963) showed that the haemolytic toxins, streptolysins O and S, liberated β-glucuronidase and acid phosphatase from the large granule fraction of rabbit liver, heart,

spleen and lymph-nodes. Degradative enzymes such as cathepsin, deoxyribonuclease, ribonuclease, phosphatase, and β-glucuronidase are liberated from lysosomes in the tissues of the host by haemolytic bacterial toxins and these liberated enzymes may be responsible for the harmful, often shock-like, effects. In gas gangrene the organisms produce toxin in the tissues of the host and, though the patient is in a state of shock, the gas gangrene toxin is not detectable systemically. Specific antitoxin therapy may be ineffective. The shock-producing substance is apparently removed by amputation of the affected limb. It is conceivable, therefore, that the shock-producing substances may be degradative enzymes or may arise from the action of such enzymes derived from lysosomes in the tissues of the host.

Endotoxins are found in cell-free autolysates of bacterial cultures. Delafield (1932), Olitzki, Avinery and Koch (1942) showed that the washed dead bodies of a number of Gram-negative organisms are toxic whereas those of Gram-positive bacteria are not. Although the bacterial endotoxins have been studied by many competent investigators during the past several decades, the exact relationship between their chemical structure and biological properties remained largely unknown. As ordinarily isolated, the endotoxins are complexes of lipid, polysaccharide and protein or peptide-like substances. It was observed by Morgan and Partridge (1942) that the protein moiety was necessary for endotoxic potency. Webster et al. (1955) and Landy et al. (1955) demonstrated that the protein-free lipopolysaccharide retained the ability to stimulate all of the characteristic

physiological effects. This view was further confirmed by Ribi et al.(1960).

Boivin and Mesroboanu (1935) and Freeman (1942) demonstrated the loss of antigenicity and most of the endotoxic potency of endotoxins by hydrolysis with dilute acetic acid leaving the serologically specific polysaccharide hapten and a lipid component which was extractable with chloroform. Some of the lipids obtained from endotoxin by hydrolysis were shown to retain certain of the endotoxic properties by Westphal and Luderitz (reviewed by Ribi et al., 1961). In order to find out the minimal chemical structure necessary for elicitation of biological properties, Ribi et al. (1961) tried to strip away extraneous material from the endotoxin prepared from Salmonella enteritidis. These workers all but eliminated protein and lipid components in which the ultimate toxic principle was thought to reside. Their most highly refined product was composed of polysaccharide complexes together with small amounts of fatty acids, nitrogenous material and phosphorus.

The type of toxin produced by V. cholerae and the nature of toxic activity relevant to the disease that the organism produces in man have not yet been satisfactorily ascertained. In describing the aetiological relationship of this microorganism to Asiatic cholera, Koch (cited by Pollitzer and Burrows 1955) thought of the disease as "a condition of toxicosis due to specific poison". He further continued, "the sympton complex of the cholera attack proper, usually considered to be due to dehydration of the blood, is in my opinion to be considered essentially as an

intoxication". Efforts were thereafter made to demonstrate a specific cholera toxin experimentally. Nicati and Rietsch (1884) were the first to make an attempt by intravenous injection of filtrates of broth cultures of V. cholerae of more than a week old in dogs and they succeeded in producing symptoms of poisoning characterized by vomiting, dyspnoea, and paralysis of the extremities leading in some of the animals to death within 12 hr. They found, however, that filtrates of young broth cultures were incapable of producing signs of toxicosis in these animals. Apparently they were dealing with an endotoxin liberated after autolytic disintegration of the organisms. Further experiments by Cantani in 1886 (Pollitzer, 1959) showed that broth cultures of vibrios killed at 100°C and injected intraperitoneally into dogs produced signs of intoxication not dissimilar from those observed in human cholera. Cantani attributed the toxic signs to the action of an endotoxin liberated after death of the organisms. His assumption was emphasised by Pfeiffer in 1892 (cited by Pollitzer, 1959) who 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 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".

A continuous drop of body temperature in guinea-pigs following intraperitoneal administration of cholera toxin was observed by Pfeiffer in 1892 (Pollitzer, 1959). This was one of the

remarkable symptoms of cholera intoxication in these animals and it was apt to commence as early as 1½ to 2 hr after the administration of the toxin. Associated with the temperature drop, the animals became prostrated with paralysis of the hind limbs; fibrillary convulsion of the muscles could be observed. Death generally occurred within 12-16 hr of administration of a high dose of the toxin. With a lesser dose the animal showed signs of intoxication but it became well after 24 hr.

Several workers including Kolle and Schurmann (1912) and Kolle and Prigge (1928) observed that the agent responsible for the pathogenesis of cholera was not an endotoxin; it was a soluble exotoxin secreted by the growing organisms. Pollitzer (1959) on the authority of Kolle and Prigge (1928) stated that the above claims "deserve no credence" and agreed with the following statement of Wilson and Miles, 1946. "The cholera vibrio does not secrete a true soluble exotoxin but . . . . 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 micrococcus - another organism that readily undergoes autolysis - is very close, though the cholera vibrio is far more toxic".

Boivin and Mesrobeanu (1935, 1936) in their study of endotoxin reported the extraction of toxic substances of glucolipid nature from various Gram-negative bacteria including V. cholerae. Equal volumes of washed bacterial cell suspension in distilled water and 0.5N trichloracetic acid were mixed and kept for 3 hr in the refrigerator for toxin extraction. The bacterial cells were then

removed by centrifugation and the supernate was precipitated with 4-5 volumes of alcohol or acetone. The precipitate dissolved in water was biuret negative. Digestion of the organisms with trypsin prior to extraction resulted in increased production of toxin. The toxin killed mice weighing 20 g. on intraperitoneal inoculation of a dose of about 0.1 mg. A similar kind of toxic material was also found in some of the non-pathogenic bacteria. After further studies on the glucolipid complex obtained by extraction with trichloracetic acid, it was concluded that the compound containing the principal part of the endotoxin of the organisms represented their "complete antigen"; this included the somatic antigen.

Burrows (1944) obtained endotoxin from V. cholerae by high speed grinding of the cells with sand. The toxin was also isolated by dissolving the cells in 6 M urea or by digesting with trypsin. It could be obtained from intact cells by extraction with 0.5 M trichloracetic acid, methyl or ethyl alcohol, chloroform or ethyl ether in a soxhlet apparatus. It could not be extracted in glycols. Addition of 3-5 volumes of alcohol to the trichloracetic acid extract resulted in precipitation of the polysaccharide while the toxic factor remained in solution. On evaporating the filtrate from alcohol-precipitated trichloracetate solution, a yellow oil containing most of the toxicity was separated out. A similar substance could be prepared by alcohol precipitation of the ground cells or of urea-dissolved or trypsin-digested cells, and also by direct extraction of the intact cells with methyl or

ethyl alcohol or chloroform. On addition of 10 volumes of chilled acetone to the alcoholic solution of the toxin, a white amorphous lipid substance precipitated out. It gave negative results in the Molisch, Millon and Biuret tests for protein and it contained 5 per cent. nitrogen and 1.7 per cent. phosphorus. It was insoluble in water, stable in acid, and thermostable, but it was unstable in 0.1N NaOH at room temperature. It was closely associated with a phospholipid. The mouse lethal dose was 0.03 mg.

Burrows, Wagner and Mather (1944) exposed excised strips of small intestine of guinea-pig and rabbit to their cholera toxin and observed increased flow of Ringer-Locke solution across the stretched strip under a hydrostatic pressure of 8-10 in. of the solution. A similarly increased permeability produced by a suspension of living vibrios was abolished by agglutinating the cells with antiserum, but the effect of the toxin could not be neutralized by the addition of hyperimmune serum even in a concentration of 10 per cent. to the toxin-Ringer Locke solution. If, however, the strips of intestine were taken from immunized animals they were found to be resistant to the action of the toxin.

Bernard and Gallut (1943) obtained maximum yields of endotoxin by growing the cholera vibrio in glucose broth for 4 hr, centrifuging the culture and reinoculating the supernate. The process was repeated 3-4 times. The acidity that developed as a result of the successive cultures killed the organisms; there was then diffusion of endotoxin which accumulated in the medium. The extensive studies of Gallut and Grabar (1943, 1947) showed evidence of production of 2 toxins by V. cholerae. One toxic component appeared to be low

in molecular weight, dialyzable, antigenic, thermostable and glucolipid in nature; it produced vascular congestion and this was its prominent histopathological effect. The other toxic factor was a non-dialyzable, thermolabile, protein-like substance and it produced a characteristic hypothermic effect.

Banerjee (1942) separated cholera toxin without the use of chemicals. The toxin was produced in Ramon's (1933) broth in which a cellophane bag containing sterile medium or normal saline was kept immersed. The toxin dialyzed from the surrounding broth culture into the bag. The toxic fluid thus obtained was found to kill guinea pigs in doses of 1-2 ml. when injected intraperitoneally and the minimum lethal dose for mice was 0.25 ml. given intravenously.

Freter (1953) stated that the endotoxin of V. cholerae grown in 1 per cent. glucose peptone water could be partially extracted with trichloracetic acid or pyridine, while 70-80 per cent. remained in the cell. He then reported better extraction of 30-40 per cent. of the soluble toxicity from vibrios with dilute acid at pH 3.8 for 4 hr. The soluble toxin thus obtained could be purified by coprecipitation with calcium phosphate or calcium carbonate. The purified substance contained 4.5 per cent. N and 1.7 per cent. P and it had an LD50 for mice of about 0.2 mg. The residual toxin from the acid-extracted vibrios could further be extracted at neutral pH if the organisms were dried in acetone before the second extraction process. This substance was not

toxic at pH 3.8. Freter (1956) characterized in greater detail the differential properties of the 2 toxic fractions of V. cholerae, one of which was acid-soluble and the other was insoluble in dilute HCl at pH 3.5. The acid-insoluble part of the toxic material had a mouse LD50 of approximately 0.35 to 0.75 mg. and appeared to be lipoprotein, whereas the acid-soluble moiety of the toxin had a mouse toxicity of 0.05 to 0.10 mg. and resembled Boivin's antigen.

A toxic protein was isolated by Jenkin and Rowley (1959) from Inaba and Ogawa strains of V. cholerae and from water vibrios by dissolving the bacteria in 2.5M urea and then precipitating with ammonium sulphate. These workers suggested that there was a similarity between the protein isolated by them and the protein component of the Boivin antigen. The lipopolysaccharide endotoxin of the strains examined was found to be less toxic than the lipoplysaccharides from other Gram-negative bacteria.

Ghosh (1938) reported extraction of cholera toxin in trichloracetic acid from tryptic digested vibrio cells. He also obtained toxins from autolysates of cholera vibrios kept in distilled water in the refrigerator for 1 month. These toxins killed rabbits within $\frac{1}{2}$ - 2 hr after intravenous injection and they also produced local skin lesions on intracutaneous inoculation in animals.

De and his co-workers (1951) found that there was exudation of protein-rich fluid into the peritoneum of rabbits after intraperitoneal injection of cultures of V. cholerae killed by heat

at 56°C for 15 min. The fluid was poor in polymorphonuclear leucocytes and its accumulation was associated with haemo-concentration and a fall of blood pressure. De and Chatterje (1953) devised an experiment with adult rabbits in which a small loop of the intestine was isolated from the rest of the gut by 2 ligatures around the gut wall. When an inoculum of 1 ml. of a peptone water culture of V. cholerae was injected into the lumen of this loop, a large amount of fluid accumulated there within 24 hr. Congestion and oedema of the wall of the loop and necrosis of the summits of the villi were also seen. De (1959) claimed that sterile filtrates of 6-12 hr cultures of recently isolated strains of V. cholerae grown in 5-10 per cent. Difco Bactopeptone would induce a typical loop lesion. The active fraction was inactivated at 56°C within 30 min. and it could be precipitated with ammonium sulphate. It was not found in phage lysates of vibrios and seemed to be a true exotoxin. The requirements for growth were critical (De, Ghose and Chandra, 1962) and aeration was essential. The production of the toxin was inhibited by the presence of mucin and bile in the medium.

Dutta, Panse and Kulkarni (1959) produced diarrhoea in baby rabbits by feeding a crude acid-lysate of vibrios (Gallut, 1954). Panse and Dutta (1961) found that when filtrates of bacteriologically positive stools of clinically diagnosed cases of cholera were fed to baby rabbits, they succumbed to a disease similar to experimental cholera. It was thus suggested that a choleragenic factor was excreted in the stools of patients with cholera during the active stage of the disease.

Jenkin and Rowley (1959) could not produce loop lesions by injecting streptomycin-sterilised loop fluid into fresh loops and these workers concluded that active growth of the vibrios in the loop was essential for the development of a positive loop. Various metabolic products of bacterial growth were thought to play an important role in the production of a positive loop. Jenkin and Rowley were able to induce accumulation of fluid after an injection of 25 mg. of sodium lactate into a loop. They also observed a similar effect when 5 mg. of lactate was inoculated into a loop of a rabbit that simultaneously received endotoxin intravenously. Formal et al.(1961) could not produce a positive loop with even 66 mg. of lactate along with intravenous endotoxin. These workers also demonstrated that loop fluid contained only traces of lactate till degeneration of the mucosa began. Barely detectable amounts of lactate were found in the intestinal fluids of choleraic baby rabbits.

De (1961) was unable to produce positive loops by injection of ultrasonic lysates of whole vibrios but Osa and Dutta (1963) could produce diarrhoea in baby rabbits by feeding large amounts of whole culture lysates of V. cholerae strain 569B. In confirming this, Finkelstein, Norris and Dutta (1964) observed that filtrates of aerated cultures in Difco Brain Heart Infusion Broth (BHIB) or in a synthetic medium (Finkelstein and Lankford, 1955) supplemented with 1 per cent. casaminoacid (Syncase medium) produced greater yields of toxin. Filtrates of these cultures were also active in man (Finkelstein, 1965). Two components, designated as Procholeragen A and Procholeragen B were separated by dialysis of BCL and syncase culture filtrate but not from

HIB cultures. Procholeragen A was heat labile and nondialyzable whereas Procholeragen B was heat stable and dialyzable. Neither was diarrhoeagenic when fed alone to baby rabbits; for full choleragenic activity, a mixture of the 2 components was essential.

Schafer and Lewis (1965) and Ghosh (1965) could demonstrate thermolabile non-dialyzable enterotoxin in loop fluid and culture filtrates produced by several strains of V. cholerae. The toxin could be precipitated by zinc sulphate and ammonium sulphate. The fluid accumulating in ileal loops of rabbits18 hr after injection of V. cholerae culture was centrifuged at room temperature for 15-20 min. at 2000 g to deposit the debris. The supernate was separated and excess of chloroform was added. The mixture was allowed to stand at room temperature for 1 hr and the supernatant fluid was decanted from the chloroform. A loopful was streaked on to a nutrient agar plate to ensure freedom from live vibrios. The rest was recentrifuged for 30 min. to precipitate the killed vibrios. By inoculating this sterile loop fluid into fresh loops, Ghosh (loc. cit.) was able to produce positive loops in adult rabbits. The loop lesions so produced were indistinguishable morphologically from those induced by infection with cultures of V. cholerae. One of these loop fluids was again inoculated into a third set of 4 loops. There was no lesion after 18 hr. The fluid was thus shown to be free of a transmissible agent.

Craig (1965) detected a heat-labile, formalin-inactivated, acid-sensitive non-dialyzable "skin toxin" in the filtrates of stools from cholera patients and from young V. cholerae cultures

grown in 5 per cent. Difco Bacto-peptone. The skin toxin caused induration and increased capillary permeability following intracutaneous inoculation in rabbits and guinea-pigs. Such toxic activity was not detected in stools from other kinds of diarrhoeal disease nor in the culture filtrates of other enteric pathogens. The skin toxin was neutralized by convalescent sera from cholera patients but not by antibacterial sera containing agglutinins produced in rabbits in response to inoculations of living or killed vibrios.

Burrows (1965) suggested that there are 3 types of cholera toxins that may occur:

(i) The toxin that is lethal for mouse and 10-day egg;

(ii) The toxic activity showing intradermal reactivity, giving a toxic reaction in infant rabbits and in ligated bowel loops, and producing a cytopathic effect on Earle's L cells in culture; and

(iii) The factor that shows toxicity for anurian epithelium and inhibits p-aminohippurate concentration by kidney slices in vitro.

The Rabbit Loop.

Experimental work in cholera research has been limited because the disease is not readily reproducible in animals. The baby rabbit provides a model in which the pathology of the experimental infection approximates that of natural cholera. Dutta and Habbu (1955) confirmed that classical cholera vibrios cause a disease in infant rabbits that closely resembles clinical

cholera in man. Some authorities maintain that this is the best and most reliable model currently available.

The rabbit loop is an alternative model in which the pathogenicity of the vibrios is judged by the evidence of an inflammatory reaction and the accumulation of a large amount of fluid in the inoculated loop that has the gross, microscopic, and cultural appearances of cholera stool. As the writer has based many of his observations on this latter model, a short review of its development follows.

In a brief "note on experimental cholera" Violle and Crendiropoulo (1915) mentioned the rabbit loop model. A loop of about 10 cm. long was made by these workers in the upper part of the small intestine of rabbits. The animals died overnight, perhaps due to intestinal obstruction. These workers could not infect rabbits by injecting vibrios into the lumen of the intestine above or below a single ligature. Thus intestinal obstruction was not thought to predispose the ileum to infection. Accumulation of watery fluid in a loop and desquamation of the mucosal epithelium was considered to be the sign of a typical loop lesion.

De and Chatterje (1953) tried this technique and injected living V. cholerae suspended in 1 ml. of peptone water into the lumen of a 10-cm. long loop of rabbit small intestine. After 24 hr there was an accumulation of a large amount of blood-stained fluid within this loop. The fluid contained mucous flakes, epithelial cells and live vibrios. Variable numbers of red cells and a few pus cells were seen but no macrophages

were observed. The albumen content of the fluid was high. The columnar epithelium was found to be necrotic, especially near the tip of the villi. There was evidence of oedema in the submucosal tissue spaces and lymphatics. The larger blood vessels were markedly engorged. Hydropic changes were seen in stromal cells but not in the villar epithelium. There was no cellular infiltration. Dutt, Mondal and De (1964) placed more emphasis on degenerative changes, especially denudation of the epithelium of the loop.

The rabbit loop procedure was claimed to be a general method of assessing the pathogenicity of enteric bacteria by De, Bhattacharya and Sarkar (1956). Esch. coli strains isolated from human stools were tested for their ability to produce loop lesions. It was observed that only 3 of 20 strains from healthy men caused loop lesions whereas 9 of 20 strains from patients with chronic diarrhoea and 15 of 20 strains from patients with acute diarrhoea were pathogenic. All of 3 strains isolated from children with infantile diarrhoea were also pathogenic, i.e. they produced loop lesions.

Namioka, Urushido and Sakazaki (1958) and Taylor, Maltby and Payne (1958) confirmed the value of the loop test for assessing the enteropathogenicity of strains of Esch. coli. McNaught and Roberts (1958) confirmed the statements of the above authors and also recorded rare false positive reactions in uninoculated loops. False positive loops were observed by De and Ghose (1959) only when the amount of fluid in the inoculum exceeded 1 ml. in a 10 cm.-loop. False positive reactions tended

to occur if material was injected too rapidly or if the loop was too short. Taylor, Maltby and Payne (1958) working on enteropathogenic Esch. coli observed that the proximal part of the intestine was more sensitive to infection.

The technique of the loop experiments of De and Chatterje was modified by Kasuga et al. (1963) and Hattore et al. (1965) who washed the loops in normal saline to remove debris and mucin. The American workers including Leitch (1965) and Schafer and Lewis (1965) also tried to modify the technique by the use of long loops that were washed with saline. These modifications did not avoid some false positive reactions.

The suitability of animals other than rabbits for loop experiments was also studied. De (1961) observed that rats were unsatisfactory for the purpose. Fresh, Vertage and Reyes (1964) noted that monkeys and goats could be used but there was no advantage. Guinea pigs were considered to be better than rabbits by Nikonov et al. (1958) for these workers could induce loop lesions in the former animals with only 2-3 colony-forming units (CFU) of V. cholerae and the guinea pig ileum was thought to harbour fewer commensal organisms. Florey (1933) used loops of guinea pig intestine to study the reaction of the mucous membrane to irritants. He observed copious inflammatory exudation within a few hours of inoculating a dense suspension of Salmonella typhimurium. There is no record of mice being used for loop experiments.

The observations of McNaught and Roberts (1958) and Taylor, Maltby and Payne (1958) confirmed that about 60 per cent. of the

Esch. coli strains isolated from patients with diarrhoea induced positive loop lesions in rabbits. The production of positive loop lesions in rabbits and guinea pigs had been obtained by injecting enteropathogenic staphylococcus aureus (Jenkin and Rowley, 1959), S. typhimurium (Florey, 1933; Singh, 1964) and Shigellae (Arm, Floyd and Farber, 1962; Kasuga et al., 1963). Taylor, Wilkins and Payne (1961) found that strains of Esch. coli causing enteritis in animals were unable to induce loop lesions in rabbits. Namioka, Urushido and Sakazaki (1958) obtained positive loop lesions in rabbits with 14 of 15 Esch. coli strains from the stomach and ileum of pigs dying of transmissible viral enteritis, while 9 of 11 strains from the colon were non-pathogenic.

Sarkar and Tribedi (1958) produced positive loops in rabbits with 19 of 30 strains of Alkaligenes faecalis isolated from the blood of patients thought to be suffering from enteric fever. Gupta et al. (1956) produced loop lesions in rabbits with 35 of 36 NAG vibrios isolated during an outbreak of gastroenteritis at Kumbh fair. Sayamov (1963) found strains of V. eltor from the Middle East and Celebes equally infective in both rabbits and guinea pigs, whereas Mukerjee (1963) found only 1 of 4 El Tor strainsfrom the Middle East to be pathogenic in rabbits.

De (1959) utilized the ligated rabbit gut segment to show that bacteria-free culture filtrates of V. cholerae could produce changes in the ligated loop identical with those given by the organisms themselves. For this purpose he inoculated 200 ml. of 5 per cent. Difco Bactopeptone medium with 5 ml. of a heavy growth of V. cholerae in Dunham's peptone water medium and

incubated in a Roux flask for 18-24 hr at 37.5°C. The culture was then clarified by high speed centrifugation and it was filtered through an Oxoid membrane filter. A 1-ml. volume of the sterile culture filtrate was injected slowly into a 4-in. ligated loop of small intestine of rabbit. After 18-24 hr, the loop was distended with fluid which resembled cholera stool. The histological changes in the wall of the loop were similar to those found in human cholera, namely, lymphocytic infiltration of the mucosa, dilation of blood vessels and lymphatics and a variable degree of desquamation of the surface epithelium and loss of villi.

A brief review of the problems to be solved.

Cholera is one of the most important public health problems of many countries in Asia and elsewhere. Some references to the incidence of the disease in India, Pakistan and Indonesia are quoted below (see Tropical Health, 1962).

The immediate area of Calcutta may be considered hyperendemic with case rates of 30-60 per 100,000 annually. Moderately endemic areas with case rates of 15-30 per 100,000 are the districts of Hoogly, Burdwan, Midnapur and Nadia. The case rates of hypoendemic areas are 10-15 per 100,000 of the population.

Cholera is of considerable importance in East Pakistan where the number of deaths between 1948 and 1959 varied between 7,002 and 29,582 yearly.

In Indonesia, the island of Celebes seems to be the major focus of the disease. An epidemic of cholera caused by the El Tor vibrios was reported in 1957 with high pathogenicity and

a case mortality of 88-90 per cent. The disease is spreading and now it is becoming endemic in a vast territory between the Pacific Ocean and the Caspian Sea. This aspect of the problem is reviewed in detail elsewhere (see p. 160).

Despite extensive research in cholera, workers are still confronted with a number of unsolved problems and their elucidation is essential for the satisfactory control and ultimate eradication of the disease. The great engineering advances in water supply and sewage disposal that have been achieved in the Western world have contributed to the eradication of cholera epidemics there. It is thus reasonable to consider that the control of cholera will ultimately depend on adequate supplies of pure water and the safe disposal of excreta. But this is not a realistic approach for the developing countries of Asia where it may still take decades to achieve the same standards of water supply and sanitation as now exist in the developed countries of the West. The problem is further complicated by the pattern of distribution of population in the Asian countries in which large numbers of people live in villages and draw their water supplies from rivers, wells and tanks.

Cholera is spread by patients during the acute stages of their illness and within a week or so of their incubation period and convalescence. Further, the cholera vibrios do not multiply in water; they remain viable in this environment for a few days only. These facts suggest that it should be possible to contain cholera locally, provided that intensive measures are taken to prevent infection of water sources during an epidemic and to

control the movement of temporary carriers by adequate quarantine measures.

Another important approach to control of the disease is mass immunization against cholera. Prophylactic immunization of the susceptible population forms one of the most important measures of epidemic control available to under-developed countries with inadequate funds and equipment to implement sanitary measures within a limited time. But several observations, including those of recent years in Calcutta, Dacca and Bangkok, have been made that cast doubt on the protective value of the currently available cholera vaccines. These observations point to the need for improving the protective potency of the existing cholera antigens. This will take time and satisfactory immunization is unlikely to be developed until we know more of the mechanisms that are involved in the pathogenicity of cholera organisms.

The present work has therefore been done in an attempt to elucidate some possibly significant toxic mechanisms that may operate in cholera. In particular, the writer has investigated the relationship of the skin toxin of V. cholerae to the enterotoxin.

Materials and Methods.

Materials and Methods

The experiments described in this section were designed to investigate the *in vitro* production of skin toxin of *V. cholerae*, to characterize the cutaneous-reactive factor involved, and to compare it with enterotoxin elaborated in experimentally infected loops of rabbit ileum.

This necessitated careful measurements and attention to detail in the preparation of various media and suspending fluids.

Enzyme preparations were used in attempts to decompose the toxic factor.

Toxin preparations were concentrated by fractional precipitation with ammonium sulphate.

Intestinal loops were made in experimental animals (see p. 46) and these were used in the production of enterotoxin.

Agar gel precipitation studies were of use in indicating antigenic relationships between different factors.

The experimental procedures and the reagents used are accordingly listed in detail as stated below.

Measurements. Percentage concentrations of solutions are expressed as volume/volume for 2 liquids and as weight/volume for solids in liquids. British Standard volumetric glassware of grade B (Pregl, 1951) was used. Pipettes of 1, 2, 5 and 10-ml.

volumes were graduated "to contain" whereas 0.1-ml. pipettes were calibrated "to deliver". A rubber teat was used for pipetting infective materials. An instrumental error, less than 0.02 ml., was therefore involved in measuring volumes greater than 0.1 ml. with the use of a teat. It has been shown by Lorenz (1962) that the overall error in pipetting microbial suspensions can be kept below 2 per cent. if the volume measured is at least half the capacity of the pipette. A 1-ml. Turner automatic pipette with 0.1-ml graduations was employed for serial doubling dilutions.

A spring or pan balance was used to determine approximate weights; for accurate weighing an Oerting Single pan Release-o-Matic balance with a sensitivity of 0.2 mg. per vernier division was used.

Strains investigated. The vibrio strains are detailed in Section II, p.226. Two of the V. cholerae strains deserve special mention in this Section. They were used for production of skin toxin and enterotoxin. V. cholerae strain 12r was isolated from a patient in East Pakistan by Dr. J.C. Feeley and freeze dried after passage through a suckling rabbit (MacIntyre and Feeley, 1964). V. cholerae strain 569B, also passaged through suckling rabbits (Dutta and Habbu, 1955), was received from the Haffkine Institute, Bombay.

Incubation Conditions. Tubes were incubated at 37°C in a thermostatically controlled water bath for periods up to 4 hr

and in an electric incubator for longer periods. A shaking instrument with a rotatory oscillation of about 250 per min. installed in the hot room at 31°C, and a thermostatically controlled water bath fitted with a shaker, were used for shake cultures.

pH determination. The pH of fluids was determined with a Beckman electric pH meter fitted with an automatic temperature compensator. The instrument was calibrated before use with commercial standard buffer solutions.

Storage. Materials were stored at room temperature between 18-20°C or in a cold room between 6-10°C or in a deep freeze at -20°C, as indicated in the text.

Suspending fluids and culture media:

Distilled water. Tap water distilled once in a metal still was generally used. The pH of the water at room temperature was between 5-6.

Normal saline (NS). This was made by dissolving 0.85 g. sodium chloride of Analar grade in 100 ml. distilled water at pH 5-6.

Phosphate-buffered saline (PBS). This was prepared according to Cruickshank (1965, p. 858). The pH was 7.0-7.2

Citrate-phosphate and Tris buffers. These were prepared according to Cruickshank (1965, pp.851, 853).

Peptone water (PW). This consisted of :

Bacteriological Peptone (Oxoid)	10g.
Sodium chloride	5g.
Distilled water	1 l.

It was sterilized by autoclaving at 121°C for 15 min. The pH of the medium was adjusted to 7.4.

Alkaline peptone water (APW). The pH of the sterile peptone water was adjusted to 8.8-9.2 with sterile deci-normal NaOH and the required amount of the alkali was estimated by titrating 5 ml. of peptone water (PW) in a Lovibond Comparator against phenolphthalein indicator.

Nutrient broth (NB). A 25-g. amount of Oxoid No. 2 nutrient broth powder was dissolved in 1 l. of distilled water and sterilised by autoclaving at 121°C (15 lb. per sq. in.) for 15 min. The pH was adjusted to 7.4.

x10 Nutrient broth. A 250-g. amount of Oxoid No. 2 nutrient broth powder was dissolved in 1 l. of distilled water and sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min. The pH was adjusted to 7.4.

5 per cent. Difco-Bactopeptone water.

This contained:

Difco Bactopeptone	50 g.
Sodium chloride	5 g.
Distilled water	1 l.

The medium was sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min. and the pH was adjusted to 7.6.

Syncase Medium. This was made up as follows:

NH_4Cl	595 mg.
Na_2SO_4	89 mg.
$MgCl_2.6H_2O$	42.5 mg.
$FeCl_3.6H_2O$	4.85 mg.

$MnCl_2 \cdot 4H_2O$	3.6 mg.
Sucrose	500 mg.
Na_2HPO_4	500 mg.
Distilled water	1 l.

This was supplemented with 1 per cent. casamino acid. The medium was sterilized by autoclaving at 121°C(15 lb. per sq. in.) for 15 min. and the pH was adjusted to 8.0.

Thioglycollate broth. This contained:

Bacto yeast extract	5 g.
Bacto Casitone	15 g.
Bacto dextrose	1 g.
NaCl	2.5 g.
L-cystine, Difco	0.05 g.
Thioglycollic acid	0.3 g.
Distilled water	1 l.

It was sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min.; pH was adjusted to 7.1.

Nutrient agar (NA). 40g. of blood agar base (Oxoid) was dissolved in 1 l. of distilled water and sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min. The pH was adjusted to 7.4.

Soft agar. 6g. of Davis Agar (Type P) was added to 1 l. of nutrient broth No. 2 (Oxoid) and sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min.; the pH was adjusted to 7.4.

Streptomycin agar. 40g. blood agar base (Oxoid) was dissolved in 1 l. of distilled water and sterilized by autoclaving at 121°C (15 lb. per sq. in.) for 15 min.; the pH was adjusted to 7.4.

Dilutions of streptomycin sulphate B.P. (Glaxo) containing the required concentrations were added aseptically to the cooled agar at 46°C.

2-1 medium (Lankford, 1958).

1.5 g. agar was added to 100 ml. nutrient broth. The pH was adjusted to 8.2 and the medium was sterilized by autoclaving at 121°C for 15 min. While the medium was still hot, 10 ml. of 10 per cent. glucose and 2 ml. glycerol (sterilized separately by autoclaving for 10 min. at 115°C) were added.

Gelatine-tellurite-taurocholate agar (TTGA).

This was prepared as described by Monsur (1961), tellurite being added to a concentration of 1:20,000 and the pH adjusted to 8.4-8.6.

Minimal liquid medium (Bhaskaran and Rowley, 1956).

This contained:

Ammonium sulphate	0.1 per cent.
Glucose	0.1 per cent.
$MgSO_4.7H_2O$	0.02 per cent.
NaCl	0.5 per cent.
K_2HPO_4	0.1 per cent.

in distilled water, adjusted to pH 8.0 and sterilized in the autoclave at 121°C (15 lb. per sq. in.) for 15 min.

The medium was solidified with 2 per cent. Ion agar to make minimal solid medium.

Peptone saline (PS). A 10-ml. volume of sterile peptone water was added to 100 ml. of sterile physiological saline; the pH was 7.0-7.2.

Enzyme preparations.

The following were obtained commercially:

Trypsin-(Bovine pancreas) A crystalline, salt-free preparation with a potency of 2,000 u/mg. was supplied by Light & Co. Ltd., Colnbrook, England.

Pancreatin - B.D.H. Biochemical from pancreas, conforming to BP 1958, was obtained from The British Drug HousesLtd., B.D.H. Laboratory Chemicals Division, Poole, England.

Linase (Wheatgerm, Worthington), was supplied by Light & Co. Ltd., Colnbrook, England.

Phosphorylase-a-from Rabbit Muscle. A 2x crystallized suspension in 0.0015M EDTA, 0.00192M. sodium glycerophosphate, 0.1M sodium fluoride, at pH 6.6 was obtained from Sigma Chemical Company, 3,500 Dekalb Street, St. Louis 18, Missouri., U.S.A.

Procholerasen A. A sample was obtained from Dr. H.K. Ghosh.

Freeze-drying mixture: One part of commercial horse serum was mixed with 1 part of 30 per cent. sucrose in broth, each component being sterilized separately by membrane filtration.

Initial treatment of bacterial strains.

The strains were received freeze-dried in ampoules from field laboratories and type culture collections (See Table 42). Enquiries revealed that most of the strains had been maintained on agar slopes covered with liquid paraffin before being freeze-dried.

On receipt, the contents of each ampoule were rehydrated with 0.2 ml. of broth and streaked on to a NA plate. The inoculated plate was then incubated overnight. Subcultures from smooth colonies were made on another NA plate and also in peptone water. A 3-4 hr growth from the PW tube was used for characterizing the strains (see below) and the over-night growth from the plate was suspended in 2 ml. of freeze-drying mixture; 0.1 ml. of this suspension was introduced into each of 20 sterile ampoules of 6 mm. internal diameter. These were then processed for 18-20 hr in an Edwards Centrifugal freeze-drier according to Greaves' method (Cruickshank, 1965, p. 806). The ampoules were then sealed in vacuo. The contents of 1 of these ampoules were rehydrated as before in broth and streaked on to a NA plate. If a pure heavy growth was obtained on the plate after overnight incubation, then the rest of the ampoules were stored in the cold room.

Characterization of strains.

1. Morphology of the colony. This was studied by obliquely illuminating cultures grown on 2-1 medium.
2. Motility. A hanging drop preparation was made from a 3-hr peptone water culture of the strain under test and motility was observed microscopically.
3. Sugar fermentation was observed in peptone-sugar tubes (Cruickshank, 1965, p. 813).
4. Reduction of tellurite. This was indicated by blackening of the colonies on a plate of gelatin-tellurite-taurocholate agar (Monsur, 1961) after incubation for 48 hr.

5. Oxidase test. An overnight agar culture was flooded with 1 per cent. tetramethyl-p-phenylenediamine and the excess fluid was drained off. A purple colour, usually pale, was developed by the vibrio colonies and persisted for at least 20 min. (Kovács, 1963).

6. Liquefaction of gelatin. This test was performed as recommended by Minor and Piéchaud (1963).

7. Haemolysin production was tested according to the technique of Feely and Pittman (1963).

8. Cholera Red reaction. A peptone water tube was inoculated with the test strain and incubated overnight. Five drops of concentrated sulphuric acid were then added. The reaction was considered positive if a pink colour developed within 15 min.

9. Voges-Proskauer reaction (VP). This was done by Barritt's method (1936) on 48-hr cultures. The result was said to be strongly positive (2+) if a distinct pink colour developed in 20 min. or weakly positive (1+) when the colour developed within 40 min.

10. Slide agglutination with antiserum (Inaba and Ogawa). This was done by mixing a loopful of antiserum with a drop of saline suspension from an overnight agar culture of the strain on a microscope slide. The specific Inaba and Ogawa antisera were supplied by Burroughs Wellcome & Co. The specificity of each serum was checked with 2 Inaba and 2 Ogawa strains.

11. Sensitivity to polymyxin B. This was tested by adding 1 drop of a 3-hr PW culture to 1 ml. of peptone water containing

20 units of polymyxin B sulphate (Burroughs Wellcome & Co.). The strain was considered to be sensitive to the drug if no turbidity was observed in the tube after overnight incubation. Control cultures were included.

12. Phage sensitivity. Lysis of strains at Routine Test Dilution of the Group IV cholera phage was determined according to the method of Mukerjee (1961).

Estimation of protein content of precipitated fractions by Folin-Ciocalteu phenol test for protein.

Crude skin toxin precipitated with ammonium sulphate from 5 per cent. Bacto-peptone culture filtrate of *V. cholerae* strain 12r and dialyzed against distilled water was tested for its protein content by the Folin-Ciocalteu Phenol test. This determines protein by measuring the blue colour produced on addition of the Folin reagent to an alkaline solution of the protein (Kabat and Mayer, 1961, p. 556). The method was made more sensitive by the addition of a minute amount of Cu^{++} to enhance development of the colour. Thus, Lowry *et al.* (1951) employed solutions of 2 per cent. Na_2CO_3 in 0.10N NaOH and 0.5 per cent. $CuSO_4.5H_2O$ in 1 per cent. sodium or potassium tartrate. Just before use, 1 ml. of the copper solution was added to 50 ml. of the Na_2CO_3 - NaOH mixture.

A 1-ml. volume of this reagent was added to 0.2 ml. of the test protein solution. After 10 min. at room temperature, 0.1 ml. of commercial Folin reagent, previously titrated with

phenolphthalein and diluted to an acidity of 1N, was added with vigorous mixing and the test solution was assayed spectrophotometrically after 30 min. or longer at a wavelength of 720 mμ as detailed in the text (p.64).

Animal experiments

Experimental animals were obtained from the Animal Breeding Station of the University and from external dealers. Guinea pigs were kept in groups of 2-4 in wire cages and rabbits were kept singly in cages. Both of these species were fed with commercial pellets. They were also supplied with green vegetables thrice weekly. Water was allowed ad libitum, even to animals being starved.

Preparation of intestinal loops in adult rabbits.

Rabbits between 5-12 months of age and of either sex were used. The test animal was deprived of solid food for 16-18 hr before the challenge. It was then anaesthetized with intravenous Nembutal, the dose being 1 ml. per 5 lb. of body weight. A 0.5-ml. volume of the total calculated dose was injected at a time. All of the injections were made very slowly. Failure to observe this precaution may kill the animal during anaesthesia. When completely anaesthetized, the animal was secured to an operating board and the hair of the abdomen was shaved. A central midline incision 4-5cm. long was made in the abdomen. The tip of the appendix could

easily be recognised; it is attached to the terminal part of the ileum by a short mesenteric fold and at a fairly constant point about 25 cm. from the ileocaecal junction. Starting at about 15 cm. proximal (oral) to this point, i.e. about 40 cm. from the ileocaecal junction, ligatures were placed around the ileum at intervals of 9-11 cm. Thus, 5-7 closed segments or "loops" were created along the middle third of the ileum. The toxin and the culture doses were injected into different loops of the gut in different animals. Each alternate loop served as a control.

The length of the gut in living animals is difficult to measure because of peristalsis. It has been observed in dead animals that the terminal "straight arteries" of the mesentery enter the gut wall at an average interval of 0.5 cm. Thus, 20 vessels enter over a length of about 10 cm. and this was chosen as the standard length of a loop. In living animals the ligatures were therefore placed around the gut wall after every 20 vessels to obtain loops of standard length.

After administering the challenge doses, the ileum was replaced; the peritoneum and the muscle coats were stitched in one layer. The skin was closed either with Mitchell clips or it was stitched with cotton threads. The operation took about 30-40 min. and the animal regained consciousness within 2-3 hr. No food was given and each animal was killed after about 11 hr by intravenous injection of 2 ml. of a saturated solution of $MgSO_4$.

A <u>postmortem</u> examination was then made. The animal was placed on its back. The chest and abdomen were swabbed with

2 per cent. lysol and the excess was wiped off. With sterile forceps and scissors, the skin was incised along the midline and reflected laterally. The muscle and peritoneal coats were dealt with similarly. The loop lesion was recorded as "weak positive" (1+) when the loop contained at least 5 ml. of fluid, and "strong positive" (2+) when there was also congestion and haemorrhage in the gut wall.

Agar gel precipitation studies

Explanatory note. Oudin in 1946 showed that when a concentrated antigen is layered over an agar-antiserum mixture in a tube a precipitin band is formed in a position that is directly related to the concentration of the antigen and its diffusion co-efficient, and inversely proportional to the concentration of the antibody. Thus, dilution of the antisera or antigen causes displacement of the band towards the antiserum or antigen respectively. Following these observations, methods were developed so as to allow the antigen and antibody to diffuse towards each other in agar when one or more precipitin bands were formed according to the number of specific reactants present. This method is known as double diffusion. It may be carried out in tubes or in petri dishes. The most suitable temperature for the immuno-diffusion test is 4°C because although the formation of bands is rapid at higher temperatures, there is decreased resolution of the precipitin bands and the precipitation is less complete.

Moreover, there is less risk of denaturation of labile reactants at the lower temperature. The time required for a visible precipitin band to form depends upon the diffusion rates of the two reactants and their relative concentrations. The precipitin bands are sharpest when they are first formed and they tend to broaden with time.

Procedure. The double diffusion agar plates were prepared by pouring 10 ml. of a 1 per cent. Ion agar in 0.85 per cent. NaCl (or 0.2 M phosphate buffer at pH 7.2) containing 0.1 per cent. sodium azide into a small flat-bottomed petri dish of about 6cm. diameter to give a perfectly level surface. Wells were cut in the agar when it had solidified with a cork borer or more conveniently with a gel cutter. (Different sizes, shapes and arrangements of wells have been used in this technique. The circular central well with equidistant satellite wells is the most popular.) After filling up the wells with antigen and antiserum using separate Pasteur pipettes the plate was covered and placed in a damp chamber such as a plastic lunch box with a piece of wet filter paper in the base. Diffusion was allowed to occur in the cold (4°C), or at room temperature (18°C), or at 37°C, overnight or longer if necessary. The plate was examined under incident light (Cruickshank, 1965, p. 948). Precipitin bands may form in one of 3 different patterns; (i) in the reaction of identity, the band lines are continuous from one well to the next; (ii) in the reaction of non-identity, the lines cross each other; and (iii) in the reaction of partial identity, the pattern is similar to (i) except for a spur formation at the junction of the

precipitin bands.

In the present gel-precipitation experiements with cholera toxin, double diffusion plates were incubated in most cases at room temperature (18°C) for 3-4 days. The toxin was placed in the central well and the antitoxin in the peripheral wells and vice-versa. The reactions obtained in different experiments are described in the Results section.

Results.

Experimental Results

Cutaneous activities of soluble products of V. cholerae.

Explanatory note.

Filtrates of stools from patients with cholera and from young cultures of V. cholerae grown according to the methods for production of cholera enterotoxin outlined by De Ghose & Sen (1960) and De, Ghose and Chandra (1962) were found by Craig (1965) to contain heat-labile substances that on intracutaneous injection in guinea pigs and rabbits induced identical skin lesions characterized by erythema, induration and a prolonged increase in capillary permeability. The term "skin toxin" has been applied to these skin-reactive substances of the culture filtrates of V. cholerae by Craig (loc. cit.). As in the case of the entero-toxin, maximum production of skin toxin was found to be related to surface/volume ratio of the culture media. The influence of time of incubation of skin toxin production by V. cholerae grown in shallow cultures was also studied by Craig (loc. cit.). He found that the concentration did not reach its maximum until 24-48 hr.

Results of present study.

In vitro production of skin toxin from V. cholerae has been achieved in the present study by culturing in 5 per cent. Difco Bactopeptone. Equally good yields were obtained from cultures in 1 per cent. peptone water. V. cholerae strain 12r (Ogawa) was grown in the peptone water media at 37°C for 24 hr in shallow cultures with a surface/volume ratio of 2.5. The ratio was calculated by dividing the surface area of the container in sq.cm.

with the volume (ml.) of the media used for growing the culture. The bacterial growth was centrifuged at 4,000 *g* for 1 hr in the refrigerated M.S.E. centrifuge at 5°C. The supernatant fluid, sterilized either by membrane filtration or with chloroform, gave skin lesions on intracutaneous injections in albino guinea pigs. A similar skin-reactive substance was also obtained with equal success from Inaba strain 569B grown in 5 per cent. Difco Bacto-peptone cultures. The presence of skin-reactive substances in these preparations was demonstrated by injecting a volume of 0.1 ml. from each preparation intracutaneously in partially-randomized sites on the backs of albino guinea pigs or rabbits. The guinea pigs or rabbits were prepared by clipping and shaving the hairs off their backs. Each of the guinea pigs were given 16 injections in 4 rows of 4 while each rabbit accommodated 50-60 injections. Most of our materials were tested on guinea pigs. It was noted that filtrates of positive loop fluid and *V. cholerae* cultures produced skin lesions characterized by marked induration and erythema beginning 2 to 3 hr after intracutaneous injection and becoming maximal at 18-24 hr, but persisting for 4-5 days in both guinea pigs and rabbits.

The effects of loop fluid and culture filtrates on skin capillary permeability were investigated by intravenous injection of Pontamine Sky Blue 6 x B (0.12 ml. of a 5 per cent. solution per 100 g. body weight of the guinea pig) at varying times after the intracutaneous injection of the test material (Elder and Miles, 1957; Craig and Miles, 1961). Experiments showed that filtrates of positive loop fluid and *V. cholerae* cultures capable

of evoking induration also caused an increase in skin capillary permeability. Immediately after injection of the dye there was a marked increase in permeability of skin capillaries followed by recovery within 1 hr. During the next few hours the permeability gradually increased with maximum intensity of blueing at 18-24 hr. Between 24 to 48 hr the intensity faded and the skin appeared to return to normal by 72 to 96 hr. The induration developed more gradually during the first 24 hr. A graph of the time-course of increased induration following intracutaneous injection of 0.1 ml. of $(NH_4)_2$ SO_4-precipitated cholera toxin in guinea pigs is shown in the upper 2 curves of Fig. 1. Induration and blueing were maximal at about the same time but the induration subsided more gradually over a period of 4-5 days. Residual induration was palpable at 6 - 7 days. The results of intradermal injections of filtrates of V. cholerae cultures and positive rabbit gutloop fluid in guinea pigs are shown in Table 2.

Fig. 1.

TIME-COURSE OF INCREASED INDURATION FOLLOWING INTRACUTANEOUS INJECTION OF 0·1ml OF $(NH_4)_2SO_4$-PRECIPITATED CHOLERA TOXIN IN GUINEA PIGS.

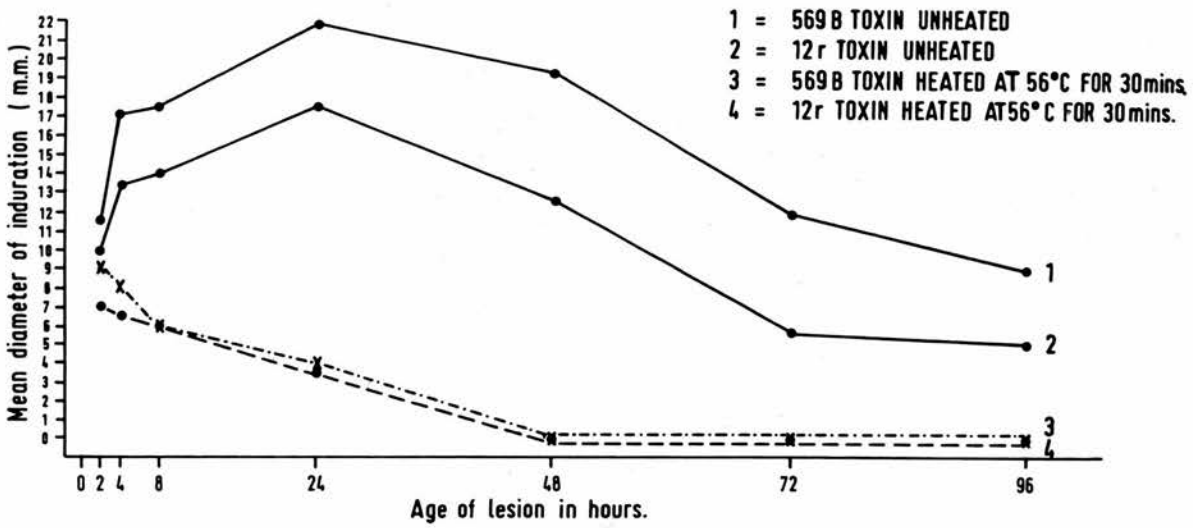


Table 2.

Skin lesions in Guinea Pigs following Intradermal Injections of Loop Fluid and Culture Filtrates.

Source of Toxin	Mean diameter of skin lesion per 0.1 ml. dose of inoculum	
	Induration (mm.)	Blueing (mm.)
Culture filtrates of *V. cholerae* Strain 12r grown in 5 per cent. Bacto-peptone media.	13.4	13.1
Culture filtrate of *V. cholerae* Strain 569B grown in 5 per cent Bacto-peptone media.	14.3	14.0
Culture filtrate of *V. cholerae* Strain 12r grown in 1 per cent. Peptone water media.	9.5	9.8
Culture filtrate of *V. cholerae* Strain 569B grown in 1 per cent. Peptone water media.	10.0	9.9
V. cholerae Strain 12r induced positive rabbit gut loop fluid filtrate.	16.6	16.2
Controls		
5 per cent. Bacto-peptone water	Nil	Nil
1 per cent. Peptone water	Nil	Nil

The production of skin toxin by strains 569B and 12r cultured in 5 per cent. Bacto-peptone water at 37°C under the conditions described above was studied in samples taken at intervals from 2 to 72 hr of incubation. The toxin was first demonstrable after incubation of strain 569B for 4 hr and after

6 hr with strain 12r. Thereafter the concentration increased gradually with maximal production at 24-48 hr. The results are shown in Table 3 and the data are represented graphically in Fig. 2.

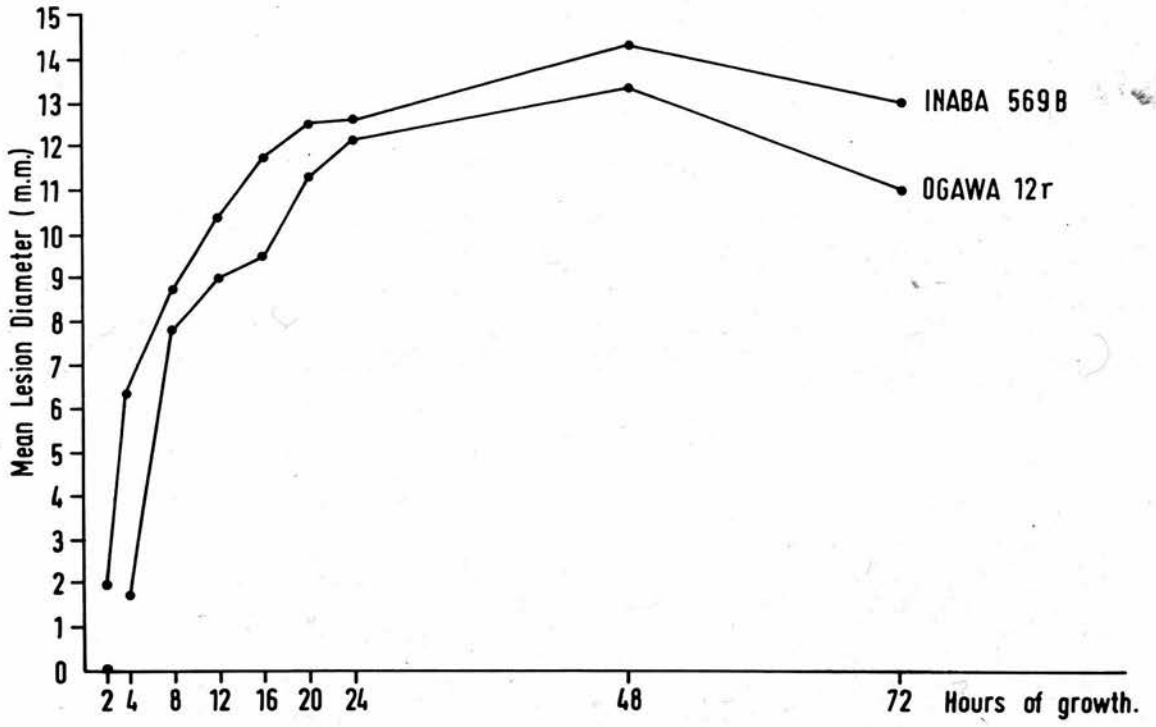
Table 3.

Time-course of production of induration evoking factor in 5 per cent. Bacto-peptone water at pH 7.3 by 2 strains of V. cholerae.

Hours of growth at 37°C	Mean diameter (mm.) of induration following intracutaneous injection of 0.1 ml. of sample of culture filtrate of:	
	V. cholerae Strain 12r	V. cholerae Strain 569B
2	Nil	2
4	1.8	6.4
6	7.0	8.0
8	7.9	8.8
12	9.0	10.4
16	9.5	11.8
20	11.3	12.5
24	12.1	12.6
48	13.4	14.3
72	11.0	13.0

Fig. 2.

TIME-COURSE OF PRODUCTION OF INDURATION-EVOKING FACTOR
IN 5% BACTO-PEPTONE, pH 7·3, BY TWO STRAINS OF VIBRIO CHOLERAE.



The skin reactions evoked by loop fluid and culture filtrates were compared by determining both dose-response and time-course of induration and increased capillary permeability in parallel tests on the same sets of guinea pigs. For these determinations, positive rabbit-gut loop fluid filtrate produced in response to V. cholerae strain 12r was compared with a filtrate of a 24-hr 5 per cent. Bacto-peptone culture of the same strain. The relative potencies of loop fluid and culture filtrates as regards their production of (i) induration, (ii) erythema, and (iii) capillary permeability are shown in the table. The time-course of duration activity of both loop fluid and culture filtrates in the same animals displayed the same sequence of events as shown in Table 4 and Fig. 3. Thus the skin responses evoked by loop fluid and culture filtrate suggest that the active principle in the 2 materials may be similar.

Fig. 3.

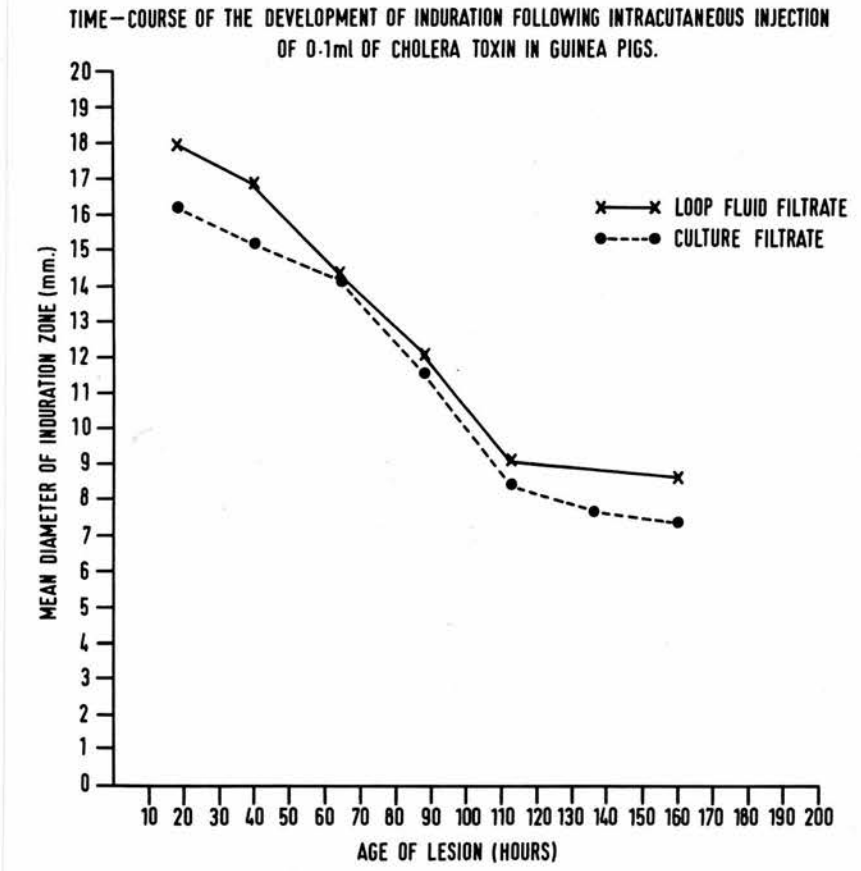

TIME—COURSE OF THE DEVELOPMENT OF INDURATION FOLLOWING INTRACUTANEOUS INJECTION OF 0·1ml OF CHOLERA TOXIN IN GUINEA PIGS.
MEAN DIAMETER OF INDURATION ZONE (mm.)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
AGE OF LESION (HOURS)
LOOP FLUID FILTRATE
CULTURE FILTRATE

Table 4.

Induration, erythema and capillary permeability produced by intradermal injections of loop fluid and culture filtrate.

Age of lesions	Mean diameter (mm.) of skin lesion produced by 0.1 ml. of			
	Loop fluid		Culture filtrate	
	Induration and Erythema	Blueing	Induration and Erythema	Blueing
18 hours	17.9	18.0	16.2	16.5
40 "	16.8	16.9	15.2	15.0
64 "	14.3	14.5	14.2	14.5
88 "	12.0	12.3	11.5	11.9
112 "	9.0	9.5	8.3	8.5
136 "	8.8	9.0	7.7	7.9
160 "	8.6	8.9	7.3	7.5

Effects of culture filtrates of enteric organisms other than V. cholerae on guinea pig skin.

Cultures of Shigella flexneri type 6, enteropathogenic Escherichia coli type O 26 and El Tor and non-agglutinable vibrios in 5 per cent. Difco Bacto-peptone at pH 7.3 were tested for the production of skin-

reactive substances similar to those produced by *V. cholerae*. The cultures were grown under identical conditions to those followed with *V. cholerae* for production of toxin. Positive skin lesions of varying diameter were obtained with culture filtrates of *Esch. coli*, El Tor vibrios and some NAG vibrios; the other organisms did not produce skin-reactive substances. The results are shown in Table 5.

Table 5.
Induration in Guinea pigs following intradermal injection of culture filtrates.

Culture filtrates of strain	Mean diameter (mm.) of induration following intradermal injection of 0.1 ml. of culture filtrates
Esch. coli type O 26	6.0
El Tor vibrios	
H1	13.5
H2	14.5
H4	14.5
H5	15.0
H6	13.0
H7	13.5
H8	16.0
D67	15.0
NAG vibrios	
172 (group V)	14.4
W255 (")	10.0
W343 (" I)	12.4
658 (" II)	8.9
586 (" II)	12.0
H9 (" I)	11.3
696 (" II)	12.8
H11 (" I)	9.5
965 (" I)	Nil
449 (" I)	Nil
8042 (" I)	Nil
H10 ("III)	Nil
1035 (" II)	Nil
H12 ("III)	Nil
Shigella flexneri type 6	Nil
Control 5 per cent. Difco Bacto-peptone water at pH 7.3	Nil

Fractional Precipitation of V. cholerae skin toxin with ammonium sulphate.

Solid ammonium sulphate was added in amounts calculated to give a series of precipitates at 0-20, 20-40, 40-60 and 60-80 per cent. ammonium sulphate saturation (Dixon and Webb, 1958). Before collecting the precipitate at each stage, the mixture was kept for 1 hr at 5°C. The precipitates were collected at each stage by centrifugation at 4,000 g for 30 min. at 2°C and they were each resuspended in a known minimal volume of distilled water. These samples were dialyzed in cellophane sacs for 72 hr against large excess volumes of distilled water at 4°C. The water was changed twice in 24 hr. and agitated 3-4 times between each change. Each fraction was sterilized by membrane filtration and assayed for its induration effect and capillary permeability activity by intracutaneous injection of a dose of 0.1 ml. from each fraction in guinea pigs; 18-20 hr after intracutaneous injection the skin lesions were measured and the capillary permeability was investigated by the intravenous dye procedure. The results are shown in Table 6.

Table 6.

The skin lesions produced following inoculation of ammonium sulphate-precipitated skin toxin in guinea pigs.

Precipitate at percentage $(NH_4)_2SO_4$ saturation of	Mean diameter (mm.) of lesion produced per 0.1 ml. of inoculum prepared from precipitate	
	Induration	Blueing
0 - 20	10.3	8.0
20 - 40	19.3	18.0
40 - 60	10.3	8.0
60 - 80	7.5	4.0

Estimation of protein content of fractionated toxin.

In the present test a series of twofold dilutions of the toxin obtained by precipitation with 60 per cent. saturated $(NH_4)_2SO_4$ were treated by the Folin-Ciocalteu phenol test procedure (see Methods) and the absorption values of the products were compared at a wavelength of 720 mμ in a Unicam SP.1300 colorimeter against a known standard prepared from bovine serum albumen. From the colorimetric readings of the sample and the standard control at 720 mμ wavelength, the protein content of the sample was estimated by calculation as stated below.

Standard Control

Concentration of Bovine Serum albumen	Colorimetric readings of the F - C product
50 ug/ml.	0.060
100 ug/ml.	0.141
200 ug/ml.	0.289
400 ug/ml.	0.520
Sample dilutions	**Colorimetric readings of the F - C product**
1/40	0.176
1/20	0.300
1/10	0.520

It is evident that the $1/_{10}$ test sample dilution gives a result equivalent to the control that contained 400 ug of protein per ml. A more elegant evaluation is obtained by a regression technique which is as follows:

Let $X_1, X_2, X_3, \ldots$ be the concentrations of known dilutions of bovine serum albumen and let $Y_1, Y_2, Y_3, \ldots$ represent the colorimetric readings obtained from the treated samples of test and control materials. For convenience in handling these data, the X series

is divided by 100 and the Y series is multiplied by 100; this avoids the use of awkward decimal values. The coded values for the standard bovine serum control test results are therefore tabulated as follows:

X	Y	XY	X^2
0.5	6.0	3.0	0.25
1.0	14.1	14.1	1.0
2.0	28.9	57.8	4.0
4.0	52.0	208.0	16.0

$\leq Y = 101.0$ $\leq X = 7.5$ $\leq XY = 282.9$ $\leq X^2 = 21.25$

We know the equation for the Regression line, $Y = MX + C$ where M gives the slope of the Regression line and C is constant.

Now the values of M and C are determined by putting the values of our standard control from the above table in the following equations:

$$M = \frac{\leq XY - \frac{(\leq X)(\leq Y)}{N}}{\leq X^2 - \frac{(\leq X)^2}{N}}$$ (N representing the number of items)

$$= \frac{282.9 - \frac{757.5}{4}}{21.25 - \frac{56.25}{4}}$$

$$= \frac{282.9 - 189.37}{21.25 - 14.06}$$

$$= \frac{93.53}{7.19}$$

$$= 13.0$$

and C $= \frac{(\Sigma X)(\Sigma XY) - (\Sigma Y)(\Sigma X^2)}{(\Sigma X)^2 - N(\Sigma X^2)}$ (N representing the number of items)

$= \frac{7.5 \times 282.9 - 101 \times 21.25}{56.25 - 4 \times 21.25}$

$= \frac{24.5}{28.75}$

$= 0.85$

Putting these values for M and C in the Regression equation, $Y = MX + C$ we have $Y = 13X + 0.85$.

Now putting the colorimetric value of the sample for Y in the above equation, we have for $1/_{10}$ dilution of the sample

$52 = 13X + 0.85$

or $X = 3.93$

Multiplying this coded value of X by 100 we get the value of protein content in ug/ml. in $1/_{10}$ dilution of the sample, i.e. 393 ug/ml.

Similarly $1/_{20}$ and $1/_{40}$ dilutions contain 224 and 129 ug/ml. respectively. Thus the undiluted toxin sample contained 3.9 mg. of protein per ml.

The effect of dialysis

The effect of dialysis upon the cutaneous activities of enterotoxin and skin toxin of V. cholerae was compared in a number of experiments conducted in parallel.

The test sample of toxin was held in a sac of cellophane tubing of diameter 1 cm. and dialyzed at 5°C for 48-72 hr. The water was changed daily and stirred 2-3 times daily. The

effect of dialysis upon the cutaneous activities of enterotoxin and skin toxin was then determined by comparing the skin lesions produced by injecting intracutaneously in guinea pigs a volume of 0.1 ml. from each of the dialyzed and undialyzed materials. Dialysis of both materials for 48-72 hr at 5°C against large volumes of distilled water resulted in no loss of skin reactivity. The results are recorded in Table 7.

Table 7.

The effect of dialysis upon the enterotoxin and skin toxin of V. cholerae.

Material	Undialyzed sample		Dialyzed sample	
	Mean diameter (mm.) of		Mean diameter (mm.) of	
	Induration	Blueing	Induration	Blueing
Enterotoxin	13.3	14.5	13.2	13.9
Skin toxin	10.0	9.5	9.8	9.4

Effect of pH

The skin induration activity of the skin toxin and enterotoxin was tested over the pH range 4-9. Equal volumes of the respective toxin and buffers at different pH values as shown in Table 8 were mixed and incubated at 37°C for 30 min. prior to intracutaneous inoculation of a volume of 0.1 ml. from each mixture in guinea pigs. It was observed from the skin lesions that the skin-reactive principles

in both the skin toxin and enterotoxin were stable over the range of pH values tested.

Table 8.

The effect of pH on cutaneous activities of skin toxin and enterotoxin.

Buffer	pH of the buffer	Mean diameter (mm.) of induration following intracutaneous injection of a volume of 0.1 ml. of a mixture of		
		equal volumes of skin toxin and diluent at various pH values.	equal volumes of enterotoxin and diluent at various pH values.	diluent only at various pH values.
Citrate phosphate	4	12.6	14.6	Nil
"	5	12.5	14.5	Nil
Phosphate buffer	6	12.8	14.6	Nil
"	7	12.7	14.7	Nil
"	8	12.6	14.4	Nil
Tris buffer	9	12.7	14.7	Nil

Effect of temperature on cholera toxin.

The effect of heat upon the skin-reactive content of enterotoxin and skin toxin of V. cholerae was studied in a series of experiments. Both the induration-evoking factor and capillary permeability factor of both materials are completely destroyed after exposure to heat at 56°C for 30 min. or at 100°C for 2-3 min. but they are stable at 37°C at least for 72 hr. In a typical experiment the toxin under test was heated in a thermostatically controlled water bath at 56°C and samples were withdrawn after varying periods. A volume of 0.1 ml. from each of these samples was then injected intracutaneously on the shaved skin of the back of albino guinea pigs. The skin lesions were measured 18-20 hr after the intracutaneous injection. The capillary permeability was investigated by intravenous injection of Pontamine Sky Blue 6 x B (0.12 ml. of a 5 per cent. solution/100 g.). The result of a time-course study of progressive inactivation of enterotoxin and skin toxin of V. cholerae held at 56°C for varying periods of time are shown in Table 9 and the data are presented graphically in Figs. 4 and 5.

Fig. 4.

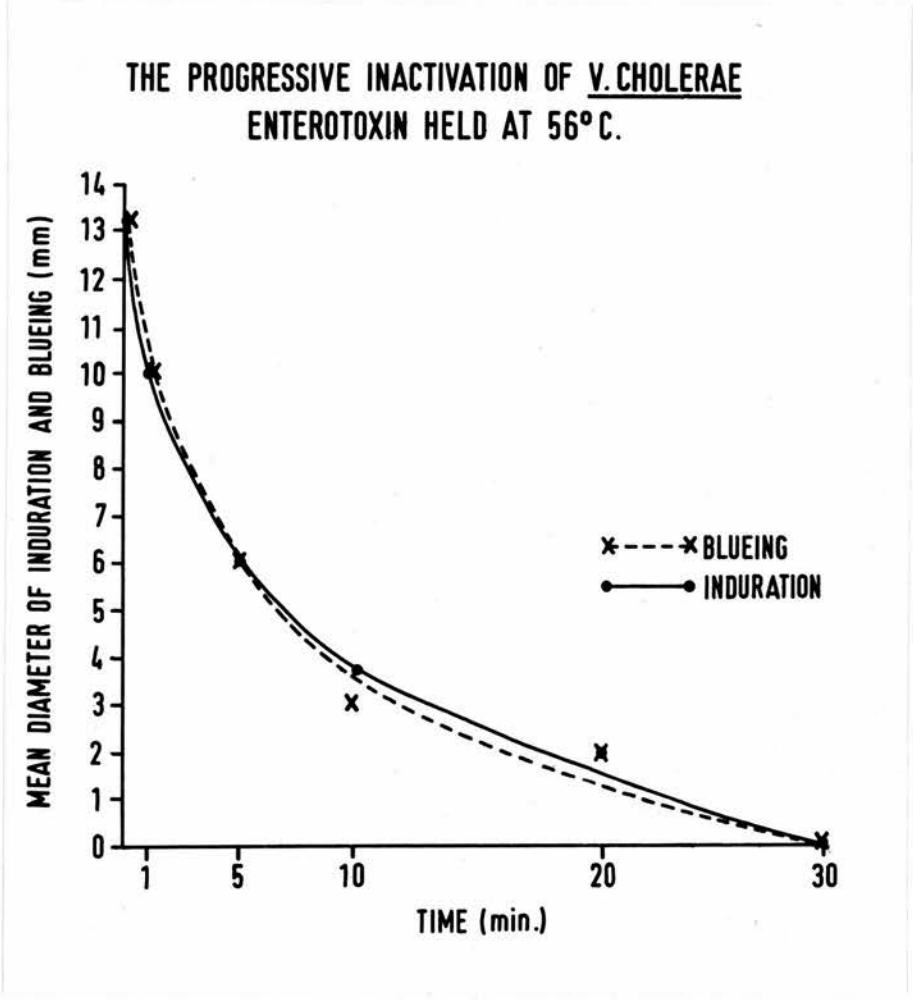

THE PROGRESSIVE INACTIVATION OF V. CHOLERAE ENTEROTOXIN HELD AT 56° C.
MEAN DIAMETER OF INDURATION AND BLUEING (mm)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
1
5
10
20
30
TIME (min.)
BLUEING
INDURATION

Fig. 5.

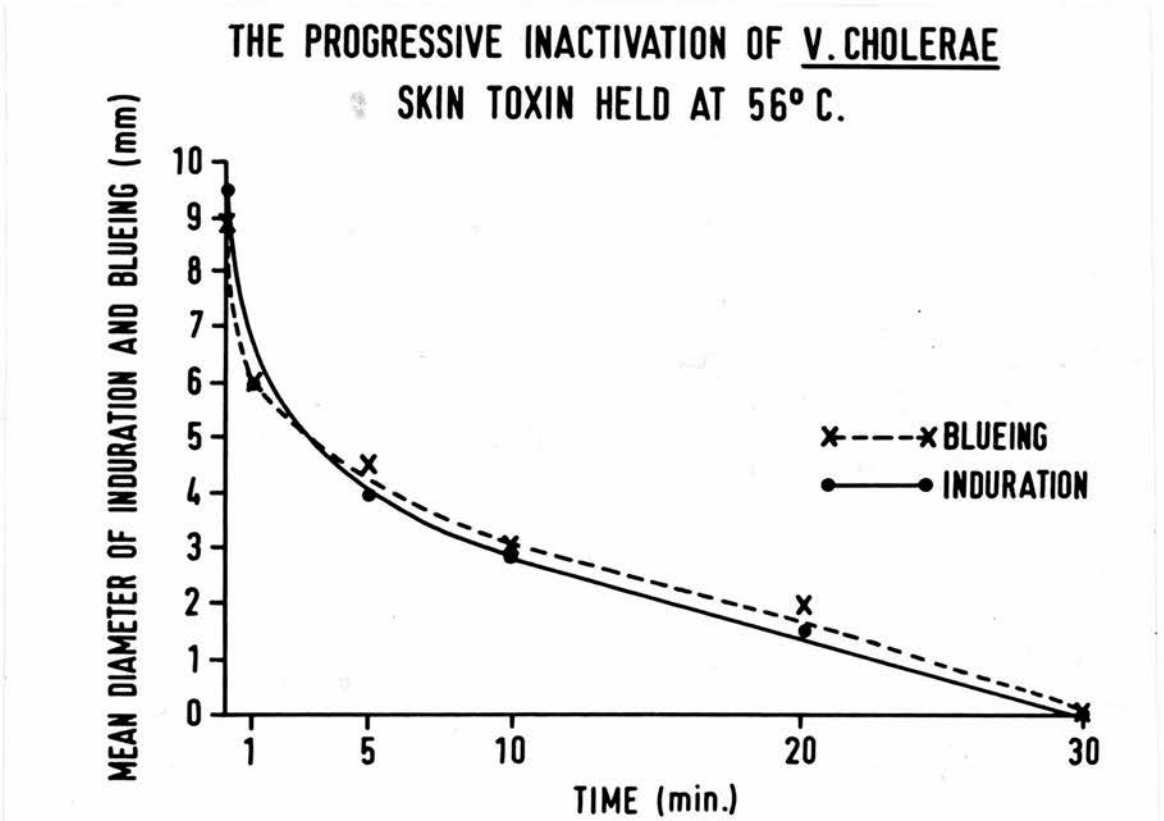

THE PROGRESSIVE INACTIVATION OF V. CHOLERAE
SKIN TOXIN HELD AT 56° C.
MEAN DIAMETER OF INDURATION AND BLUEING (mm)
10
9
8
7
6
5
4
3
2
1
0
1
5
10
20
30
TIME (min.)
BLUEING
INDURATION

Table 9.

The effect of heating at 56°C on the cutaneous activities of enterotoxin and skin toxin.

Time of heating at 56°C	Enterotoxin		Skin toxin	
	Mean diameter (mm.) of		Mean diameter (mm.) of	
	Induration	Blueing	Induration	Blueing
1 min.	10.0	10.0	6.0	6.0
5 "	6.0	6.0	4.0	4.5
10 "	3.8	3.0	2.9	3.0
20 "	2.0	2.0	1.5	2.0
30 "	-	-	-	-
Unheated Control	13.3	13.3	9.5	8.9

Stability of cutaneous factor of enterotoxin and skin toxin of V.cholerae held at room temperature (18°C) and 37°C for 72 hr.

The stability of both enterotoxin and skin toxin was determined by comparing the skin lesions produced by intracutaneous injection of 0.1 ml. of test samples and controls that were stored in a deep-freeze at -20°C. The mean values obtained in different experiments with the same batches of the toxins are shown in Table 10.

Table 10

The effect of storage at various temperatures on the cutaneous activities of skin toxin and enterotoxin.

Material	Held for 72 hr at room temperature 18°C		Held for 72 hr at 37°C		Control	
	Mean diameter (mm.) of		Mean diameter (mm.) of		Mean diameter (mm.) of	
	Induration	Blueing	Induration	Blueing	Induration	Blueing
Enterotoxin	16.0	15.0	12.6	14.0	16.9	18.0
Skin toxin	13.0	12.5	11.3	10.5	15.5	16.5

It is evident that very slight reduction of skin effect has occurred with both skin toxin and enterotoxin being held at 18 or 37°C for 72 hr.

The effect of diluent on skin toxin

This was studied by mixing 1 part of the skin toxin with 1 part of each of saline, phosphate-buffered saline at pH 7.2, and 0.01 per cent. calcium chloride in saline at pH 7.2. Thereafter a volume of 0.1 ml. from each of the mixtures was injected intracutaneously in guinea pigs. No difference was observed in the skin lesions produced by these inoculations. Thus it does not appear that the toxin is dependent upon calcium ions. The results of the experiment are shown in Table 11.

Table 11.

The influence of various diluents on the skin induration activity of the skin toxin of V. cholerae.

Material injected Mixture of equal volume of		Mean diameter (mm.) of induration produced/ 0.1 ml.
Diluent	Toxin	
Saline	Skin toxin	12.3
Phosphate-buffered saline	"	12.3
Saline with Calcium chloride	"	12.3
Saline	Nil	Nil
Phosphate-buffered saline	Nil	Nil
Saline with Calcium chloride	Nil	1.5

Oxidation of skin toxin and enterotoxin with hydrogen peroxide.

Hydrogen peroxide (H_2O_2) was used for oxidation of skin toxin and enterotoxin in a final concentration of 0.1 per cent. H_2O_2. The H_2O_2 was initially 6 per cent. and was diluted (1 part to 5 parts) in phosphate-buffered saline at pH 5.9; thereafter 1 ml. of this

dilution was added to 9 ml. of the toxin under test and the mixture was incubated at 37°C for 1 hr.

Reduction of oxidized toxin with thioglycollic acid.

The reducing agent, thioglycollic acid, was prepared to reduce the oxidized toxin by adding 1 drop of BDH Universal Indicator to 0.1 ml. of 90 per cent. thioglycollic acid, diluted with phosphate-buffered saline at pH 5.9, and then 4 per cent. sodium hydroxide was added, drop by drop until the solution became blue-green. The volume was then made up to 5 ml. with phosphate-buffered saline. This neutral thioglycollic solution should be prepared fresh each day. Four parts of the oxidized toxin were added to 1 part of the neutral thioglycollic solution and the mixture was left to stand at room temperature (18°C) for 15 min. before it was considered to be completely reduced.

Direct reduction of the toxin was also done by mixing 4 parts of the toxin under test with 1 part of the neutral thioglycollic solution and the mixture was left at room temperature as before for 15 min. for complete reduction. Immediately after the oxidation and reduction procedures all of the preparations and the reagent controls were transferred seperately into cellophane tubing of diameter 1 cm. and dialyzed against large volumes of distilled water for 48 hr at 4°C in order to eliminate any possibly injurious reagents before the samples were tested for effects on guinea pig skin. The samples were then tested for their skin induration

and capillary permeability activities by injecting 0.1-ml. volumes of each intracutaneously.

Effect of Oxidation and Reduction

The skin induration and capillary permeability activities of the skin toxin and enterotoxin were not inactivated when they had been oxidized by H_2O_2 nor when they were reduced with thioglycollic acid. In a typical experiment, either the skin toxin or the enterotoxin producing skin indurations of 16 mm. and 16.5 mm. respectively per 0.1 ml. on guinea pig skin was incubated with H_2O_2 in a final concentration of 0.1 per cent. at pH 5.9. The oxidized toxins were tested for their skin induration and capillary permeability activities and were found to have the same titres as the original untreated toxin in either case. The treated toxins were again reduced with thioglycollic acid and tested thereafter for their production of similar skin lesions. The extent of the various skin reactions that resulted is recorded in Table 12. (It should be noted that the design of this experiment does not exclude the possibility that reduced components would be re-oxidized during the period of dialysis).

Table 12.

The effects of oxidation and reduction on the cutaneous activities of skin toxin and enterotoxin.

Treatment	Skin toxin		Enterotoxin		Reagent Controls	
	Mean diameter (mm.)		Mean diameter (mm.)		Mean diameter (mm.)	
	Induration	Blueing	Induration	Blueing	Induration	Blueing
Original	16.0	15.5	16.5	16.3		
Oxidized	16.0	14.5	16.3	15.8	2.0	2.5
Indirect reduction	16.1	14.3	16.3	16.0	2.0	2.0
Direct reduction	15.5	14.5	16.0	15.5	2.5	2.2

Effect of formaldehyde on cholera toxin contained in rabbit gut loop fluid filtrate.

The effect of formaldehyde on cholera toxin contained in loop fluid was studied in the following experiment:

To whole loop fluid, different amounts of 5 per cent. formalin were added to make final concentrations of formaldehyde over the range of 1.4 - 0.2 per cent. Three sets of each series were prepared in parallel. One was incubated at 37°C, another was left at room temperature, and the third was held at 4°C. After 29 hr all of the samples including the reagent controls were dialyzed

against large volumes of distilled water for 72 hr at 4°C so as to eliminate the formaldehyde. These samples were then tested for skin induration and capillary permeability activities by injecting a volume of 0.1 ml. intracutaneously from each of the samples. The presence of toxin in the samples was indicated by production of zones of induration and erythema of varying diameter. The test was prepared according to the following protocol:-

Tube Numbers	I	II	III	IV	V	VI
Formalin 5% in saline (ml.)	0.7	0.5	0.3	0.1	Nil	0.7
Loop fluid filtrate (ml.)	0.3	0.3	0.3	0.3	0.3	Nil
Saline (ml.)	0.0	0.2	0.4	0.6	0.7	0.3
Final conc. of formalin	3.5%	2.5%	1.5%	0.5%	Nil	3.5%
Final conc. of formaldehyde	1.4%	1.0%	0.6%	0.2%	Nil	1.4%

The induration zones were measured 18-20 hr after the intracutaneous injection of the test materials. The increased capillary permeability was investigated by intravenous injection of Pontamine Sky Blue 6 x B (0.12 ml. of a 5 per cent. solution per 100 g. body weight of the guinea pig) 18 hr after the intracutaneous injection; readings were made 5 hr after the dye injection. The results of tests and controls are shown in Table 13.

Table 13.

The cutaneous activities of the cholera toxin contained in rabbit gut loop fluid filtrate after being held in the presence of different concentrations of formaldehyde at different temperatures for 29 hr.

Tube Number	I		II		III		IV		V		VI	
Final conc. of formaldehyde	1.4%		1.0%		0.6%		0.2%		Nil		1.4%	
	mean diameter of lesion (mm.) per 0.1 ml. dose		mean diameter of lesion (mm.) per 0.1 ml. dose		mean diameter of lesion (mm.) per 0.1 ml. dose		mean diameter of lesion (mm.) per 0.1 ml. dose		mean diameter of lesion (mm.) per 0.1 ml. dose		mean diameter of lesion (mm.) per 0.1 ml. dose	
Series	Induration	Blueing	Induration	Blueing	Induration	Blueing	Induration	Blueing	Induration	Blueing	Induration	Blueing
held at 4°C	7.3	7.0	11.0	13.1	11.0	14.5	11.3	13.0	14.3	14.6	Nil	Nil
Series held at 18°C	6.3	4.8	6.3	5.3	7.3	5.0	8.5	10.1	13.1	12.8	Nil	Nil
Series held at 37°C	5.0	4.3	7.5	5.9	5.4	5.6	5.9	4.8	14.5	13.1	Nil	Nil

In a similar experiment, the activity of the skin toxin (produced *in vitro* by growing *V. cholerae* Ogawa strain 12r in 5 per cent. peptone water) after exposure to different concentrations of formaldehyde at different temperatures was recorded as shown in Table 14.

Table 14.

The cutaneous activities of the skin toxin after being held in the presence of different concentrations of formaldehyde at different temperatures for 29 hr.

Number of Tube	I	II	III	IV	V	VI
Final conc. of formaldehyde	1.4%	1.0%	0.6%	0.2%	Nil	1.4%
	Mean diameter of induration & erythema (mm.) per 0.1 ml.	Mean diameter of induration & erythema (mm.) per 0.1 ml.	Mean diameter of induration & erythema (mm.) per 0.1 ml.	Mean diameter of induration & erythema (mm.) per 0.1 ml.	Mean diameter of induration & erythema (mm.) per 0.1 ml.	Mean diameter of induration & erythema (mm.) per 0.1 ml.
Series held at 4°C	6.5	10.0	10.3	10.3	13.5	Nil
Series held at 18°C	6.3	6.3	7.5	8.0	13.5	Nil
Series held at 37°C	5.0	5.3	5.3	5.5	13.3	Nil

The skin-reactive component of cholera toxin contained either in loop fluid filtrate or in culture filtrate thus seemed to be stable despite exposure for 29 hr to concentrations of formaldehyde up to 1 per cent. at 4°C, but it was considerably inactivated at both 18°C and 37°C even in the presence of 0.2 per cent. formaldehyde for 29 hr. The toxin

was significantly inactivated by exposure to 1.4 per cent. formaldehyde for 29 hr at 4°C, 18°C or 37°C.

Enzymic-decomposition studies

Effect of trypsin on skin toxin

The effect of trypsin was tested and it was found that skin toxin of V. cholerae retained its full activity after exposure to trypsin 0.05 per cent. in buffer at pH 7.0 at time 0 and after 2 hr at 37°C.

In a typical experiment, 5 ml. of ammonium sulphate-precipitated toxin from strain 12r was cooled at 4°C and mixed with 5 ml. of a potent cold solution of 0.05 per cent. trypsin (bovine pancreas) in phosphate-buffered saline at pH 7.0. The mixture was held at 4°C for 30 min. and then tested for skin induration activity by injecting a volume of 0.1 ml. intracutaneously into guinea pigs. The toxin-trypsin mixture was then put in a water bath at 37°C and thereafter samples were withdrawn at 10, 20, 30, 60 and 120 min. of incubation and each sample was tested by injecting a volume of 0.1 ml. intracutaneously in guinea pigs. Positive and negative controls were also incorporated with the test by injecting only toxin and trypsin respectively under otherwise identical conditions. The skin reactions following these intracutaneous injections are recorded as shown in Table 15.

Table 15.

The effect of trypsin on skin toxin at time 0 and after incubation for varying time periods.

Toxin - trypsin mixture held at 4° and 37°C for	Mean diameter of induration (mm.) produced/0.1 ml. dose
time 0 at 4°C	13.3
10 min. at 37°C	13.4
20 min. "	13.3
30 min. "	13.3
60 min. "	13.3
120 min. "	13.2
Control:	
Toxin	13.3
Trypsin	Nil

Effect of chymotrypsin

Equal volumes of toxin and chymotrypsin in saline at pH 7.0 were mixed so that there was a concentration of 1 mg/ml. of chymotrypsin in the resultant mixture. The mixture was incubated at 37°C for 2 hr in the water bath before it was injected intracutaneously in guinea pigs in doses of 0.1 ml. to determine

the effect of chymotrypsin on the toxin.

The results are shown in Table 16.

Table 16.

The effect of chymotrypsin on skin toxin.

Materials injected intra-cutaneously (0.1 ml.)	Mean diameter of induration (mm.)
Mixture of chymotrypsin and skin toxin obtained from 12r strain.	12.9
Mixture of chymotrypsin and skin toxin obtained from 569B strain.	15.0
Controls: Mixture of skin toxin obtained from 12r strain and saline.	13.0
Mixture of skin toxin obtained from 569B strain and saline.	14.9
Mixture of chymotrypsin and saline	Nil
Saline alone	Nil

It is evident from the results that the skin toxin retained its full activity after exposure to chymotrypsin 1 mg/ml. for 2 hr at 37°C at pH 7.0.

Effect of pancreatin

This was tested by mixing skin toxin and enterotoxin separately with solutions of pancreatin in Tris buffer at pH 8.2 having 5, 2.5 and 1 mg. of pancreatin per ml. in the resultant mixtures. These were held at 37°C in the water bath for 2½ hr prior to intracutaneous

inoculation of a volume of 0.1 ml. from each of the mixtures in guinea pigs. Both of the toxins were found to be completely inactivated in the presence of 0.5 and 0.25 per cent. pancreatin. The results are shown in Table 17.

Table 17.

The effect of pancreatin on skin toxin and enterotoxin

Equal volume of		Mean diameter of induration (mm.)
Toxin	Solution of pancreatin in Tris Buffer, pH 8.2	
Loop fluid (Enterotoxin)	10 mg/ml.	Nil
"	5 mg/ml.	Nil
"	2 mg/ml.	13.8
Skin toxin	10 mg/ml.	Nil
"	5 mg/ml.	Nil
"	2 mg/ml.	10.5

Controls

Equal volume of		Mean diameter of induration (mm.)
Loop fluid (Enterotoxin)	Tris buffer at pH 8.2	14.0
Skin toxin	"	11.0
Tris buffer pH 8.2	Pancreatin 10 mg/ml. in Tris buffer at pH 8.2	Nil
"	Pancreatin 5 mg/ml. in Tris buffer at pH 8.2	Nil
"	Pancreatin 2 mg/ml. in Tris buffer at pH 8.2	Nil
Tris buffer only		Nil

A control at 4°C should have been done but it has not been done in the present study.

Effect of lipase

The effect of lipase on skin toxin and enterotoxin was studied by mixing 1 part of a solution of lipase in citrate-phosphate buffer at pH 5.0 to an equal volume of either of the toxins so as to get a·5 per cent. lipase concentration in the resultant mixtures. Each mixture was incubated at 37°C for 2 hr in the water bath and thereafter the effect of lipase upon the toxins was determined by observation of the skin lesions following intracutaneous injections of a volume of 0.1 ml. from each of the mixtures in guinea pigs. The results are shown in Table 18.

Table 18.

The effect of lipase on skin toxin and enterotoxin

Equal volume of		Mean diameter of induration (mm.) produced
Toxin	Lipase in citrate-phosphate buffer at pH 5.0	
Enterotoxin	10 mg/ml.	6.0
Skin toxin	10 mg/ml.	6.0
Controls:		
Enterotoxin	Citrate-phosphate buffer at pH 5.0	14.0
Skin toxin	" " "	10.5
Citrate-phosphate buffer at pH 5.0	Lipase 10 mg/ml. in citrate-phosphate buffer.	6.0
Citrate-phosphate buffer only		Nil

It appeared that lipase itself produced some degree of skin reaction. The toxin-lipase mixtures certainly showed reduced cutaneous activity so that there was partial or complete inactivation of skin toxin by lipase.

Effect of phosphorylase.

The effect of incubation of skin toxin with phosphorylase in concentrations of 1 mg/ml. and 0.5 mg/ml. both in Tris buffer at pH 7.9 and in acetate buffer at pH 5.6 was studied.

To 1 part of each of these concentrations of phosphorylase in each of the buffers, 1 part of the skin toxin was added. The mixtures were then incubated at 37°C for 5 hr (Dutta and Oza, 1963) and a volume of 0.1 ml. of each mixture was then injected intracutaneously into guinea pigs. The results are shown in Table 19.

Table 19.

The effect of phosphorylase on skin toxin

	Material injected in guinea pig	Mean diameter of induration (mm.) following 0.1 ml. of intracutaneous injection
Test	1 Part of Phosphorylase 1 mg/ml. in Tris buffer + 1 part of skin toxin.	13.8
	1 part of Phosphorylase 0.5 mg/ml. in Tris buffer + 1 part of skin toxin.	14.3
	1 Part of Phosphorylase 1 mg/ml. in acetate buffer + 1 part of skin toxin.	14.4
	1 Part of Phosphorylase 0.5 mg/ml. in acetate buffer + 1 part of skin toxin.	14.4
Control	1 Part of Tris buffer + 1 part of skin toxin.	14.8
	1 Part of acetate buffer + 1 part of skin toxin.	14.0
	1 Part of Phosphorylase 1 mg/ml. in Tris buffer + 1 part of Tris buffer.	2.0
	1 Part of Phosphorylase 1 mg/ml. in acetate buffer + 1 part of acetate buffer.	3.0
	Tris buffer only.	Nil
	Acetate buffer only.	Nil

It is evident that phosphorylase did not inactivate or enhance the skin toxin of V. cholerae.

Adsorption studies with skin toxin

A 0.5-g. amount of activated charcoal was mixed well with 5 ml. of skin toxin of V. cholerae strain 569B and left at room temperature for 30 min. Aliquots of the mixture (1 ml.) were then transferred into 4 small tubes and centrifuged at 2,000 g for 15 min. The supernatant fluids from all of the tubes were pooled. The deposits in the tubes were treated separately as follows by resuspension in :

(i) 1 ml. saline

(ii) 1 ml. citrate-phosphate buffer at pH 5.0.

(iii) 1 ml. phosphate-buffered saline at pH 7.0.

(iv) 1 ml. phosphate-buffered saline at pH 8.0.

All of the above suspensions were re-centrifuged and the supernatants were collected separately. The various fractions thus collected were tested on guinea pig skin to detect the presence of skin-reactive substances by injecting a volume of 0.1 ml. from each preparation. The results are shown in Table 20.

Table 20.

Effect of activated charcoal on skin toxin of *V. cholerae* strain 569B.

Material inoculated intracutaneously in guinea pigs	Mean diameter of induration (mm.) produced/0.1 ml. of the inoculum
1, skin toxin of *V. cholerae* strain 569B	15.0
2, supernatant after treatment of skin toxin with activated charcoal (4 such preparations)	5.3
3, supernatant obtained by resuspending the deposit (i) from 2 in saline.	Nil
4, supernatant obtained by resuspending the deposit (ii) from 2 in citrate-phosphate buffer at pH 5.0	Nil
5, supernatant obtained by resuspending the deposit (iii) from 2 in phosphate-buffered saline at pH 7.0	Nil
6, supernatant obtained by resuspending the deposit (iv) from 2 in phosphate-buffered saline at pH 8.0	Nil

It appeared from these results that more than 50 per cent. of the skin toxin was adsorbed by the activated charcoal but it could not be eluted by saline, by citrate-phosphate buffer at pH 5.0, or by phosphate-buffered saline at pH 7.0 or pH 8.0.

In another experiment, 2 ml. of the same skin toxin of strain 569B was mixed with 0.5 g. of activated charcoal and kept at room temperature for 1 hr. The mixture was then centrifuged at 2,000 g for 15 min. The supernatant fluid was collected and the deposit was resuspended in 2 ml. of 2M NaCl. The charcoal was then precipitated by centrifugation and the resulting supernatant was dialysed against an excess of physiological saline for 24 hr. The various fractions were tested on guinea pigs to detect the presence of skin reactive substance. The test and results are summarized in Table 21.

Table 21.

Effect of activated charcoal on skin toxin of *V. cholerae* strain 569B

Fraction tested by intracutaneous injection in guinea pigs	Mean diameter of induration (mm.) produced/ 0.1 ml. of the inoculum
1 - skin toxin of *V. cholerae* strain 569B	15.8
2 - supernatant after treatment of skin toxin with activated charcoal	6.0
3 - supernatant obtained by resuspending the deposit from (2) in 2M NaCl and dialysed against physiological saline	Nil
4 - equal volume (1 ml.) of toxin and 2M NaCl mixture dialysed against physiological saline	14.0

The above results show that if some of the toxin is indeed

adsorbed to charcoal the adsorbed toxin could not be eluted by 2M NaCl, but the findings do not exclude other possible reasons for the partial inactivation of skin toxin following exposure to charcoal.

Adsorption and elution of skin toxin

A 5-ml. volume of skin toxin of V. cholerae strain 12r was mixed with an equal amount of Brushite suspension in 0.001M phosphate buffer at pH 6.8 and the mixture was left at room temperature for 30 min. The mixture was then equally distributed into 4 small tubes. The supernatant fluids from all of the tubes were pooled after centrifugation at 2,000 g for 15 min. The deposit from the first tube was re-suspended in 2 ml. of 0.001M phosphate buffer at pH 6.8. The deposit from the second tube was suspended in 2 ml. of citrate-phosphate buffer at pH 5.0, and the third deposit was suspended in 2 ml. of phosphate-buffered saline at pH 7.0. The fourth deposit was suspended in 2 ml. of phosphate buffered saline at pH 8.0. All of the above suspensions were re-centrifuged and the supernatants were collected separately. The various fractions were then tested for skin reactivity in guinea pigs. The experiment and the results are summarized in Table 22.

Table 22.

Adsorption and elution of skin toxin of V. cholerae strain 12r.

Fraction tested by injecting intra-cutaneously in guinea pigs	Mean diameter of induration (mm.) produced by 0.1 ml. of inoculum.
1 - Skin toxin of V. cholerae strain 12r.	13.5
2 - Supernatant after adsorption with equal amount of Brushite suspension in 0.001M phosphate buffer. (Four such preparations).	6.8
3 - Supernatant obtained by resuspending the deposit (i) from 2 in 2 ml. 0.001M phosphate buffer at pH 6.8.	5.0
4 - Supernatant obtained by resuspending the deposit (ii) from 2 in 2 ml. citrate-phosphate buffer at pH 5.0	7.5
5 - Supernatant obtained by resuspending the deposit (iii) from 2 in 2 ml. phosphate-buffered saline at pH 7.0.	6.0
6 - Supernatant obtained by resuspending the deposit (iv) from 2 in 2 ml. phosphate-buffered saline at pH 8.0.	5.0
Controls (diluents)	
0.001M phosphate buffer at pH 6.8.	Nil
Citrate phosphate buffer at pH 5.0.	Nil
Phosphate-buffered saline at pH 7.0	Nil
Phosphate-buffered saline at pH 8.0	Nil

It is evident from the results that some adsorption of skin toxin occurred and that this was readily eluted. The adsorbed component was not inactivated and retained full activity after elution.

Haemolytic test with skin toxin and enterotoxin of V. cholerae strain 12r.

The effects of skin toxin and loop fluid (enterotoxin) of V. cholerae strain 12r on 1 per cent. sheep cell suspension in normal saline were observed in the following experiment.

A volume of 0.5 ml. of skin toxin or enterotoxin was added to an equal volume of 1 per cent. sheep cell suspension. In the control mixture the toxin was replaced by normal saline. Both the test and control mixtures were incubated at 37°C in the water bath for 2 hr. The results were recorded at intervals of 15 min. as shown in Table 23. The tests were thereafter kept in the refrigerator overnight and final readings were then made.

Table 23.

The haemolytic effect of skin toxin and enterotoxin on sheep red cell suspension

Results recorded after (min.)	Skin toxin obtained from		Enterotoxin	Control
	5 per cent. Bacto peptone water	1 per cent. peptone water	present in loop fluid	
15	-	-	-	-
30	+	-	-	-
45	+	-	-	-
60	+	-	-	-
75	+	±	-	-
90	+	+	-	-
105	+	+	-	-
120	+	+	-	-
After overnight in refrigerator	+	+	+	-

+ = Haemolysis. - = No haemolysis.

The effect of skin toxin and enterotoxin on guinea pigs treated with mepyramine maleate

The drug mepyramine maleate is a powerful antihistaminic and its activity in the experimental animal is fairly specific in this

respect (Dr. Brocklehurst, personal communication). A volume of 1 ml. of a solution of mepyramine maleate in saline in a dose of 10 mg./Kilo body weight of the animal was injected subcutaneously 8 hr before the administration of the toxin and every 8 hr during the experiment. A control was also incorporated in the test by injecting the toxin into normal untreated guinea pigs. The effect of the antihistamine on the response to the toxin was then compared by observing the skin lesions produced in test and control animals; the results are shown in Table 24. It is evident that the cutaneous effects of the skin toxin and enterotoxin were not inhibited by prior and sustained treatment of the animals with mepyramine maleate.

Table 24.

The mean diameters (mm.) of the lesions produced after intracutaneous injection of a volume of 0.1 ml. of skin toxin and enterotoxin on mepyramine maleate-treated and untreated control guinea pigs.

Toxin	Mean diameter (mm.) of induration	
	Test guinea pig	Control guinea pig
Skin toxin from *V. cholerae* strain 12r.	13.5	13.2
Skin toxin from *V. cholerae* strain 569B.	11.3	11.8
V. cholerae strain 12r-induced positive rabbit gut loop fluid filtrate.	15.0	15.0

Effect of skin toxin and enterotoxin of *V. cholerae* on charcoal gelatin disks

To 2 ml. of each of the cholera toxins a charcoal gelatin disk was added. Two sensitivity discs containing Kanamycin sulphate ('Kannasyn') were also added into each tube to prevent bacterial contamination. A control was also incorporated in which the toxin was replaced with saline. The test and the control tubes were incubated at 37°C for 4 days. Liquefaction of the gelatin was observed after overnight incubation when carbon particles were seen to settle at the bottom of the tubes. Complete disintegration of the disks occurred after 4 days of incubation. The results are shown in Table 25.

Table 25.

Results of liquefaction of charcoal gelatin disks

Material added to charcoal gelatin disk	Disintegration of charcoal gelatin disks in cholera toxins on incubation at 37°C for				Result of sterility check of the material
	24 hr	48 hr	72 hr	4 days	
Skin toxin of *V. cholerae* strain 12r	++	+++	++++	CD	Sterile
Skin toxin of *V. cholerae* strain 569B	++	+++	++++	CD	Sterile
Loop fluid (enterotoxin) of *V. cholerae* strain 12 r	++	+++	++++	CD	Sterile
Control	-	-	-	-	Sterile

CD = complete disintegration of disk;

++, +++, ++++ = increasing degrees of disintegration;

- = no disintegration.

Titration of skin toxin in ileal loops of rabbits

A sample of skin toxin of *V. cholerae* strain 12r, which produced a zone of induration of 14-15 mm. following intracutaneous inoculation of a volume of 0.1 ml. in guinea pigs, was injected in ileal loops of rabbits in varying amounts, undiluted and after dilution in saline, to determine the minimal concentration that could produce a positive loop. The results of this titration, summarized from experiments in 5 rabbits, are shown in Table 26. The appearances of the loops can also be seen in figures 6, 7, 8 and 9. Each animal was killed 11 hr after the administration of toxin and the reaction was then assessed at post mortem examination.

Table 26.

The results of titration of skin toxin in ileal loops

Rabbit No.	Loop No.	Dose per loop	Materials injected	Result (Loop effect)
1	L1	2 ml.	12r skin toxin	++
"	L2	2 ml.	"	++
2	L2	2 ml.	"	++
"	L3	1 ml.	"	++
"	L4	0.5 ml.	"	+
3	L2	2 ml.	"	++
"	L3	1 ml.	"	++
"	L4	0.5 ml.	"	+
4	L1	0.5 ml.	1 in 2 dilution of 12r skin toxin in saline	+
"	L2	0.5 ml.	1 in 5 dilution of 12r skin toxin in saline	±
"	L3	0.5 ml.	1 in 10 dilution of 12r skin toxin in saline	-
"	L4	0.5 ml.	1 in 20 dilution of 12r skin toxin in saline	-
7	Between L1 and L2	0.5 ml.	1 in 4 dilution of 12r skin toxin in saline	+

\+ = weak positive ;

++ = strong positive ;

\- = no loop lesion.

Fig. 6.

Ilealloops in normal rabbit No. 1.

Loop No.	Dose per loop	Materials injected	Result (loop effect)
L_1	2 ml.	Skin toxin 12r (S T 12r)	++
L_2	"	" "	++
L_3 Distal	"	Skin toxin 569B (S T 569B)	+
C_1	"	Sterile normal saline	-
C_2		Not inoculated	-

++ = Strong positive

+ = Weak positive

- = No loop lesion

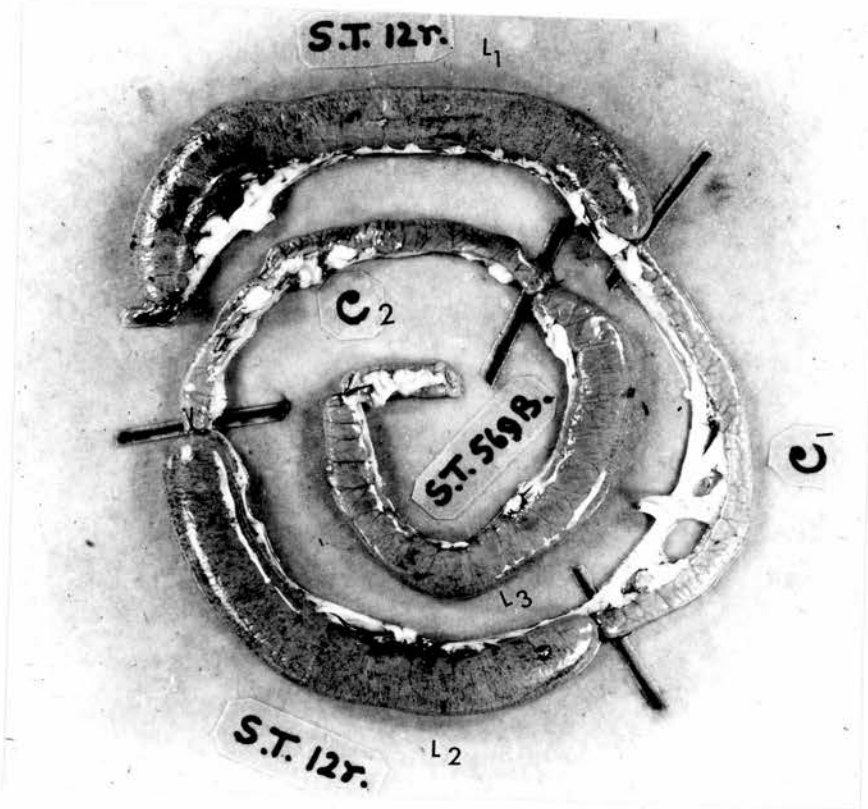

S.T. 12r.
L1
C2
S.T. 569B.
C1
L3
S.T. 12r.
L2

Fig. 7.

Ileal loops in normal rabbit No. 2.

Loop No.	Dose per loop	Materials injected	Result (loop effect)
L_1 Distal	0.5 ml.	12r culture suspension (10^5/ml.) in peptone saline.	++
L_2	2 ml.	Skin toxin 12r (S T 12r)	++
L_3	1 ml.	Skin toxin 12r (S T 12r)	++
L_4	0.5 ml.	Skin toxin 12r (S T 12r)	+
C_1	2 ml.	Sterile normal saline	-
C_2		Not inoculated	-
C_3		"	-

++ = Strong positive

+ = Weak positive

- = No loop lesion

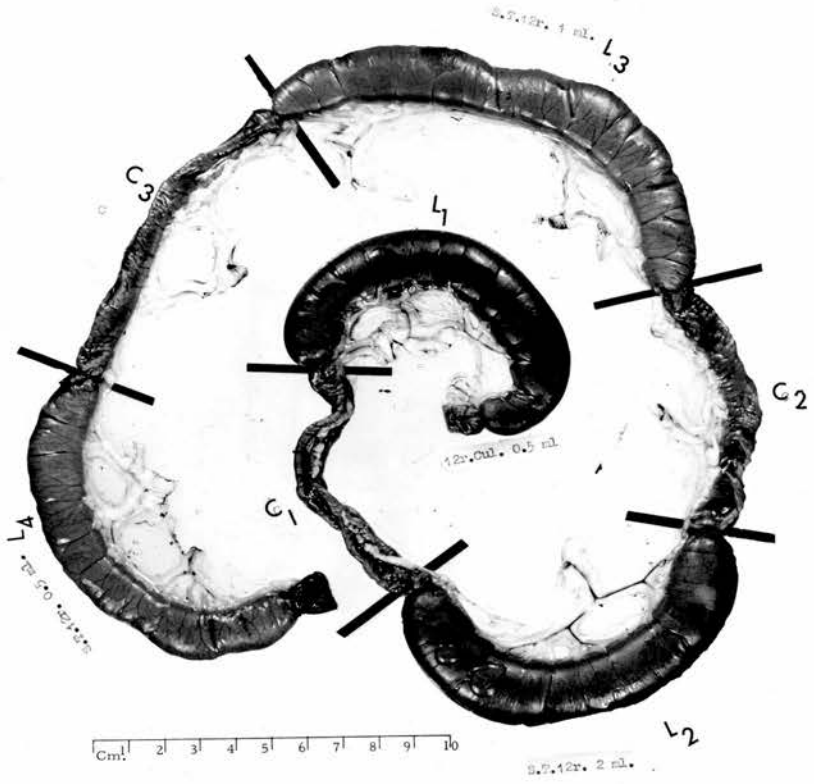

L3
C3
L1
C2
12r.Cul. 0.5 ml
C1
L4
L2
Cm. 1 2 3 4 5 6 7 8 9 10
8.P.12r. 2 ml.

Fig. 8.

Ileal loops in normal rabbit No. 4.

Loop No.	Dose per loop	Materials injected	Result (loop effect)
L_1 Distal	0.5 ml.	1 in 2 dilution of 12r skin toxin in saline	+
L_2	0.5 ml.	1 in 5 dilution of 12r skin toxin in saline.	±
L_3	0.5 ml.	1 in 10 dilution of 12r skin toxin in saline.	-
L_4	0.5 ml.	1 in 20 dilution of 12r skin toxin in saline.	-
C_1		Not inoculated	-
C_2		"	-
C_3	0.5 ml.	Sterile normal saline	-

+ = Weak positive

- = No loop lesion

Normal Rabbit

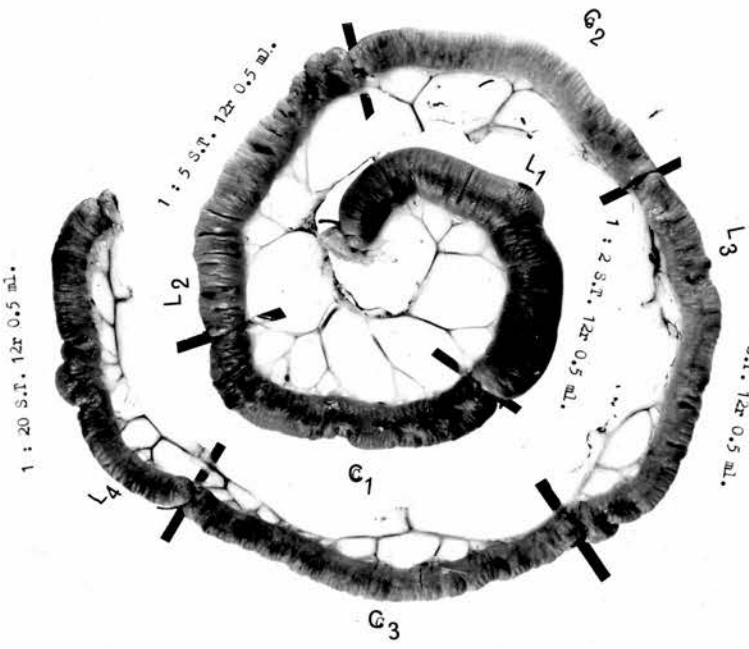


Fig. 9.

Ileal loops in normal rabbit No. 7.

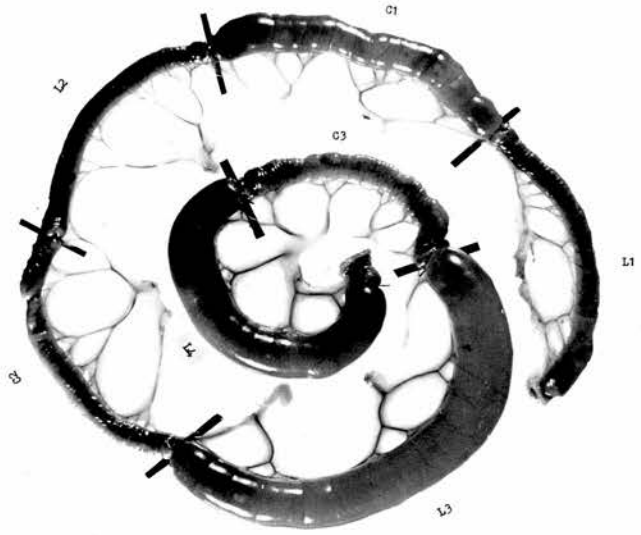
Loop (L) No.	Materials injected	Result (loop effect)
L_1	0.5 ml. of 12r culture (1.8×10^8/ml.) + 0.5 ml. serum against 12r toxin.	-
L_2	0.5 ml. of 12r culture (1.8×10^8/ml.) + 0.5 ml. serum against 12r culture.	-
L_3	0.5 ml. of 12r culture (1.8×10^8/ml.) + 0.5 ml. peptone saline.	++
L_4 Distal	0.5 ml. of 569B culture (6.8×10^7/ml.) + 0.5 ml. peptone saline.	++
C_1	0.5 ml. of 1 in 4 12r skin toxin.	+
C_2	1 ml. saline.	-
C_3	0.5 ml. of serum against 12r culture + 0.5 ml. saline.	-

++ = Strong positive

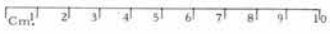
+ = Weak positive

- = No loop lesion

Normal Rabbit



L1 = 0.5ml 12r cult. of 1.8 x 10^8 cells/ml + 0.5ml serum/12r toxin
L2 = " " + 0.5ml serum/12r cult.
L3 = " " + 0.5ml Peptone saline
L4 = 0.5ml 569B cult. of 6.8 x 10^7 cells/ml + 0.5ml Peptone saline
C1 = 0.5ml of 1:4 12r toxin.
C3 = 0.5ml serum/12r cult. + 0.5ml saline.



It is evident from the above results that a sample of skin toxin of strain 12r, which produces a zone of induration of 14-15 mm. diameter following intracutaneous injection of a volume of 0.1 ml. in the guinea pig, was also capable of producing a positive loop lesion in a normal rabbit when a 0.5 ml. volume of a dilution up to 1 in 4 in saline was injected into the lumen of the loop.

The effects of skin toxin and enterotoxin of V. cholerae in mice

The effects of the skin toxin and enterotoxin of V. cholerae were studied by intraperitoneal and intravenous inoculations in Swiss white mice, each weighing 20-25 g. The animals were observed for 7 days after inoculation. There was no death during this observation period and all the animals looked healthy throughout the period. The toxic activities of both the skin toxin and enterotoxin of V. cholerae for mice were thus recorded as negative as shown in Tables 27 and 28.

Tables showing the effects of parenteral administration of skin toxin and enterotoxin in Swiss white mice

Table 27 : test dose = 0.5 ml. intravenously

Material injected	Weight of each mouse	Sex	Number of mice per sample	Number of deaths							Diameter (mm) of induration produced on guinea pig skin after intracutaneous injection of 0.1 ml.
				1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	
Skin toxin contained in culture filtrate of V.cholerae strain 12r grown in 5 per cent. Difco Bacto peptone water medium.	20 gm	Female	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	15.5
Skin toxin contained in culture filtrate of V.cholerae strain 12r grown in 1 per cent. Peptone water medium.	25 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	10.0
Enterotoxin contained in V.cholerae strain 12r-induced positive rabbit gut loop fluid filtrate.	20 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	18.5
Media control 5 per cent Difco Bacto peptone water.	20 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Media control 1 per cent. peptone water.	25 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 28 : test dose = 1 ml. intraperitoneally

Material injected	Weight of each mouse	Sex	Number of mice per sample	Number of deaths							Diameter of induration produced on guinea pig skin after intracutaneous injection of 0.1 ml.
				1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	
Skin toxin contained in culture filtrate of V.cholerae strain 12r grown in 5 per cent. Difco Bacto peptone water medium.	20 gm	Female	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	15.5
Skin toxin contained in culture filtrate of V.cholerae strain 12r grown in 1 per cent. Peptone water medium.	25 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	10.0
Enterotoxin contained in V.cholerae strain 12r-induced positive rabbit gut loop fluid filtrate.	25 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	18.5
Media control 5 per cent. Difco Bacto peptone water.	25 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Media control 1 per cent. Peptone water medium.	20 gm	"	5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

The effects of skin toxin and enterotoxin of *V. cholerae* on chorioallantoic membrane

Explanatory note:

The toxic effects of products of *V. cholerae* on the embryonated egg are characterized by extravasation of blood in 4-5 hr and death of the embryo within 10-20 hr after inoculation of toxic fractions directly on to the dropped chorioallantoic membrane of a 10-day chick embryo. The reactions may be observed directly when a window is cut in the shell. The toxicity for the embryonated egg was found by J.K. Read (cited by Burrows, 1965) to be present in both intracellular and cell wall preparations of the vibrios but only traces were found in centrifuged supernatants of culture in which the vibrios had been grown.

The toxicities of the skin toxin and enterotoxin of *V. cholerae* for the 9-day chick embryo were assayed in our laboratory by inoculating a volume of 0.1 ml. of the respective sterile toxin directly on to the dropped chorioallantoic membrane. The inoculated eggs were incubated at 37°C and observed daily aseptically for 4 days and examined for extravasation of blood or death of the embryo. There was no extravasation of blood nor death in any of the 4 eggs inoculated with skin toxin; 1 embryo out of 4 eggs was found dead within 24 hr of inoculation of enterotoxin. The skin toxin is thus relatively non-lethal; the enterotoxin is probably non-lethal, and certainly not markedly lethal.

Table 29.

The effects of skin toxin and enterotoxin of V. cholerae on chorioallantoic membrane

Material inoculated	Number of eggs inoculated per sample	Dose of toxin per egg	Period of observation for detection of extravasation of blood and death of embryo				Diameter of induration produced on guinea pig skin after intracutaneous injection of 0.1 ml. of the toxin (mm).
			1st day	2nd day	3rd day	4th day	
Skin toxin (0-60% $(NH_4)_2SO_4$ -precipitated 12r toxin)	4	0.1 ml.	Nil	Nil	Nil	Nil	16.5
Enterotoxin contained in V. cholerae strain 12r - induced positive rabbit gut loop fluid filtrate	4	0.1 ml.	1 D	Nil	Nil	Nil	18.0
Control: Distilled water	4	0.1 ml.	Nil	Nil	Nil	Nil	Nil

D = Dead

Toxicity of products of *V. cholerae* for cells in tissue cultures

Explanatory note. Study of the pathological effects of bacterial toxins on mammalian cells in tissue culture may help in understanding the toxic effects observed in the intact animal. Recently, the toxic effects of diphtheria toxin on cell cultures were reviewed by van Heyningen and Arseculeratne (1964). The endotoxin of *Esch. coli* has been studied by Gabliks and Solotorovsky (1962) using continuous cell lines as well as primary cell cultures derived from a number of animal species.

The localized nature of infection in cholera suggests that an important element in the pathogenesis of the disease is a primary toxic effect in the bowel, with a generalized toxaemia possibly contributing to the disease process. Studies on the toxicities of intracellular (IC) and cell wall (CW) preparations of *V. cholerae* (Burrows, 1965) for mammalian cell cultures have been carried out, largely with a clone of Earle's L cell, by Read (1965). Both fractions were toxic to cells in tissue cultures, the former killing the cells and the latter producing a transient cytopathic effect when the substances were introduced into the culture fluid in a concentration of 250-500 µg/ml.

Results. In the present work the toxicities of the skin toxin

and enterotoxin of V. cholerae for tissue cell monolayers of HEp 2 cells have been studied. A volume of 0.2 ml. of each toxin was inoculated in to each of 4 tubes of the cell cultures, and the mixtures were incubated at 37°C in a revolving drum for 7 days. The culture tubes were examined carefully every day for detection of cytopathic effect. There was no difference between the cells in inoculated and control uninoculated culture tubes.

The experiment is summarized in Table 30.

Table 30.

The effect of skin toxin and enterotoxin on tissue cell monolayers with HEp 2 cells.

Material inoculated	No. of culture tubes per sample	Dose of inoculum per tube	Cytopathic effect observed on							Diameter of induration (mm.) produced on guinea pig skin after intracutaneous injection of 0.1 ml. of the toxin
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	
Skin toxin (0-60% $(NH_4)_2SO_4$ precipitated 12r toxin).	4	0.2 ml.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	16.5
Enterotoxin contained in V. cholerae strain 12r induced positive rabbit gut loop fluid filtrate	4	0.2 ml.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	18.0
Diluent control (distilled water)	4	0.2 ml.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Media control (HEp2 cells)	4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Not done

Agar gel precipitation studies with skin toxin and enterotoxin of V. cholerae strain 12r

Attemps were made to identify the skin toxin and enterotoxin of V. cholerae strain 12r by means of a double-diffusion precipitation technique with ion agar (for details see Methods). The central well contained antitoxic serum against 12r toxin and in the peripheral wells 1, 2, 3 and 4 were samples of undiluted skin toxin and samples diluted 1 in 2, 1 in 4 and 1 in 20 respectively. Wells 5 and 6 contained enterotoxin diluted 1 in 10 and undiluted respectively.

The results (Fig. 10) indicate that undiluted and diluted samples of the skin toxin precipitate with the homologous antitoxic serum. On the other hand, no such precipitin bands were observed with any preparations of enterotoxin, although this was of comparable potency in producing cutaneous effects. This result was thought to be due to the presence of some inhibitory factor that prevented the enterotoxin from forming precipitin bands in the double diffusion plate. Accordingly, the enterotoxin was mixed separately with skin toxin 12r, 569B, and Procholeragen A in equal proportions. These mixtures were then allowed to diffuse against the same antitoxic serum in an agar gel plate as shown in Fig. 11.

Fig. 10.

Double diffusion precipitation of cholera toxin.

Central well	=	Antitoxic serum against skin toxin, 12r.
Well No. 1	=	Undiluted 12r skin toxin.
Well No. 2	=	1 in 2 dilution of 12r skin toxin.
Well No. 3	=	1 in 4 " "
Well No. 4	=	1 in 20 " "
Well No. 5	=	1 in 10 dilution of enterotoxin (loop fluid).
Well No. 6	=	Undiluted enterotoxin produced *in vivo* in response to *V. cholerae* strain 12r.


6
1
5
2
4
3

Fig. 11.

Double diffusion gel precipitation analysis of enterotoxin.

Central well = Antitoxic serum against skin toxin, 12r.

Well No. 1 = Enterotoxin. (Loop fluid produced *in vivo* in response to *V. cholerae* strain 12r)

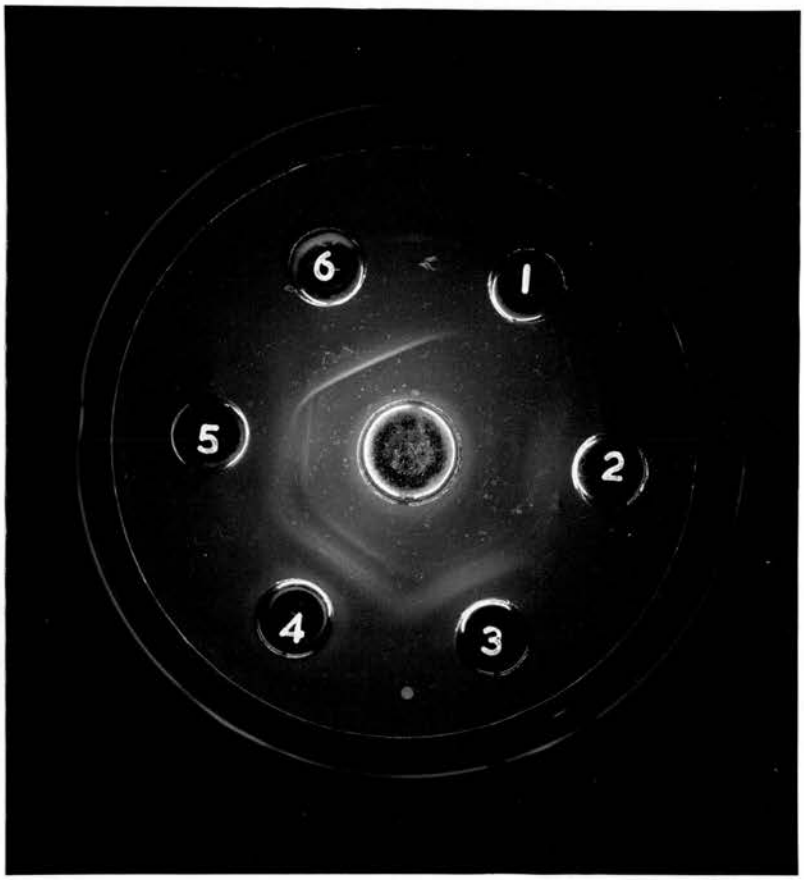
Well No. 2 = Equal volume of enterotoxin and skin toxin, 12r.

Well No. 3 = Skin toxin, 12r.

Well No. 4 = Skin toxin, 569B.

Well No. 5 = Equal volume of enterotoxin and skin toxin, 569B.

Well No. 6 = Equal volume of enterotoxin and procholeragen A.


6
1
5
2
4
3

It was observed that these mixtures gave precipitin bands whereas no band appeared between the wells containing the enterotoxin alone and the antitoxin. Had there been an inhibitory factor present in the enterotoxin, no precipitin bands would be expected to form between the central antitoxin and any of the peripheral wells containing the respective skin toxin-enterotoxin mixtures. Thus, it seems that there is no inhibitor in the enterotoxin that prevents the development of precipitin bands when it encounters antitoxic serum in the double diffusion plate.

Gel diffusion studies with culture media and normal serum of rabbit

Tests confirmed that the culture medium that is used for production of skin toxin does not give any precipitin bands with serum of rabbits immunized with skin toxin. Further, it has also been observed that normal sera of rabbits obtained before immunization with skin toxin do not produce precipitin bands against skin toxin on an agar gel diffusion plate. The results are shown clearly in Figs. 12 and 13.

These results indicate that the precipitin bands that appeared between the skin toxin and antitoxin on double diffusion plates indicate a specific antigen-antibody reaction related to the products of the organism.

Fig. 12.

Gel diffusion study with culture media.

Central well = Antitoxic serum against skin toxin 12r.

Well No. 1 = Skin toxin 12r.

Well No. 2 = Enterotoxin. (Loop fluid produced *in vivo* in response to *V. cholerae* strain 12r)

Well No. 3 = Skin toxin 569B.

Well No. 4 = 20 per cent. Bacto Peptone water.

Well No. 5 = Same as Well No. 1.

Well No. 6 = Same as Well No. 4.


6
1
5
2
4
3

Fig. 13.

Gel diffusion study with normal serum of rabbit.

Central well = Skin toxin 569B.

Well No. 1 = Normal serum of rabbit No. R_1.

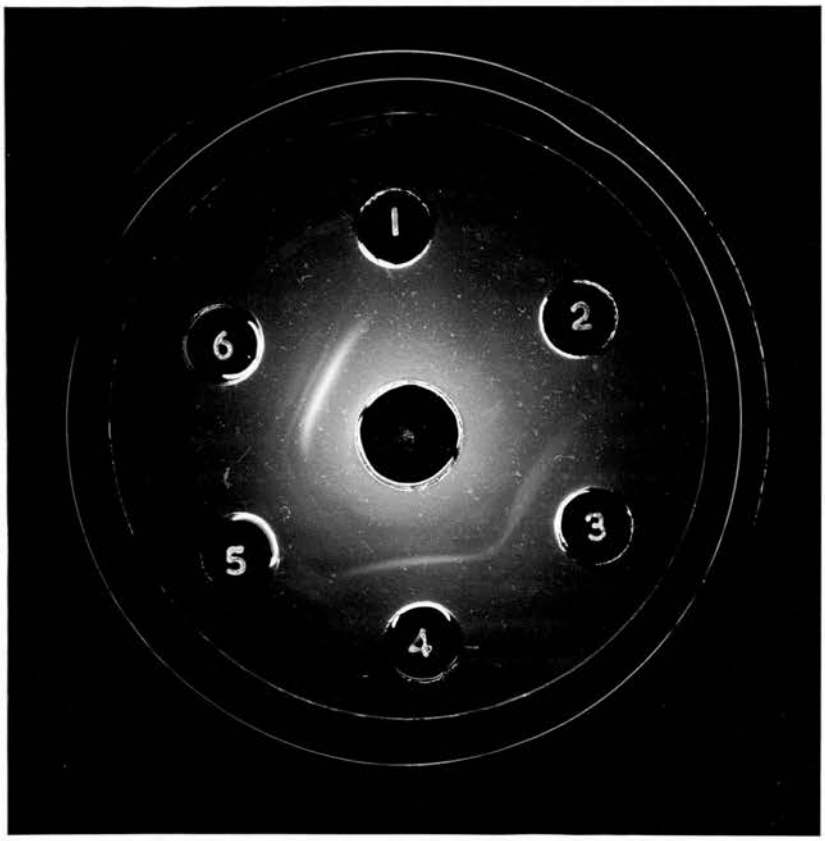
Well No. 2 = Normal serum of rabbit No. R_2.

Well No. 3 = Antitoxic serum against skin toxin 569B from rabbit No. R_1.

Well No. 4 = Antitoxic serum against skin toxin 569B from rabbit No. R_2.

Well No. 5 = Antitoxic serum against skin toxin 12r from rabbit No. R_3.

Well No. 6 = Antitoxic serum against skin toxin 12r from rabbit No. R_4.


1
2
3
4
5
6

Agglutination of V. cholerae with serum of rabbit immunized with skin toxin

Agglutination of V. cholerae strains 12r and 569B was studied in tube agglutination tests using immune sera obtained from rabbits immunized with bacteria-free soluble products of either of these strains.

To 1 part of a saline suspension (matching Brown's opacity tube No. 2) of V. cholerae strains 12r or 569B, 1 part of each of the doubling dilutions of the serum under test was added and mixed well. The mixture was incubated at 37°C in the water bath for 4 hr and thereafter kept in the cold room overnight. The results of the agglutination tests are shown in Tables 31 and 32.

Table 31.

The agglutination of suspensions of V. cholerae by dilutions of "antitoxic" sera prepared against soluble products of V. cholerae

Cell suspension in saline of V. cholerae strain	Agglutination occurring in the presence of serum against skin toxin of V. cholerae strain 569B diluted in saline 1 in									
	5	10	20	40	80	160	320	640	1280	Control
12r	+	+	+	+	+	+	+	-	-	-
569B	+	+	+	+	+	+	-	-	-	-
Final dilution of serum 1 in:	10	20	40	80	160	320	640	1280	2560	

+ = Agglutination - = No agglutination

Table 32

The agglutination of suspensions of *V. cholerae* by dilutions of "antitoxic" sera prepared against soluble products of *V. cholerae*

Cell suspension in saline of *V. cholerae* strain	Agglutination occurring in the presence of serum against skin toxin of *V. cholerae* strain 12r diluted in saline 1 in									
	10	20	40	80	160	320	640	1280	2560	Control
12r	+	+	+	+	+	+	+	-	-	-
569B	+	+	+	-	-	-	-	-	-	-
Final dilution of serum 1 in:	20	40	80	160	320	640	1280	2560	5120	

+ = Agglutination - = No agglutination

Thus it is evident from the results that the antitoxic serum against 12r toxin agglutinates strains 12r and 569B at dilutions of 1 in 1280 and 1 in 80 respectively. The serum prepared against 569B toxin agglutinates strain 569B at a dilution of 1 in 320 and 12r at a dilution of 1 in 640.

Neutralizing capacity of the antitoxic sera

Rabbits were immunized with skin toxins precipitated with ammonium sulphate from culture filtrates of V. cholerae and dialyzed against distilled water. These toxins were sterilized by filtration through Oxoid membrane filters before being used for immunizing the rabbits. Two of the strains of V. cholerae were used for toxin production, namely V. cholerae Ogawa strain 12r and Inaba strain 569B. The toxins were used separately for immunization of the animals. The immunizing doses of 1 ml. each per week were administered subcutaneously in 1 group of rabbits. In another group, similar doses of 1 ml. of the toxin per week were injected intracutaneously in divided volumes at different sites. The rising titres of the sera were followed by bleeding the animals after the third dose in the former and the second dose in the latter group of animals. The sera in both cases were found to neutralize completely the skin reactive effects of loop fluid and culture filtrates of V. cholerae and the sera were effective in dilutions up to 1 in 20 in saline. Following the same weekly immunization procedures, titres of 1 in 80 were obtained in sera from each group after 6 weeks.

In a typical experiment for titration of the serum, 1 part of the culture filtrate or loop fluid filtrate was added to 1 part of each of twofold serial dilutions of the serum in saline. The mixtures were incubated at 37°C in the water bath for 2 hr prior to

intracutaneous injection of a volume of 0.1 ml. from each of the mixtures in guinea pigs, and intravenous dye was injected 18 hr later. The serum was capable of preventing both induration and permeability effects of both loop fluid and culture filtrates. The normal sera of the rabbits obtained before immunization were found to have no such neutralizing effect. The results of the titration of a serum are shown in table 33 and the data are represented graphically in figures 14 and 15.

Table 33.

Neutralization of the cutaneous activities of enterotoxin-positive loop fluid and *in vitro* prepared skin toxin of *V. cholerae* strain 12r by antitoxic serum obtained from immunization of rabbit with the same skin toxin of strain 12r

Equal volume of		Final dilution of serum in the resultant toxin-serum mixture 1 in	Mean diameter of lesions produced/0.1 ml. of inoculum (mm.)	
Toxin	Serum dilution 1 in		Induration	Blueing
Entero toxin-positive loop fluid	5	10	Nil	Nil
"	10	20	"	"
"	20	40	"	"
"	40	80	"	"
"	80	160	12.5	13.0
"	160	320	17.5	14.2
In vitro prepared skin toxin	5	10	Nil	Nil
"	10	20	"	"
"	20	40	"	"
"	40	80	"	"
"	80	160	10.0	15.8
"	160	320	13.7	18.0

Controls

Table 33 (contd.)

Equal volume of		Mean diameter of lesions produced/0.1 ml. of inoculum (mm.)	
		Induration	Blueing
Enterotoxin -positive loop fluid	Normal serum	17.8	18.0
In vitro prepared skin toxin	"	14.2	14.0
Enterotoxin -positive loop fluid	Saline	17.5	17.8
In vitro prepared skin toxin	"	14.0	14.3
Saline	Normal serum	Nil	Nil
Saline	Immune serum	"	"

Similarly skin toxin and enterotoxin of V. cholerae strain 569B were neutralized with serum of rabbits immunized with the same skin toxin of V. cholerae strain 569B. It was further observed that antitoxic serum prepared against one skin toxin is equally effective in neutralizing skin toxin prepared from a heterologous strain. Thus, the antitoxic serum prepared against 569B skin toxin was found to neutralize the skin toxin of strain 12r as shown in table 34 and fig. 16.

Table 34.

Neutralization of skin toxin of V. cholerae strain 12r with antitoxic sera prepared against skin toxin of strain 569B

Equal volume of		Final serum dilution in the resultant toxin - serum mixture 1 in	Mean diameter of lesions produced/0.1 ml. of inoculum (mm.)	
Toxin	Serum dilution 1 in		Induration	Blueing
In vitro prepared skin toxin from strain 12r	Serum No.1	Serum No.1		
	5	10	Nil	Nil
"	10	20	"	"
"	20	40	8	8.5
"	40	80	15.5	16.0
	Serum No.2	Serum No.2		
"	5	10	Nil	Nil
"	10	20	"	"
"	20	40	9	9.5
"	40	80	15.8	16.2

Controls

Equal volume of		Mean diameter of skin lesion produced/0.1 ml. of inoculum (mm.)	
		Induration	Blueing
Skin toxin of V. cholerae strain 12r	Saline	16.0	16.2
Serum No.1	Saline	Nil	Nil
Serum No.2	Saline	"	"
Saline	-	"	"

Agar gel immuno-diffusion reactions with the skin toxin derived from strain 12r against its homologous antitoxin and against the heterologous antitoxin prepared from strain 569B toxin gave both bands of identity and non-identity. Similar bands were also observed when the skin toxin from strain 569B was tested against these sera. These observations are shown clearly in the figures 17 and 18.

Fig. 14

NEUTRALIZATION OF INDURATION EFFECT OF ENTEROTOXIN-POSITIVE LOOP FLUID BY ANTITOXIC SERUM OBTAINED BY IMMUNIZATION OF RABBIT WITH SKIN TOXIN PREPARED FROM V. CHOLERAE STRAIN 12γ. (SERUM AND TOXIN MIXTURE HELD FOR 2 hr AT 37°C IN VITRO BEFORE INTRADERMAL INOCULATION IN GUINEA PIG.)

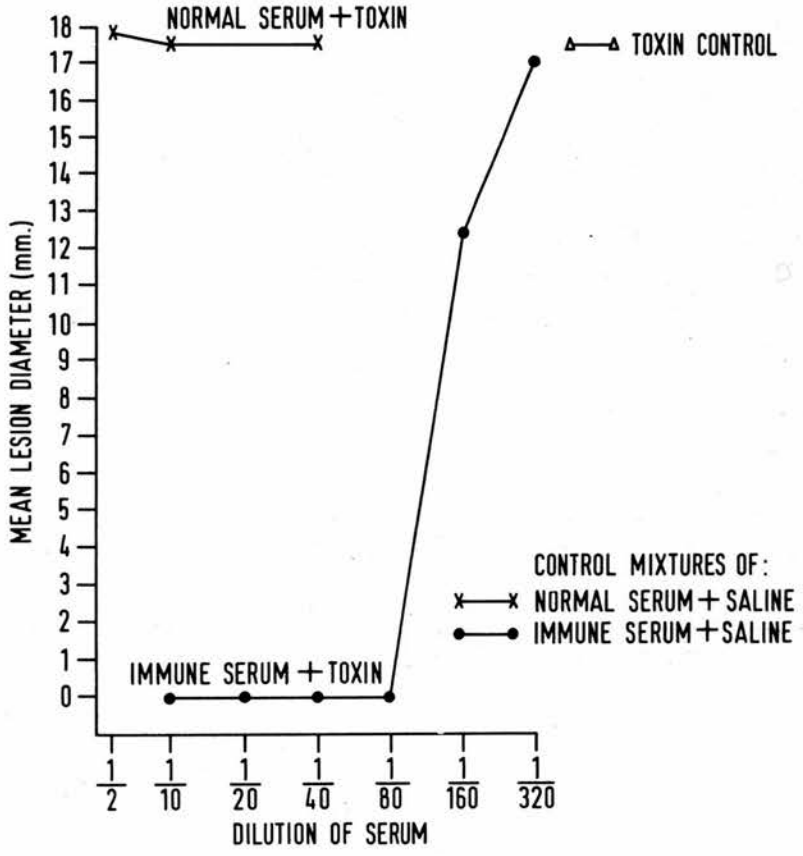


Fig. 15.

NEUTRALIZATION OF INDURATION EFFECT OF SKIN TOXIN OF V. CHOLERAE STRAIN 12γ BY ANTITOXIC SERUM OBTAINED BY IMMUNIZATION OF RABBIT WITH THE SAME TOXIN. (SERUM AND TOXIN MIXTURE HELD FOR 2hr AT 37°C IN VITRO BEFORE INTRADERMAL INOCULATION IN GUINEA PIG)

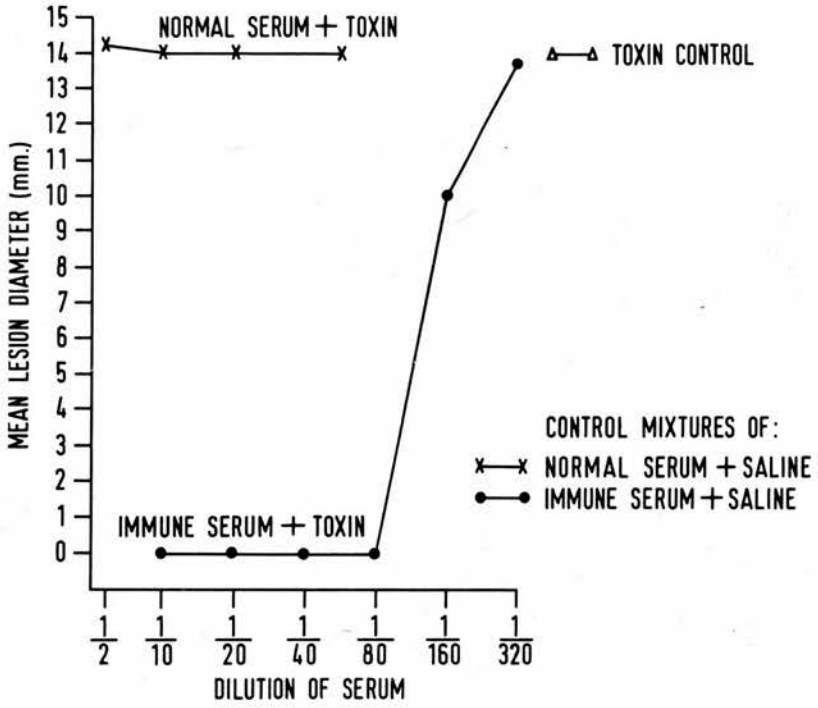


Fig. 16.

Figure showing that antitoxic serum prepared against one toxin is effective in neutralizing skin toxin prepared from a heterologous strain.

Thus, the skin toxin of strain 12r is neutralized by antitoxic serum against skin toxin 569B.

Effects of intracutaneous injection of a volume of 0.1 ml from mixtures containing equal amounts of:

1 = 1 in 5 dilution of antitoxic serum No. 1 prepared against skin toxin 569B + skin toxin 12r.

2 = 1 in 10 dilution of the above serum + skin toxin 12r.

3 = Above serum (undiluted) + saline.

4 = Antitoxic serum No. 2 against skin toxin 569B (undiluted) + saline.

5 = 1 in 5 dilution of the above serum No. 2 + skin toxin 12r.

6 = 1 in 10 dilution of the above serum No. 2 + skin toxin 12r.

7 = Skin toxin 12r + saline.

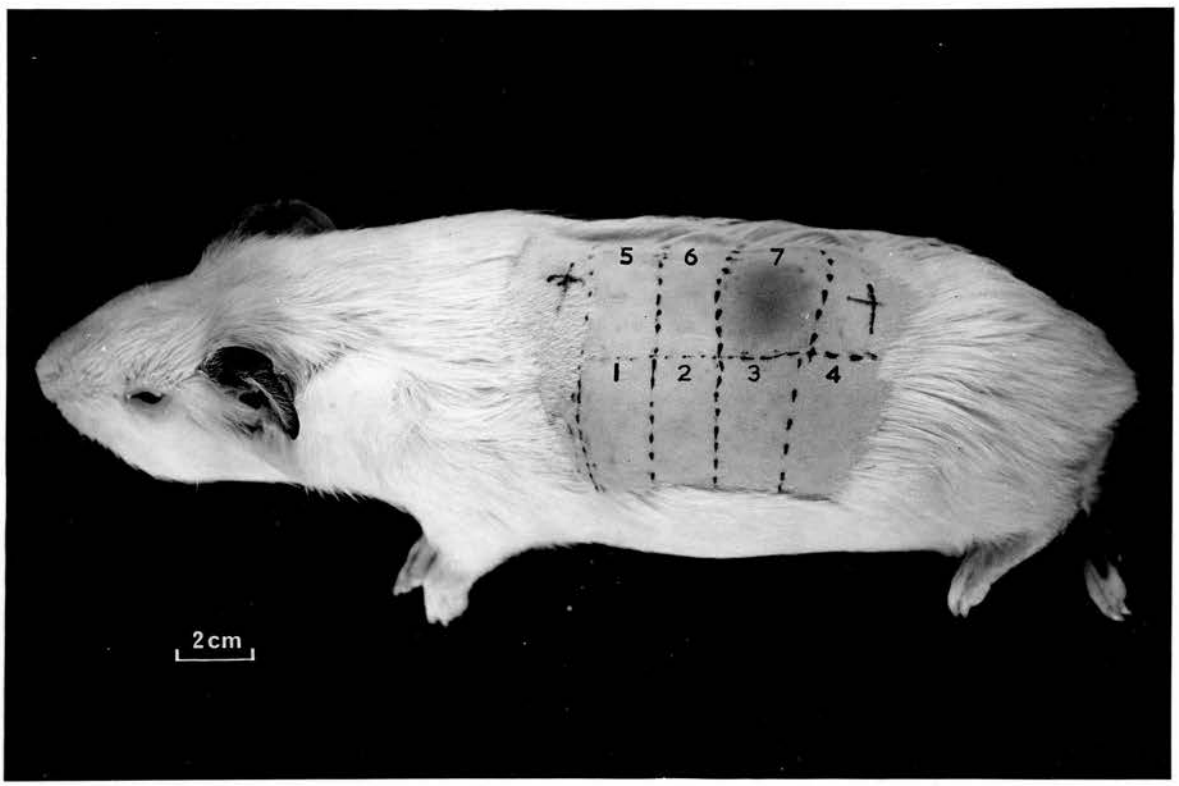

5
6
7
1
2
3
4
2cm

Fig. 17.

Agar gel immuno-diffusion reactions with skin toxin derived from strain 12r against its homologous antitoxin and against the heterologous antitoxin prepared from strain 569B toxin.

Well No. 1 = Skin toxin 12r.

Well No. 2 = Antitoxic serum against skin toxin 569B.

Well No. 3 = Antitoxic serum against skin toxin 12r.


2
3
1

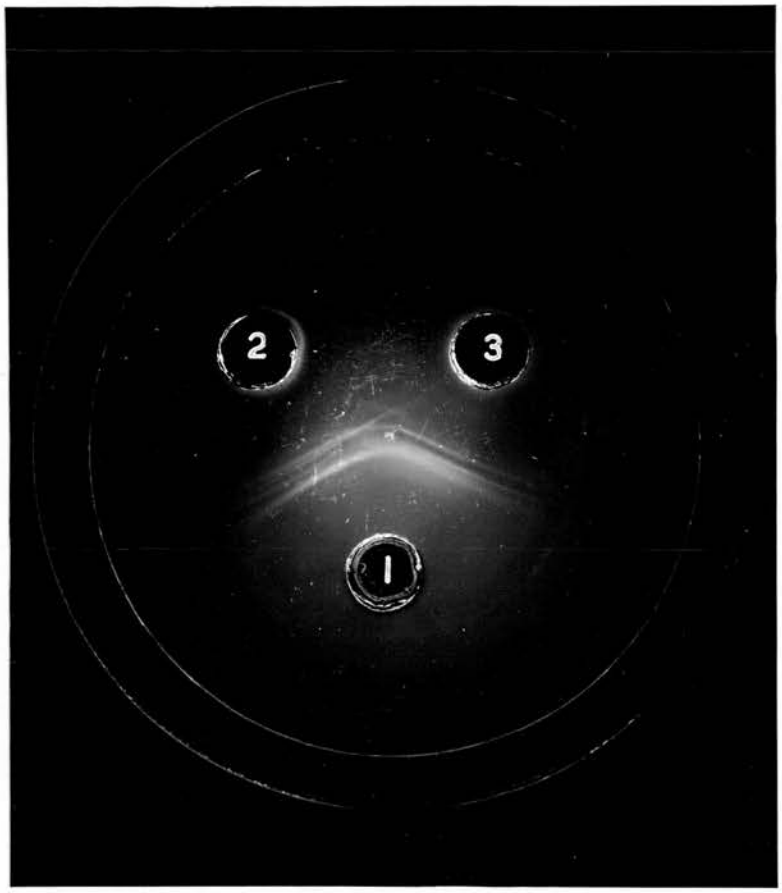
Fig. 18

Agar gel immuno-diffusion reactions with skin toxin derived from strain 569B against its homologous antitoxin and against the heterologous antitoxin prepared from strain 12r toxin.

Well No. 1 = Skin toxin 569B.

Well No. 2 = Antitoxic serum against skin toxin 569B.

Well No. 3 = Antitoxic serum against skin toxin 12r.


2
3
1

Skin and loop effects of in vitro neutralized cholera toxin

The effects of homologous serum on the cutaneous and loop activities of the skin toxin preparations of V. cholerae strain 12r were studied carefully by inoculating the toxin inactivated in vitro with homologous serum into the lumen of the ileal loops in rabbits and into skin of guinea pigs. The experimental procedures involved in the study were as follows:-

Observation of effects on ileal loops in rabbits.

Four ileal loops were made in each of 2 Californian female rabbits weighing6-8lb. The following loop inoculation procedure was then observed in both of the animals. In the proximal loop (L_1) a volume of 0.8 ml. of a mixture of equal quantities of skin toxin of V. cholerae strain 12r and saline was injected. The successive loops were also inoculated with a volume of 0.8 ml. of mixtures of equal quantities of the same toxin anddilutions of the homologous serum. Thus, the second loop (L_2) was inoculated with toxin and undiluted serum; the third loop (L_3) received toxin plus a 1 in 5 dilution of the serum; and the fourth loop (L_4) was injected with toxin and a 1 in 10 dilution of the serum. A volume of 0.8 ml. of a mixture of the serum and saline was injected into the control loop C_3 between the third and fourth loop. The control loop C_1 between the first and second loop received 0.8 ml.

of saline, while the control loop C_2 between the second and third loop received nothing. Each of the above inocula was incubated at 37°C for 2 hr prior to injection into the appropriate loop. The operation was completed within 40 min. The animals regained consciousness within 2 hr and did not show undue distress. The animals were killed 11 hr after the operation. At post mortem examination the following changes in the loops, recorded in table 35 were observed. The gross appearances of the loops in one of the animals are shown in fig. 19.

Table 35

The effects of inoculations of skin toxin and toxin-antitoxin mixture in ileal loops in 2 rabbits

Materials inoculated (0.8 ml. of a mixture of equal quantities of)	Rabbit No.	Loop No.	Loop effect
Skin toxin of V. cholerae strain 12r + saline.	1	L_1	++
Same toxin + undiluted homologous antitoxic serum.	"	L_2	-
Same toxin + 1 in 5 dilution of the same serum.	"	L_3	-
Same toxin + 1 in 10 dilution of the serum.	"	L_4	-
Saline only (0.8 ml.)	"	C_1	-
Not inoculated	"	C_2	-
Saline + undiluted serum	"	C_3	-
Skin toxin of V. cholerae strain 12r + saline	2	L_1	++
Same toxin + undiluted homologous antitoxic serum	"	L_2	-
Same toxin + 1 in 5 dilution of the serum.	"	L_3	-
Same toxin + 1 in 20 dilution of the serum.	"	L_4	+
Saline only (0.8 ml.)	"	C_1	-
Not inoculated	"	C_2	-
Saline + undiluted serum.	"	C_3	-

The results indicate that the toxin when neutralized with homologous serum is unable to produce a loop lesion in the ligated gut of rabbits.

Fig. 19.

Ileal loops in normal rabbit No. 5

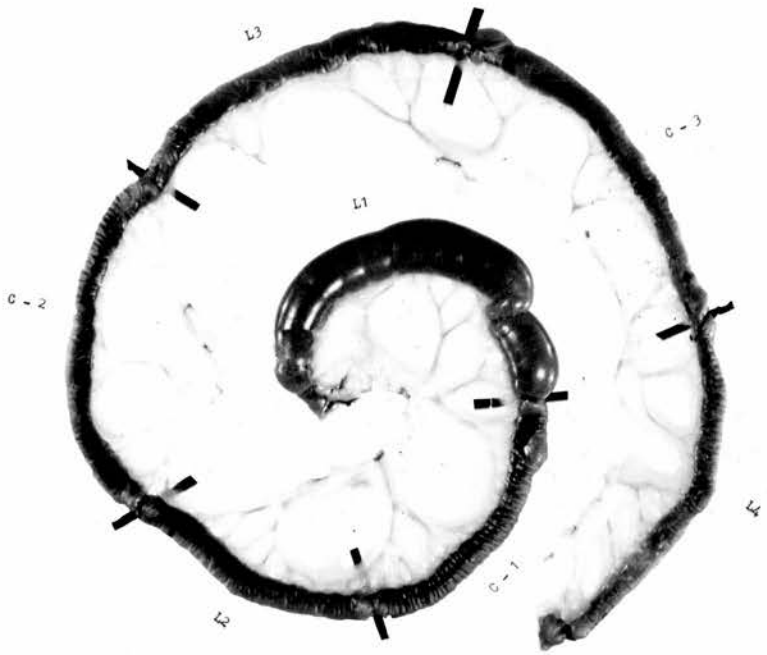
Loop No.	Materials injected	Result (loop effect)
L_1	0.4 ml. S T 12r + 0.4 ml. saline	++
L_2	0.4 ml. S T 12r + 0.4 ml. serum against 12r toxin.	-
L_3	0.4 ml. S T 12r + 0.4 ml. of 1 in 5 dilution of serum against 12r toxin.	-
L_4 Distal	0.4 ml. S T 12r + 0.4 ml. of 1 in 10 dilution of serum against 12r toxin.	-
C_1	0.8 ml. of sterile saline	-
C_2	Not inoculated	-
C_3	0.4 ml. serum against 12r toxin + 0.4 ml. saline	-

S T = Skin toxin

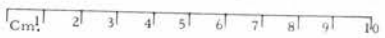
++ = Strong positive

- = No loop lesion

Normal Rabbit



L1= 0.4 ml S.T. 12r + 0.4 ml saline.
L2= 0.4 ml S.T. 12r + 0.4 ml serum/S.T. 12r
L3= 0.4 ml S.T. 12r + 0.4 ml of 1:5 serum/S.T. 12r
L4= 0.4 ml S.T. 12r + 0.4 ml of 1:10 serum/S.T. 12r
C.3= 0.4 ml Serum/S.T. 12r + 0.4 ml saline



Observations in guinea pig skin.

The cutaneous effects of the same toxin and toxin-antitoxin mixtures were also studied by intracutaneous inoculation in albino guinea pigs. The different inocula were incubated as before at 37°C for 2 hr prior to intracutaneous injections. The experiment and the results are summarized in table 36 and the effects are also shown in fig. 20.

Table 36

Effects of intracutaneous injections of toxin and toxin-antitoxin mixtures in guinea pigs

Materials injected intracutaneously (equal volumes of)	Mean diameter of induration produced/0.1 ml. of inoculum (mm.)
Skin toxin of V. cholerae strain 12r + saline.	14.0
" + homologous undiluted antitoxic serum	Nil
" + 1 in 5 dilution of serum	Nil
" + 1 in 10 dilution of serum	Nil
" + 1 in 20 dilution of serum	2.0
saline + serum	Nil

It is evident from these observations that the cutaneous and loop activities of the skin toxin preparations of V. cholerae strain 12r are effectively neutralized by the homologous antitoxic serum used in the experiments in dilutions up to 1 in 10.

Fig. 20.

Effects of intracutaneous injection of a volume of 0.1 ml. of mixtures of equal amounts of:-

1 = Undiluted antitoxic serum against skin toxin 12r + skin toxin 12r.

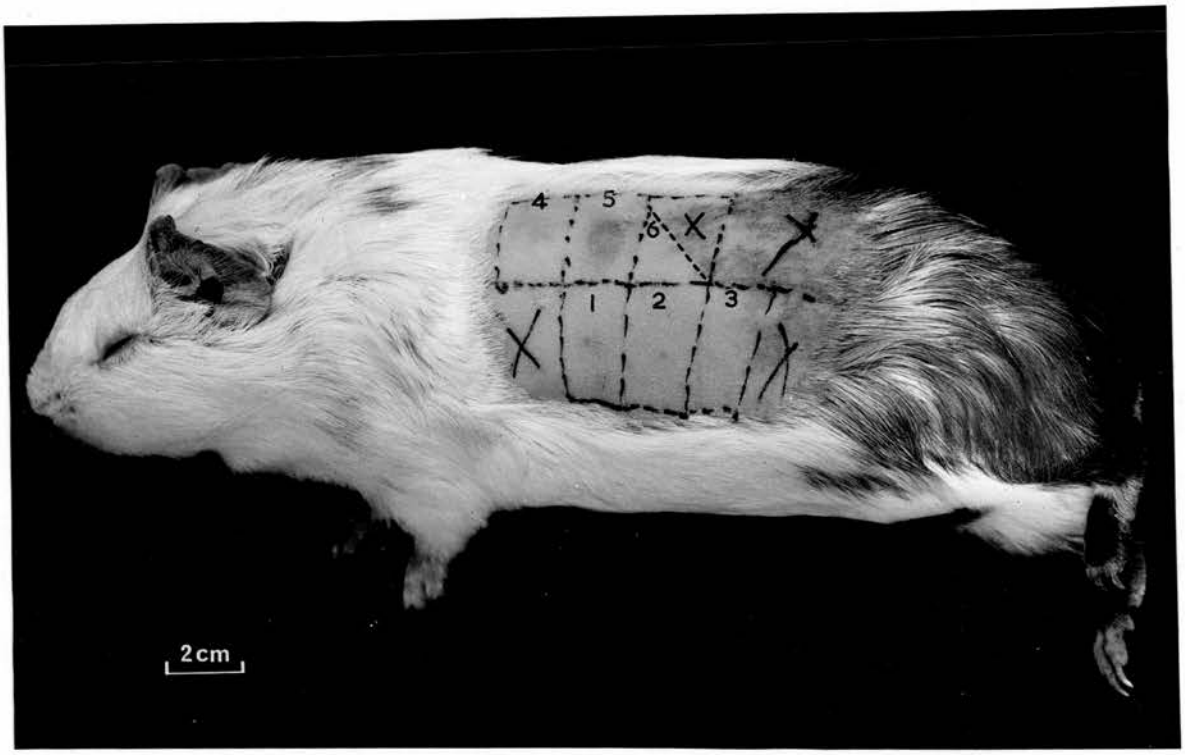
2 = 1 in 5 dilution of the above serum + skin toxin 12r.

3 = 1 in 10 dilution of the serum + skin toxin 12r.

4 = 1 in 20 dilution of the serum + skin toxin 12r.

5 = Skin toxin 12r + saline.

6 = Undiluted serum + saline.


4
5
6
1
2
3
2cm

Effect of antibacterial serum upon the ability of skin toxin of V. cholerae to produce skin lesions in guinea pigs

High-titre antibacterial serum prepared against V. cholerae strain 12r was mixed in equal amounts with different batches of skin toxin of the same vibrio strain and incubated for 2 hr at 37°C in the water bath prior to intracutaneous inoculation of a 0.1-ml. volume into guinea pigs. The tests were adequately controlled by including inocula of toxin and toxin-antitoxin mixtures in identical conditions. The results summarized from 4 different experiments are recorded in table 37.

Table 37

The result of intracutaneous injection of skin toxin treated with antibacterial serum

Experiment No.	Mean diameter of induration produced/0.1 ml. of inoculum (mm.)			
	Test	Control		
	Toxin-antibacterial serum mixture	Toxin-antitoxic serum mixture	Toxin-saline mixture	Antibacterial serum-saline mixture
1	9.5	-	9.9	-
2	12.3	-	12.0	-
3	6.3	-	14.3	-
4	14.0	-	13.9	-

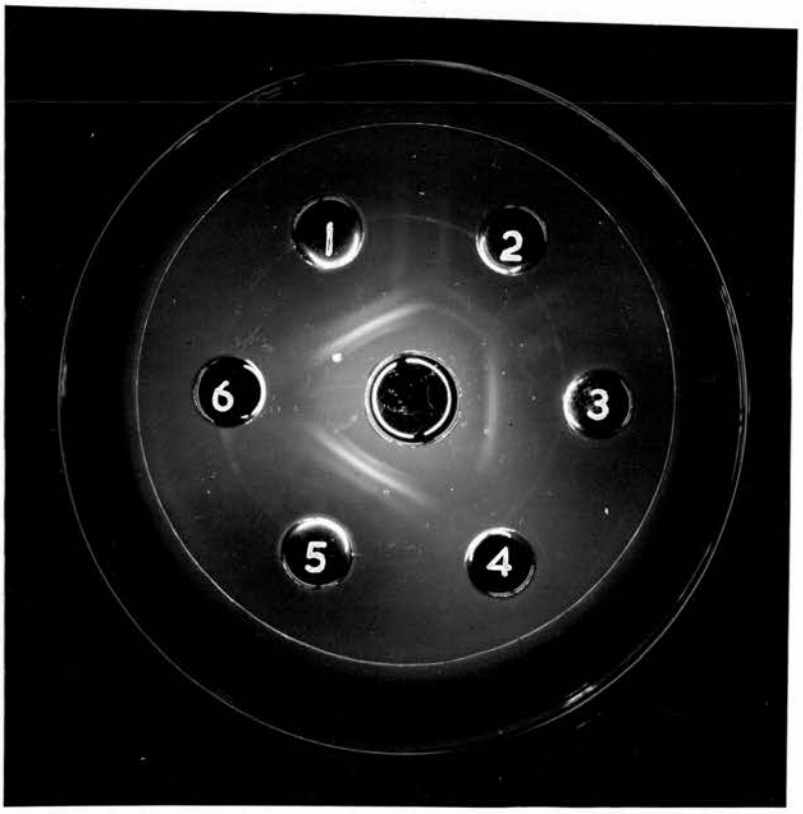
From the results of the above experiments it is noted that high titre antiserum from rabbits immunized with living vibrios had essentially no neutralizing activity against the cutaneous activity of skin toxin of V. cholerae.

The agar well double diffusion precipitation technique revealed that this high-titre antibacterial antiserum produced a precipitate with antigen contained in the skin toxin prepared from V. cholerae strain 12r. The precipitin band crossed with the band produced between the above toxin and the homologous antitoxic serum, indicating an unspecified degree of non-identity. (See fig. 21).

Fig. 21

Double diffusion precipitation of skin toxin

Central well	=	Skin toxin, 12r
Well No. 1	=	Antitoxic serum against skin toxin, 12r
Well No. 2	=	Antitoxic serum against 12r skin toxin adsorbed with living vibrios (strain 12r).
Well No. 3	=	Antibacterial serum against V. cholerae strain 12r
Well No. 4	=	Normal saline.
Well No. 5	=	Same as Well No. 1.
Well No. 6	=	Normal saline.


1
2
3
4
5
6

Effects of V. cholerae treated with homologous antitoxic or antibacterial sera on ileal loops of rabbit

A volume of 0.5 ml. of a peptone-saline suspension of an 18-hr culture of V. cholerae strain 12r with 1.8×10^8 colony-forming units per ml. was mixed with an equal amount of undiluted homologous antitoxic serum having a neutralizing titre of 1 in 80. Similarly, equal amounts of the same culture suspension and homologous antibacterial serum having an agglutination titre of 1 in 8000 were also mixed. A control was incorporated in the test by adding 1 part of peptone-saline to 1 part of the above peptone-saline suspension of the V. cholerae strain 12r.

A volume of 1 ml. of each of the test and control preparations was injected into rabbit ileal loops. The loop lesions were observed 11 hr after the administration of the test and control mixtures. The experiment and the results are summarized in table 38. The character of the lesions in rabbit No. 7 can also be seen in fig. 9 (p. 120).

Table 38

Suppression of loop lesion on inoculation of V. cholerae strain 12r treated with homologous antitoxic or antibacterial sera

Rabbit No.	Loop No.	Dose per loop	Material injected	Character of loop lesion
7	L_1	1 ml.	Equal volumes of V. cholerae strain 12r and homologous antitoxic serum.	-
"	L_2	1 ml.	Equal volumes of V. cholerae strain 12r and homologous antibacterial serum.	-
"	Control L_3	1 ml.	Equal volumes of V. cholerae strain 12r and peptone-saline.	++
6	L_1	1 ml.	Equal volumes of V. cholerae strain 12r and homologous antitoxic serum.	-
"	L_2	1 ml.	Equal volumes of V. cholerae strain 12r and homologous antibacterial serum.	-
"	Control L_3	1 ml.	Equal volumes of V. cholerae strain 12r and peptone-saline.	++

\- = No loop lesion.

++ = Strong positive (distended with accumulation of fluid, congestion and haemorrhage in the wall of the loop).

From these results it is clear that both the homologous antitoxic and antibacterial sera were capable of preventing the production of positive loop lesions by strain 12r in these normal rabbits.

In vivo neutralization of skin toxin

Rabbits were immunized with ammonium sulphate-precipitated, dialyzed and sterilized skin toxin of *V. cholerae*. The immunizing dose of 0.1 ml. of the skin toxin 12r was injected intracutaneously weekly for 4 weeks into each of 2 rabbits, A and B. The skin toxin-neutralizing serum titre in each case was found to be 1 in 20 after 4 weeks. The rabbits C and D were immunized with the skin toxin of strain 569B. Following the same immunizing procedures as mentioned above, a neutralizing serum titre of 1 in 40 was obtained after 5 weeks in each of these 2 rabbits.

These immunized rabbits were used for *in vivo* neutralization of skin toxin of *V. cholerae*. Thus, 3 ileal loops in rabbit A and 4 in each of the rabbits B, C and D were made. A volume of 0.8 ml. of a peptone-saline suspension of *V. cholerae* strain 12r having 1×10^7 colony-forming units/ml. was injected into the distal loop (L_1) of rabbit A. The proximal (L_3) and middle (L_2) loops received sterile toxin of *V. cholerae* strains 12r and 569B respectively in 2-ml. amounts. The control loop C_1 between L_1 and L_2 was inoculated with 2 ml. of sterile normal saline, while the control loop C_2 between L_2 and L_3 received no inoculum (see fig. 22).

The distal loop (L_1) of rabbit B was inoculated with 0.5 ml. of a peptone-saline suspension of *V. cholerae* strain 12r that contained 1×10^6 colony-forming units (CFU)/ml. The successive loops L_2, L_3 and L_4 received 1 ml., 0.5 ml., and 0.5 ml. volumes of a 1 in 2 dilution of the sterile skin

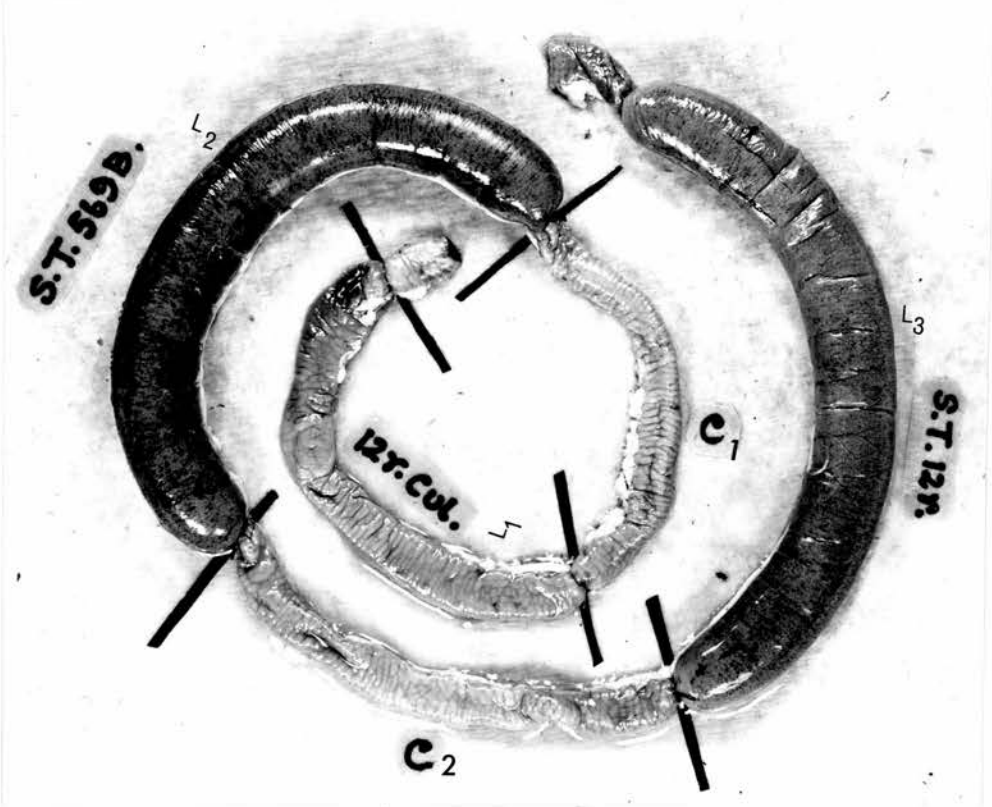
Fig. 22

Ileal loops in immunized rabbit A.

Loop No.	Material inoculated	Loop effect
L_1 Distal	0.8 ml. peptone-saline suspension of V. cholerae strain 12r (1 x 10^7/ml.).	-
L_2	2 ml. of skin toxin 569B.	++
L_3	2 ml. of skin toxin 12r.	++
C_1	2 ml. of sterile normal saline.	-
C_2	Not inoculated.	-

++ = Strong positve

- = No loop lesion


S.T. 569B.
L2
L3
S.T. 12n.
12n. Cul.
L1
C1
C2

toxin of V. cholerae strain 12r respectively. The control loop C_1 between L_1 and L_2 was inoculated with 1 ml. of sterile saline. The other 2 control loops, C_2 and C_3 were not inoculated (see fig. 23).

The proximal loop (L_1) of rabbit C received 0.5 ml. of a 1 in 2 dilution of skin toxin 12r; loop (L_2) received 0.5 ml. of 1 in 2 dilution of skin toxin 569B; loop (L_3) was inoculated with 0.5 ml. of a peptone-saline suspension of V. cholerae strain 12r with 1.8×10^8 CFU/ml.; and the last loop (L_4) received 0.5 ml. of peptone-saline suspension of strain 569B having 1.8×10^8 CFU/ml.

A volume of 0.5 ml. of a 1 in 4 dilution of the skin toxin 12r was inoculated into control loop C_3 between loops L_3 and L_4, while the control loops C_1 received 0.5 ml. of saline and C_2 was left uninoculated (see fig. 24).

The proximal loop (L_1) of rabbit D was inoculated with 0.5 ml. of a 1 in 2 dilution of skin toxin 12r while the loop (L_2) was inoculated with 0.5 ml. of skin toxin 569B diluted 1 in 2 in saline. Loop (L_3) and loop (L_4) received 0.5 ml. of peptone-saline suspension of strains 569B and 12r respectively with a dose of 1×10^7 CFU per ml. The control loops, C_1, C_2, and C_3, received a volume of 0.5 ml. of a 1 in 4 dilution of skin toxin 12r, a 1 in 4 dilution of skin toxin 569B, and saline respectively (see fig. 25). The animals were killed 11 hr after the challenge. The experiments and the results are summarized in table 39.

Fig. 23

Ileal loops in immunized rabbit B.

Loop No.	Materials inoculated	Loop effect
L_1 Distal	0.5 ml. of peptone-saline suspension of V. cholerae strain 12r (1 x 10^6/ml.)	-
L_2	1 ml. of skin toxin 12r.	++
L_3	0.5 ml. of skin toxin 12r.	++
L_4	0.5 ml. of 1 in 2 dilution of skin toxin 12r.	+
C_1	1 ml. of sterile normal saline	-
C_2	Not inoculated.	-
C_3	Not inoculated.	-

++ = Strong positive

- = No loop lesion

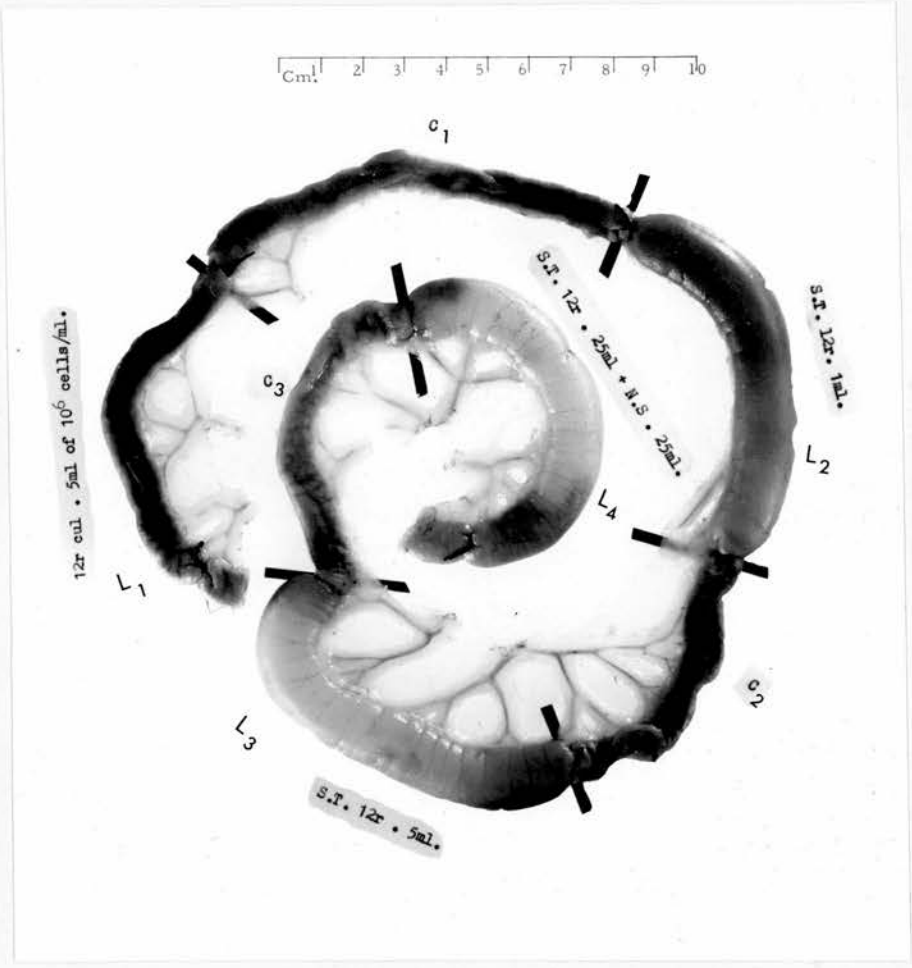

Cm. 1 2 3 4 5 6 7 8 9 10
C1
C2
C3
L1
L2
L3
L4
12r cul • 5ml of 10⁶ cells/ml.
S.I. 12r. 1ml.
S.I. 12r • 25ml + H.S • 25ml.
S.I. 12r • 5ml.

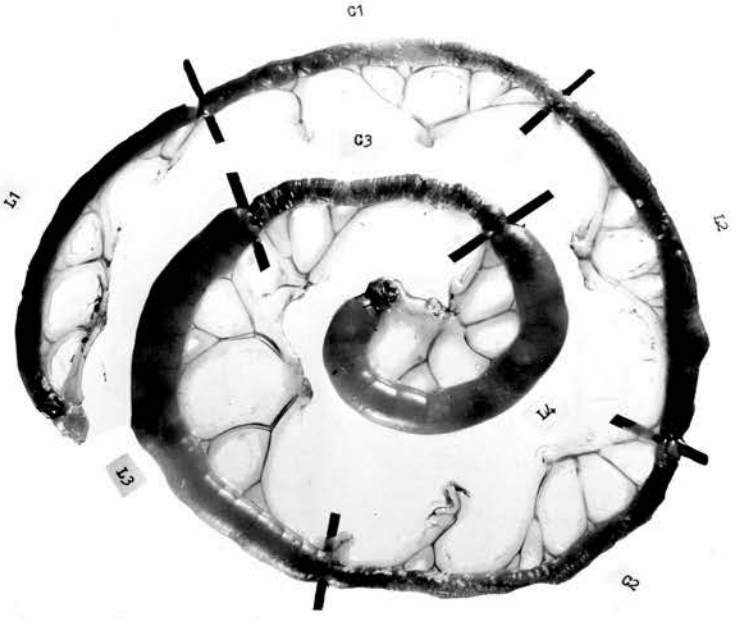
Fig. 24.

Ileal loops in immunized rabbit C.

Loop No.	Materials inoculated	Loop effect
L_1	0.5 ml. of 1 in 2 dilution of skin toxin 12r.	-
L_2	0.5 ml. of 1 in 2 dilution of skin toxin 569B.	-
L_3	0.5 ml. of peptone-saline suspension of *V. cholerae* strain 12r (1.8×10^8 /ml.)	+
L_4 Distal	0.5 ml. of peptone-saline suspension of *V. cholerae* strain 569B (1.8×10^8/ml.).	+
C_1	0.5 ml. of sterile normal saline.	-
C_2	Not inoculated.	-
C_3	0.5 ml. of 1 in 4 dilution of skin toxin 12r.	-

+ = Weak positive

- = No loop lesion



L1 = 0.5 ml of 1:2 12r toxin.
L2 = 0.5 ml of 1:2 569B toxin.
L3 = 0.5 ml 12r cult. of 1.8×10^8 cells/ml.
L4 = 0.5 ml 569B cult. of 1.8×10^8 cells/ml.
C3 = 0.5 ml of 1:4 12r toxin.

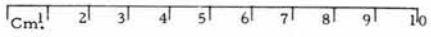


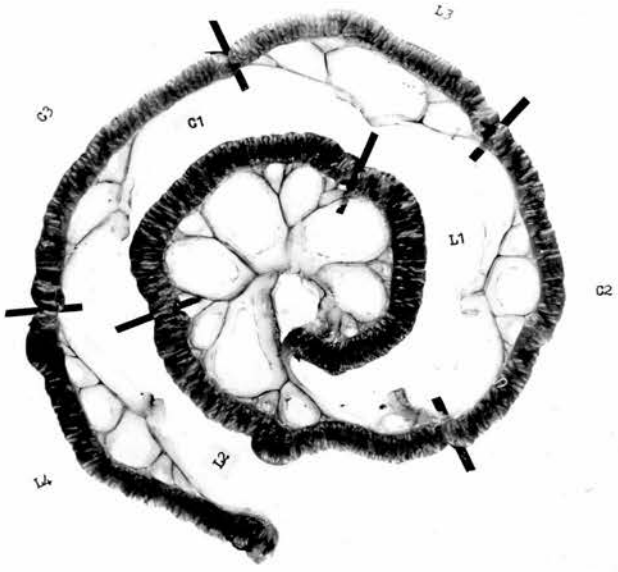
Fig. 25

Ileal loops in immunized rabbit D.

Loop No.	Materials inoculated	Loop effect
L_1	0.5 ml. of 1 in 2 dilution of skin toxin 12r.	-
L_2	0.5 ml. of 1 in 2 dilution of skin toxin 569B.	-
L_3	0.5 ml. of peptone-saline suspension of V. cholerae strain 569B (1×10^7/ml.)	-
L_4 Distal	0.5 ml. of peptone-saline suspension of V. cholerae strain 12r (1×10^7/ml.)	-
C_1	0.5 ml. of 1 in 4 dilution of skin toxin 12r.	-
C_2	0.5 ml. of 1 in 4 dilution of skin toxin 569B.	-
C_3	0.5 ml. of sterile normal saline.	-

- = No loop lesion

Immune Rabbit



L1 = 0.5 ml of 1:2 12r toxin.
L2 = 0.5 ml of 1:2 569B toxin.
L3 = 0.5 ml of 569B cult. of 10^7 cells/ml.
L4 = 0.5 ml of 12r cult. of 10^7 cells/ml.
C1 = 0.5 ml of 1:4 12r toxin.
C2 = 0.5 ml of 1:4 569B toxin.

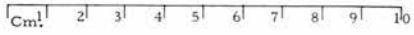


Table 39

Effects of skin toxin in ileal loops of immunized rabbits

Rabbit	Material inoculated	Loop No.	Loop effect	Serum titre
A	0.8 ml. of peptone-saline suspension of V. cholerae strain 12r (1 x 10^7/CFU/ml.)	L1	-	1 in 20
"	2 ml. of skin toxin, 569B	L2	++	
"	2 ml. of skin toxin, 12r	L3	++	
"	2 ml. of sterile normal saline	C1	-	
"	Not inoculated	C2	-	
B	0.5 ml. of peptone-saline suspension of V. cholerae strain 12r (1 x 10^6 CFU/ml.)	L1	-	1 in 20
"	1 ml. of skin toxin, 12r	L2	++	
"	0.5 ml. of skin toxin 12r	L3	++	
"	0.5 ml. of 1 in 2 dilution of skin toxin 12r	L4	+	
"	1 ml. of sterile normal saline	C1	-	
"	Not inoculated	C2	-	
"	Not inoculated	C3	-	
C	0.5 ml. of 1 in 2 dilution of skin toxin 12r	L1	-	1 in 40
"	0.5 ml. of 1 in 2 dilution of skin toxin 569B	L2	-	
"	0.5 ml. of peptone-saline suspension of V. cholerae strain 12r (1.8 x 10^8 CFU/ml.)	L3	+	
"	0.5 ml. of peptone-saline suspension of V. cholerae strain 569B (1.8 x 10^8 CFU/ml.)	L4	+	
"	0.5 ml. of sterile normal saline	C1	-	
"	Not inoculated	C2	-	
"	0.5 ml. of 1 in 4 dilution of skin toxin 12r	C3	-	
D	0.5 ml. of 1 in 2 dilution of skin toxin 12r	L1	-	1 in 40
"	0.5 ml. of 1 in 2 dilution of skin toxin 569B	L2	-	
"	0.5 ml. of peptone-saline suspension of V. cholerae strain 569B (1 x 10^7 CFU/ml.)	L3	-	
"	0.5 ml. of peptone-saline suspension of V. cholerae strain 12r (1 x 10^7 CFU/ml.)	L4	-	
"	0.5 ml. of 1 in 4 dilution of skin toxin 12r	C1	-	
"	0.5 ml. of 1 in 4 dilution of skin toxin 569B	C2	-	
"	0.5 ml. of sterile normal saline	C3	-	

The results suggest that the toxin produced during multiplication of the V. cholerae strain 12r in the lumen of the ileal loops in rabbits A and B was neutralized by the immunity developed in the mucous membrane of the intestinal tract due to immunization of these animals with sterile toxin prepared from the strain 12r; but preformed toxins in large doses were not neutralized as shown in L_2 and L_3 of both the rabbits A and B (see Table 39 and Figs. 22 and 23). When the toxin was diluted, the loop effect was reduced or suppressed completely as shown in loop L_4 of rabbit B and loops L_1, L_2 and C_3 of rabbit C (see Table 39 and Figs. 23 and 24).

In rabbit C, none of the toxins prepared from strains 12r and 569B produced a positive loop, but the living cells of each of these strains in 0.5 ml. of a suspension containing 1.8×10^8 CFU/ml. were able to produce positive loop lesions (see Table 39 and Fig. 24).

In rabbit D neither the toxins nor the living cells were able to produce any positive loop, but similar or even lesser doses of these materials have been found to produce positive loops when inoculated into the lumen of the ligated gut of normal rabbits as shown in L_1 of rabbits 2, 4 and 5 in Figs. 7, 8 and 19 respectively and C_1 of rabbit 7 in Fig. 7.

A preliminary attempt to convert skin toxin into toxoid form

The conversion of toxin into toxoid can be carried out most effectively by treating it with formalin and incubating the toxin for 2 - 3 weeks at 37°C. The change from the toxic into non-toxic state is assessed by injecting the material into susceptible animals; the change is regarded as complete when no symptoms are produced. In the present experiment, the toxin was treated with formalin or heated at 60°C for 30 min. for conversion into toxoid.

Formalin treatment - A 5-ml. volume of 5 per cent. formalin was added to a 5-ml. sample of each of the skin toxins of *V. cholerae* strains 12r and 569B. Three such mixtures of each toxin were prepared; 1 was incubated at 37°C, another was left at room temperature, and the third was held at 4°C. After 7 days, all of the above preparations were dialyzed in distilled water for 48 hr to get rid of the formalin and thereafter each was tested (i) for skin reactivity in guinea pigs by injecting a volume of 0.1 ml. intracutaneously, and (ii) for specific antigen content by the agar gel diffusion technique (Elek, 1948; Ouchterlony, 1948).

Heat treatment. A 5-ml. sample of each of the above skin toxins was heated at 60°C for 30 min. and then tested similarly for skin reactivity in guinea pigs and for the production of precipitin bands in the agar gel diffusion test. The tests and the results are summarized in table 40. The precipitin bands obtained in agar gel diffusion tests are also shown in figs 26 and 27.

Table 40.

The results of skin reactions following intradermal inoculation of heated or formalin-treated skin toxin of V. cholerae

Material injected intracutaneously in guinea pigs	Mean diameter of skin lesion produced/0.1 ml. of the inoculum (mm.)
Formalin-treated and dialyzed skin toxin of V. cholerae strain 12r	
: held at room temperature	Nil
" " 37° C	Nil
" " 4° C	6.0
The same toxin heated at 60°C for 30 min.	Nil
The same toxin unheated	13.0
Formalin-treated and dialyzed skin toxin of V. cholerae strain 569B.	
: held at room temperature	8.5
" " 37° C	8.0
" " 4° C	11.0
The same toxin heated at 60° C for 30 min.	Nil
The same toxin unheated	16.9

It is evident from the results that the formalin-treated 12r skin toxin, held at room temperature or at 37°C produced no skin reaction when injected into guinea pigs, but in the agar gel diffusion test it gave precipitin bands against antitoxic serum obtained by immunization of rabbit with 12r toxin (see fig. 28). Similar results were noted in rabbits and in agar gel diffusion tests when the toxin was heated at 60°C for 30 min. (See figs 26 and 27).

The results suggested that the skin toxin 12r was rendered non-toxic either by heating or when treated with formalin at room temperature or at 37°C incubation. Conversion was not complete at any stage of the formalin treatment of 569B and 12r toxins held at 4°C. But like the 12r toxin, the 569B toxin is found to be converted on heating at 60°C for 30 min. as is evident from the results of the skin and agar gel diffusion tests (See fig 27).

It is now necessary to use the non-toxic product to produce serum which is capable of neutralizing fresh skin toxin before we can say that we have toxoided the original without inactivating its specific skin toxin antigen.

Fig. 26

Double diffusion gel precipitation bands produced by skin toxin 12r heated at 60°C for 30 min. and also by the same toxin unheated.

Central well	=	Antitoxic serum against skin toxin 12r.
Well No. 1	=	Skin toxin 12r heated at 60°C for 30 min.
Well No. 2	=	Skin toxin 12r unheated.
Well No. 3	=	Same as Well No. 1.
Well No. 4	=	Same as Well No. 2.
Well No. 5	=	Same as Well No. 1.
Well No. 6	=	Same as Well No. 2.

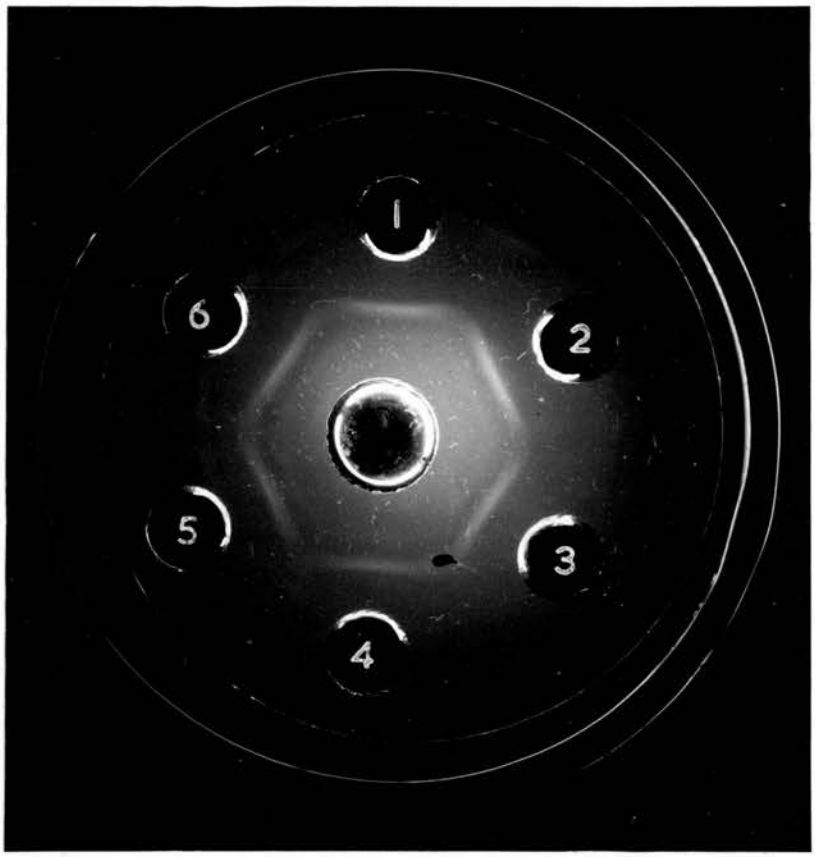

1
2
3
4
5
6

Fig. 27.

Double diffusion gel precipitation bands produced by skin toxin 569B heated at 60°C for 30 min. and also by the same toxin unheated.

Central well = Antitoxic serum against skin toxin 569B.

Well No. 1 = Skin toxin 569B heated at 60°C. for 30 min.

Well No. 2 = Skin toxin 569B unheated.

Well No. 3 = Same as Well No. 1.

Well No. 4 = Same as Well No. 2.

Well No. 5 = Same as Well No. 1.

Well No. 6 = Same as Well No. 2.


6
1
5
2
4
3

Fig. 28.

Double diffusion gel precipitation bands produced by formalin-treated skin toxin of V. cholerae

Central well = Antitoxic serum against skin toxin, 12r.

Well No. 1 = Formalin-treated skin toxin 12r and held at room temperature for 7 days.

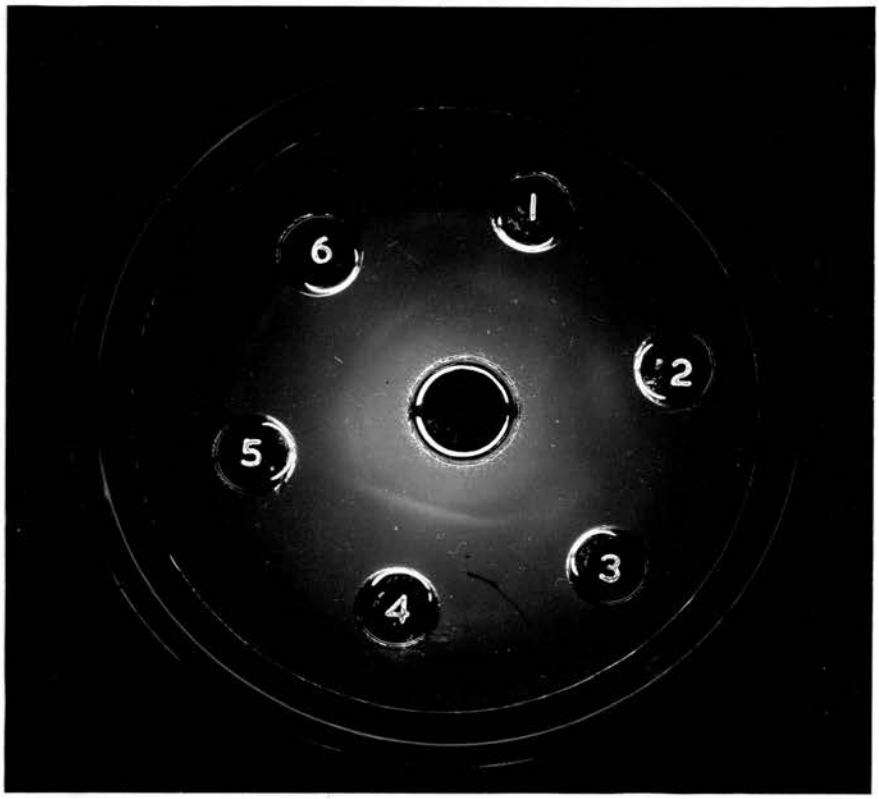
Well No. 2 = Formalin-treated skin toxin 12r and incubated at 37°C for 7 days.

Well No. 3 = Formalin-treated skin toxin 12r and held at 4°C for 7 days.

Well No. 4 = Formalin-treated skin toxin 569B and held at 4°C for 7 days.

Well No. 5 = Formalin treated skin toxin 569B and incubated at 37°C for 7 days.

Well No. 6 = Formalin treated skin toxin 569B and held at room temperature for 7 days.


1
2
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6

Comparative study of the mucosal morphology in cholera toxin-induced positive loop lesions and in uninoculated normal ileal loops in rabbits.

Normal loop.

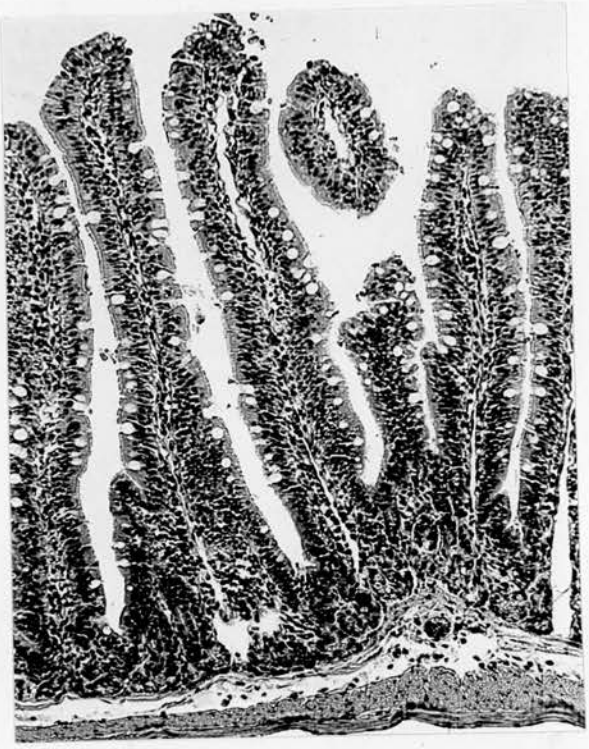
In a control specimen the mucosa of the lower ileum is found to be covered with tall finger-shaped villi of variable heights (Fig. 29a). The surfaces of the villi are covered with 2 types of cells, the absorbing cells and the goblet cells. As the area of intestine chosen for this study is the lower part of the small intestine (ileum), the goblet cells are here more numerous than in the upper part of the small intestine. The absorbing cells which cover the epithelium have a wavy refractile brush border. The bristles of this border are the microvilli of the epithelial cells, a feature recognized under the electron microscope. In a normal specimen of mucosa, the goblet cells appear full. The core of each villus is occupied by a central lacteal which is surrounded by blood vessels, nerves, and various blood cells, e.g. mononuclear cells - lymphocytes and occasionally plasma cells, occasionally eosinophils, very rarely a polymorph, and some RBC's. The crypts of Lieberkühn are situated between the villi at their bases. The mucosa is bounded by the muscularis mucosae. The submucosal blood vessels and nerves are distributed in the loose areolar tissue of the submucosa and this separates the mucosa from the serosal musculature.

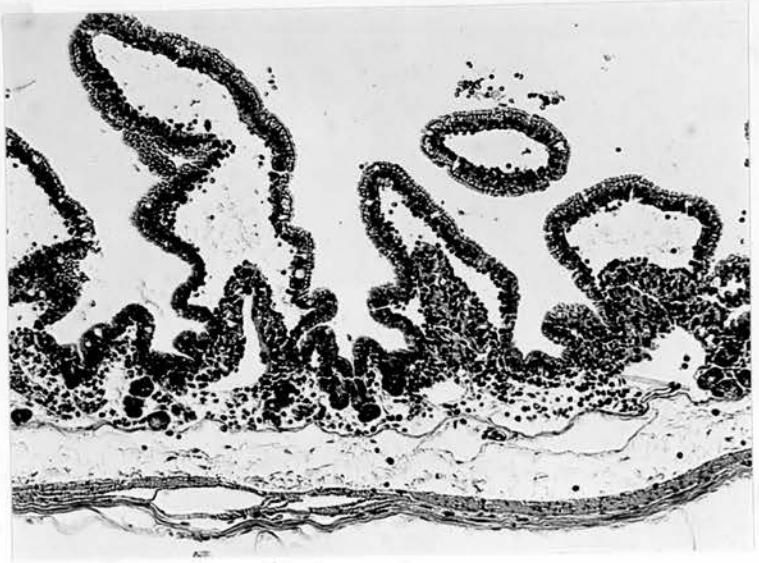
Toxin-induced positive loop.

The toxin has produced a remarkable effect on the mucosa. The villi are shorter in height. One of the most prominent features is the dilatation of the central lacteal (Fig.29b). The goblet cells appear rather small and empty. The brush border is irregular. The height of the epithelial cells is reduced in comparison with the height of the epithelial cells of the control specimen. It is difficult to determine the extent to which this morphological change of the epithelial cells is secondary to the dilation of the central lacteal. Submucosal oedema is another feature of the positive loop (Fig. 30). The blood vessels in the submucosa appear dilated (Fig. 29b). The subserosal muscle layer is thinner compared with that of the control specimen but it is difficult to say how much this apparant thinning of the muscle is secondary to submucosal oedema.

Fig. 29a. A Section of distal small intestinal mucosa obtained from a control loop. X120

Fig. 29b. The mucosa of a positive loop of ileum 12 hr after injection of cholera toxin. The villi are shorter. The stroma of the Villi shows grossly dilated areas. X120





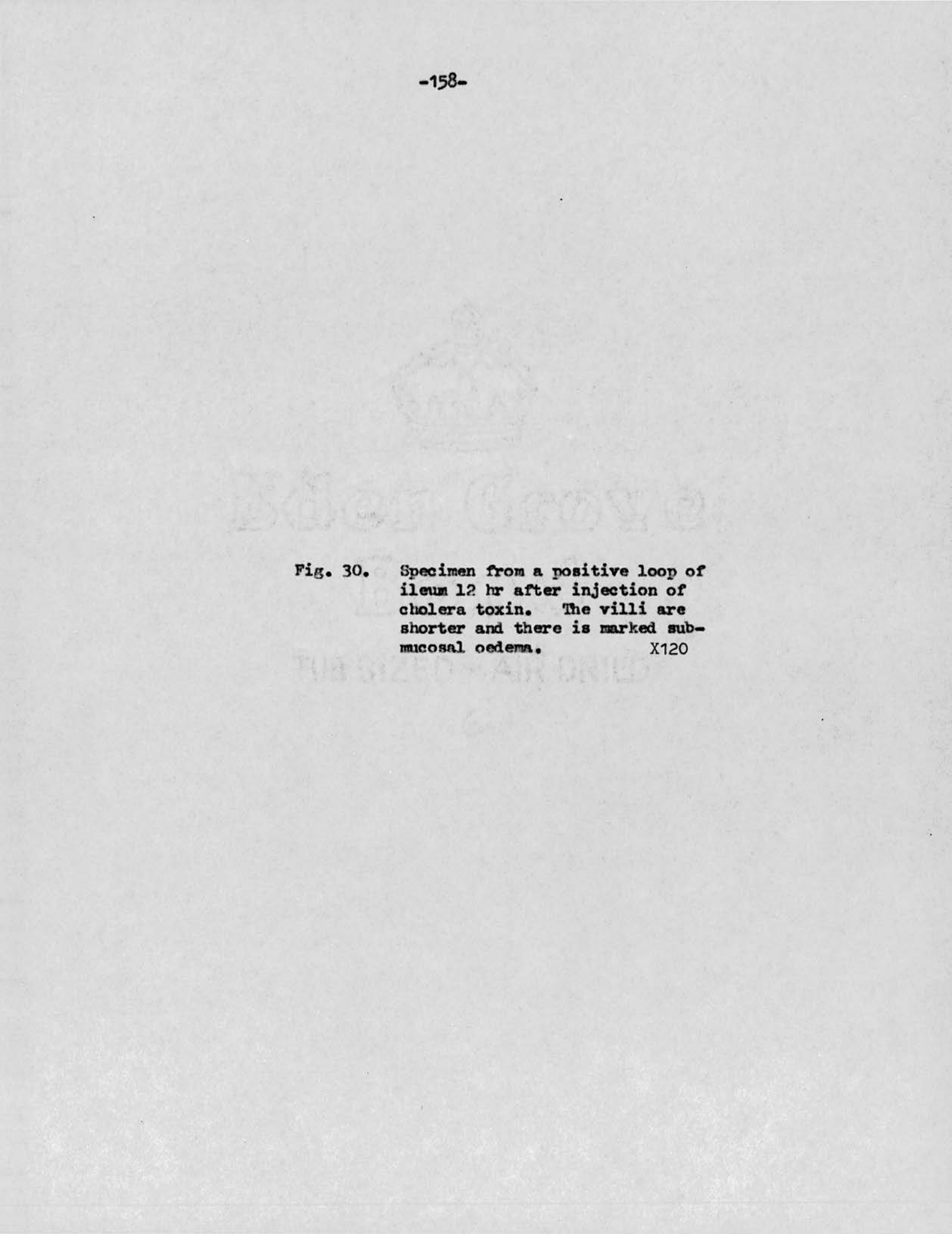
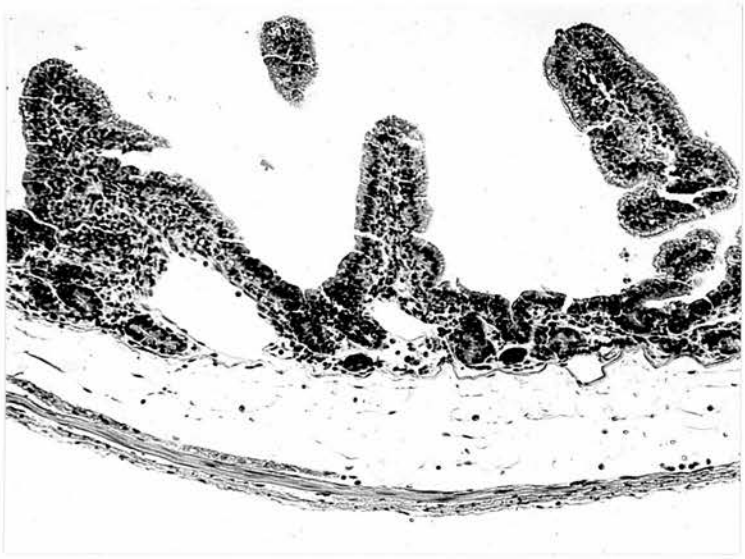


Fig. 30. Specimen from a positive loop of ileum 12 hr after injection of cholera toxin. The villi are shorter and there is marked submucosal oedema. X120



Discussion.

DISCUSSION

The need for definition.

There is much confusion in the literature regarding cholera. The word cholera has denoted distinct entities at different times in history and in different parts of the world. European physicians made the diagnosis in many forms of acute diarrhoeas occurring sporadically or in limited outbreaks and coined the names simple, sporadic and benign cholera; European cholera; English cholera; cholera nostras and cholera infantum. Outbreaks of cholera were reported in Nimes, in London and in Vienna in 1654, 1664 and 1676 respectively; these as well as the more severe outbreaks of diarrhoeal disease in London during 1679-82 described as cholera by Sydenham, probably included outbreaks of gastroenteritis caused by other organisms (Macnamara, 1876).

Asiatic cholera (syn: epidemic, pandemic, malignant, blue or algid cholera; cholera orientalis; cholera morbus) was first brought to the attention of Europeans by Portuguese seafarers returning from India. It has been pointed out by Macnamara that British physicians working in eastern India did not record the disease until the late 19th century and the severe epidemic of 1817 in Bengal was thought to be a new disease.

The correct definition of cholera is still disputed. The British Medical Dictionary (1961) defines cholera as "a severe infectious epidemic disease due to Vibrio cholerae characterized

clinically by violent purgation, vomiting and collapse". Cholera is regarded by Webster's International Dictionary (1961) as "any of several diseases affecting man and domestic animals usually marked by severe gastrointestinal symptoms". From 1961 to date, although classical cholera caused by V. cholerae continues to be almost exclusively limited to the Indo-Pakistan sub-continent, cholera El Tor caused by V. eltor with its increased potential for survival both inside and outside the human body has rapidly spread over wide territories. Cholera El Tor was originally restricted to a limited geographical area in Sulawesi Island in Indonesia. But since 1961 it has come out of its original home and the disease has spread extensively from Indonesia to Hong Kong, and to the Philippines in 1961; to Taiwan in 1962; to Burma and Indo China in 1963; to India, Pakistan and south-east Asia in 1964; and to Nepal, Afghanistan, Iran and Uzbekistan in 1965. A new factor has thus altered the scene of the world incidence of cholera.

The cause of this rapid extension has been reviewed recently (Brit. med. J., 1966). "Present studies point to the fact that the areas hitherto invaded by cholera continue to be potential foci of infection for a long time because of the presence of carriers, and with rapid means of transport the danger of the spread of cholera is great. For many years there has been a tendency to overlook the role of carriers in transmission of cholera and to accept without criticism reports on the apparent effectiveness of vaccines ..... The carrier state in cholera El Tor may last three years, and the vibrio can establish itself in the gall-bladder. Even after apparently successful treatment of

convalescent carriers with antibiotics in some cases after purging or during spontaneous diarrhoea from other causes, vibrios may be excreted. The detection and clearance of carriers is difficult and the use of antibiotics may prolong excretion of vibrios".

The role of NAG vibrios in the incidence of cholera has been described in the writer's discussion of the phage section of this thesis (see p. 326).

Dehydration and re-hydration in cholera.

When true cholera occurs, the disease usually begins with copious diarrhoea and vomiting. The speed of evacuations may be so rapid that in an hour or two a stool pan is filled and it scarcely seems possible that the body fluids are sufficient to supply the amount of fluid. The typical stool in cholera is alkaline in reaction and has a rice water appearance, being completely devoid of any biliary colouring or of any faecal matter. The vomiting starts soon after purging. It is often profuse but never so persistent as purging. Agonizing cramps attack the muscles of the limbs and the abdomen. The implicated muscles show rigid contractions as a result of depletion of chlorides and hypocalcaemia affecting neuro-muscular junctions. The patient may rapidly pass into a state of collapse. In consequence principally because of loss of fluid by the diarrhoea and vomiting, the soft parts shrink, the cheeks fall in, the eyes appear sunken with lids half open and the eyes turned up; the nose becomes pinched and thin, the lips are

cyanosed, the fingers become wrinkled and assume the appearance of what has been described as "washer-woman's fingers". The skin is cold, clammy and inelastic and the voice is whispering. The urine is suppressed; respiration is rapid and shallow; the pulse soon becomes thready, weak and rapid and finally may disappear entirely. The surface temperature falls several degrees below normal to 94 or 93°F, whilst the rectal temperature may be several degrees above normal (101 to 105°F). The blood pressure is low. The patient becomes restless, tossing about uneasily, feebly complaining of intense thirst. Although apathetic, the mind generally remains clear; sometimes the patient may pass into a comatose state. This is the "algid stage" of cholera and it may terminate in one of 3 ways - in death, in rapid convalescence or in a febrile reaction. Cholera is basically a self-limiting disease in which all symptoms appear to be due simply to loss of water and electrolyte from the gastro-intestinal tract (Phillips, 1963). For better understanding of the pathophysiological changes and for a more prompt and exact replacement of salt and water losses, studies were carried out by Carpenter (1964) in selected patients with acute, bacteriologically-proven V. cholerae infection. These studies indicate that the gastro-intestinal losses in cholera are consistently isotonic and the isotonicity of the plasma is nowadays maintained by the continued intravenous administration of isotonic fluids in a volume equal to stool losses during the course of a patient's illness.

The observation of a high concentration of bicarbonate in the cholera stool, even when the plasma carbon dioxide combining power is greatly reduced, emphasizes that acidosis poses a tremendous threat to the life of the cholera patient. As the body bicarbonate stores are small in relation to the faecal bicarbonate losses, it is obvious that its replacement is absolutely essential in the course of the treatment of the cholera patient. The potassium loss is not so great relative to total body stores and the necessity for its replenishment is less clearly defined.

The complications of pulmonary oedema, frequently reported in cholera patients, may result from (i) poor myocardial function secondary to severe metabolic acidosis or (ii) over-hydration caused by continued administration of intravenous fluids following cessation of diarrhoea in patients with superimposed acute renal failure which is another equally frequent complication of cholera. Prompt correction of shock followed by continued infusion of intravenous fluid in quantities equal to that lost via the gastro-intestinal tract will prevent the classical picture of acute renal failure in cholera patients. Thus success in the treatment of the disease has come with improved methods of re-hydration of the ill person, but we are still ignorant of the real mechanism of pathogenicity. Specific therapy must wait until we understand more of the pathogenesis. Meanwhile, it is of interest that there are similar features in Esch. coli gastroenteritis in children (cholera infantum) and diarrhoeal disease in calves. Research in these diseases may give clues of value in elucidating the underlying mechanisms that operate in cholera.

Related syndromes: (i) "Cholera infantum".

Several serological types of Esch. coli, e.g. O 26, O 55, O 111, O 119 and O 28, have been associated with epidemics of diarrhoea and gastro-enteritis affecting very young children. The disease is chiefly met with in the summer months of the year and may be attended by "mild catarrhal and atrophic changes" in the mucous membrane of the small bowel which are minor changes in contrast to the severity of the symptoms. In a severe attack there are watery stools which are foul-smelling, of yellowish colour (later greenish) and acid. They are usually frequent and they tend to be passed explosively. Vomiting occurs with acute abdominal pain and tenesmus; prostration and collapse follow and there is subnormal temperature. Rapid dehydration and wasting are seen and death may supervene after a course of a week or so, or earlier. Occasionally an infant may exhibit signs of shock and die as a result of loss of water and electrolyte into the intestinal lumen before a diarrhoea stool is passed.

(ii) "Scours"in calves.

Scours is an enteric form of colibacillosis seen in animals and caused by pathogenic Esch. coli. The disease is most common in calves but may occur in other species. It occurs chiefly during the first 3 weeks of life and particularly during the first week. The sterile alimentary tract of the calf becomes contaminated with the organism at its first feeding. It is postulated that if the antibody in the colostrum is not sufficient to inactivate the bacteria,

the latter multiply rapidly in the lumen of the gut and produce potent endotoxins. These are then absorbed from the gut and give rise to the toxic symptoms characterized by watery or pastey faeces, usually chalk-white to yellow in colour and occasionally streaked with blood. Defaecation is frequent. The faeces have an offensive, rancid smell. There is usually a systemic reaction with a temperature up to 105°F and an increase in pulse rate. The animal ceases to drink, is dull and listless and rapidly becomes dehydrated. There may be abdominal pain elicited on palpation. Sometimes tenesmus is evident and the back of the affected animal may be arched.

Animal experiments in cholera research.

Experiments designed to reproduce choleraic symptoms in animals were undertaken many years after the work of Snow (1849) who described the role of cholera evacuations in the spread of the disease. Numerous experiments with cholera stools on various animals gave contradictory results. Koch (1884) used cultures of his comma bacillus on a large variety of animals. He observed pathogenic effects only in mice which died in the course of 24-48 hr after intraperitoneal inoculation with the cultures. Rabbits inoculated intravenously with the cultures became ill but they subsequently recovered. Koch concluded "I think all the animals on which we can make experiments, and all those, too, which come in contact with human beings, are not liable to cholera, and that a real cholera process cannot be reproduced in them . . . . . Should it prove possible later on to produce anything similar to cholera in animals,

that would not, for me, prove anything more than the facts which we now have before us".

Pfeiffer (1894) observed peritonitis and death in guinea pigs following intraperitoneal inoculation of the comma bacillus. No diarrhoea was noticed and the organisms were rarely seen in the intestinal epithelium. Griffitts (1942) also found no evidence of diarrhoea or loss of fluid in his animals. Many other experimental methods tried so far without consistent success have been reviewed by Pollitzer (1959).

De and Chatterje (1953) devised an experiment with adult rabbits in which a small loop of small intestine was isolated from the rest of the gut by 2 ligatures. When 1 ml. of peptone water culture of V. cholerae was injected into the lumen of this loop, a large volume of fluid accumulated in it within 24 hr. This fluid had the gross and microscopic and cultural appearances of cholera stool. Congestion and oedema of the wall of the loop and necrosis of the summits of the villi were also noted. The development of the rabbit loop model has been reviewed in detail in the Introduction to this thesis (see p. 29). The use of the model has provided a stimulus for the re-investigation of the local effects of cholera on the dynamics of intestinal absorption. Some workers object to the use of the rabbit intestinal loop. They regard the obstructive component as unphysiological and the "positive-loop" reaction is not specific for V. cholerae. For such reasons, the baby rabbit model is often preferred.

Metchnikoff (1894) tried to infect newborn animals and found that about half of his 1-4 day old rabbits died of typical cholera-like diarrhoea when infected intraorally with a virulent cholera vibrio strain, whereas 20 out of 22 baby rabbits receiving vibrio mixed with cultures of Micrococcus, an Esch. coli strain, or a yeast, died after 36-48 hr. Schoffer (1894-95) found that the addition of a symbiotic microbe with the infecting dose of vibrio was of no advantage. Issaeff and Kolle (cited by Pollitzer, 1959) also produced manifest intestinal cholera in young rabbits. The disappearance after 6 hr of virulent vibrios placed in the mouth of baby rabbits was observed by Sanarelli (1921). The contents of the stomach were shown to kill the vibrios in a few minutes both *in vitro* and *in vivo*. This was presumed to be due to the gastric hydrochloric acid together with the fatty acid liberated from the milk. Solarino (1939) was able to produce diarrhoea in baby rabbits by placing cholera cultures in the mouth after the oesophagus of each animal had been divided and ligatured at both ends. The animals were thus infected indirectly presumably via the lymphatics. Dutta and Habbu (1955) used rabbit-passaged V. cholerae strain 569B to infect baby rabbits. This strain produced consistent choleraic diarrhoea on injecting the organism (10^4 colony-forming units) into the small intestine. The diarrhoea began 16-44 hr after inoculation and the animal usually died 10 hr later. On autopsy, some fluid was noted in the terminal ileum and a large volume was observed in the colon.

Inconstant congestion of the ileum and loss of epithelium at places were also noticed. Blood collected just before death showed definite haemoconcentration.

Research on cholera during the last 20 years appears to fall into 2 chronological stages. During the first decade modern investigations were stimulated particularly by the Egyptian epidemic of 1947 and the work has cleared the ground for further advances by destroying some ingrained notions and by defining the problems. Thereafter a beginning has been made in tackling the problems. The re-popularization of the rabbit loop model in 1953 and the baby rabbit model in 1955 re-introduced essential tools for reproducing a cholera-like disease in the laboratory.

The elusive toxic factor.

Some authorities consider that V. cholerae causes its effects by endotoxin and does not apparently produce any exotoxin. The endotoxin results from the destruction of vibrios within the bowel lumen. It is thought that this is a phospholipid that can increase intestinal permeability without producing any detectable changes in the mucosa. Although V. cholerae is now known to produce various exoproteins (see p. 14), it is true that in cholera there is no obvious toxaemia. The term toxin has, however, been widely used to denote any soluble substance liberated from the vibrio cells or formed outside the cells that can manifest adverse biological activity in the natural host or in an experimental system. The factor that

acts on the intestine to produce diarrhoea is often called enterotoxin and the term "skin toxin" has been applied to the toxin that acts on the skin (see p.28).

Although pathologists of the last century (Cohnheim, 1889) believed that there must be an enterotoxin acting on the host in cholera, all attempts to demonstrate such a toxin were unsuccessful until recently (Pollitzer, 1959). The evidence obtained by De (1959) in work with rabbit loops and Panse and Dutta (1961) in baby rabbits indicating the presence of a heat-labile enterotoxin in filtrates of cultures of V. cholerae and cholera stools has been reviewed in the Introduction to this thesis. The presence of a heat-labile skin toxin in cholera stools and V. cholerae culture filtrates as observed by Craig (1965) has also been reviewed in the Introduction. In the last 2 years several papers have appeared confirming the occurrence of enterotoxin in various preparations. The properties of the enterotoxins described by different workers are discrepant. Presenting the results of a collaborative study comparing cholera toxins, Burrows (1965) concluded that there are 3 kinds of toxic acitivity present:

(i) The toxin that is lethal for the mouse and the 10-day chick embryo;

(ii) The toxic activity showing intradermal reactivity, giving a toxic reaction in infant rabbits and in ligated bowel loops, and producing a cytopathic effect on Earle's L cells in culture;

(iii) The factor that shows toxicity for anurian epithelium and inhibits p-aminohippurate (PAH) concentration by kidney slices *in vitro*.

Burrows' data, supplemented by the more detailed information furnished by his collaborators, are summarized in the following table (table 41).

Table 41 - please see next page.

There are some differences between the views of Burrows and those of his collaborators. There are also some discrepancies between the text and his table showing the action of the groups of toxins. For example, he described cell wall (CW) as cytopathogenic but Read (1965) tabulated the effect as negative. Similarly the frog skin reaction was described as "readily reproducible" (Burrows *et al.*, 1965) but Leitch (1965) stated that "in our hands the effects of the cholera toxins on the anurian abdominal skin and bladderhave

Table 41.

The action of toxins of V. cholerae strain 569B on 8 experimental models. The data are summarised for comparative purposes from the collaborative study of Burrows (1965), Burrows et al. (1965), Leitch (1965) and Read (1965). The supernate was of centrifuged 18 hr growth in 3 per cent. Bacto-peptone solution at pH 8.0 Whole cell lysate (WCL) was made by ultrasonic lysis of Bacto-peptone-agar growth. Cell wall (CW) and intracellular substance (IC) were separated by shaking agar-grown vibrio suspension with glass beads and depositing CW by centrifugation. The figures in the last 3 columns indicate concentrations per ml.

Model of assay		Dose	Supernate	Whole cell lysate (CWL)	Cell wall (CW)	Intracellular substance (IC)
Toxin 1	Mouse toxicity	LD_{50}	Inactive	2 mg.	0.5 mg D^-HS	5 mg D^-HS
	10-day egg (CAM)	LD_{50}	Trace activity HS (i)	Active HS	70 µg D^-HS	130 µg D^-HS
Toxin 2	Rabbit skin	MRD	150 µg	0.01 µg D^-HL	1.4 ug D^-HL (i)	0.0025 µg D^-HL
	Infant rabbit	MLD	500 µg	250 mg D^-HL	Inactive	25 mg D^-
	Ligated loop of rabbit	MRD	Inactive	3 mg D^-HS	Inactive	1 mg D^-HL
	L cell cultures	0.5-1 mg/ml.	Inactive	Weak HS(ii)	Inactive (i)	1 mg D^-
Toxin 3	Anurian epithelium	RD_{50}	1 mg D^+	6 mg D^+	5 mg D^+HS	1.5 mg D^+HS
	Kidney PAH	RD_{50}	Not tested	Active	10 mg D^+	5 mg

HS/HL = Heat stable/Heat labile. D^+/D^- = Dialyzable/nondialyzable. (i) = for discrepant recordings by the authors, see text.

(ii) = unheated not tested.

not been very reproducible from one lot of prepared toxin to the next."

CW was depicted by Burrows as inactive on rabbit skin but an MRD of 1.4 µg. was recorded. Similarly, he stated that the toxicity of the supernate for chorioallantoic membrane (CAM) was barely detectable, yet he tabulated it as "(+)"; this reaction was presumably stronger than his "(±)". He did not explain why toxin 2 in WCL was heat-labile when tested in the skin test and baby rabbits but heat-stable when tested in loops and epithelial cell cultures, although he suggested that there may also be a toxin secreted extracellularly that acts on the skin and baby rabbits only.

A heuristic hypothesis was suggested by Ghosh (1965) to explain all of these discrepancies. He suggested that toxin 1 of Burrows should be called an endotoxin for which both mice and eggs are known to be highly susceptible. Ghosh postulated that toxin 2 comprised 2 components. One was choleragen and the other, though not diarrhoeagenic, was normally associated with the choleragen. The choleragen itself was heat-labile, non dialyzable, sensitive to acid and formalin, insoluble in chloroform, and precipitated by sulphates of ammonia and zinc, and thus probably a protein. The other factor was heat-stable, dialyzable, acid-resistant and biologically inactive <u>in vivo</u> or in epithelial cell cultures. It was designated as dialyzable factor (DF). From the dialyzability, heat stability and reaction with enzymes it was

presumed to be a lipid. He thought of choleragen as firmly bound inside the vibrios with DF which was also presumed to protect the choleragen to some extent from the action of acid, heat and other agents. The complex was biologically inert until dissociated *in vivo* or *in vitro* by enzymes or by physical treatment.

Ghosh thought that toxin 3 was the same as DF; their physical characters were similar. His concept of 2 components for toxin 2 was similar to the views of Finkelstein *et al.*(1964) on the procholeragens. However, the DF was considered to be a blocking and stabilizing agent rather than an essential factor for the biological action of choleragen.

Although De (1961) was unable to induce loop lesions by ultrasonic lysates of whole vibrios (WCL), Oza and Dutta (1963) induced diarrhoea in baby rabbits by feeding large amounts of WCL of *V. cholerae* strain 569B. In confirming this, Finkelstein *et al.* (1964) noted that the activity was greater in filtrates of aerated Difco Brain Heart Infusion Broth (BHIB) or a synthetic medium supplemented with 1 per cent. casamino acid (syncase medium). These filtrates were also active in man (Finkelstein, 1965b). Two fractions could be separated by dialysis of WCL and syncase filtrate, but not from cultures grown in BHIB. Procholeragen A is heat-labile (56°C for 30 min.) and nondialyzable; procholeragen B is heat stable and dialyzable. None of these was diarrhoeagenic by itself on feeding to baby rabbits, but a mixture became choleragenic. The procholeragens were not detected in other cholera strains and

El Tor vibrios, although Schafer and Lewis (1965) demonstrated a thermolabile nondialyzable enterotoxin in loop fluids and culture filtrates of several other strains of V. cholerae. The enterotoxin was precipitated with ammonium sulphate and zinc sulphate and it was inactivated by formalin.

The present study.

The present investigation has included an intensive study of apparently toxic products of V. cholerae that we have identified as skin toxin and enterotoxin on the basis of their cutaneous and intestinal activities respectively. While it is useful to use these terms, they may be misleading as the preparations have been crude and more than one factor may be involved in each case. The writer's provisional definitions of skin toxin(s) and enterotoxin(s) of V. cholerae are therefore as follows:

Skin toxin(s). (A) Skin-reactive substance produced in vitro.

(B) Skin-reactive substance derived from a positive loop fluid produced by inoculation of

(i) Organisms,

(ii) Soluble products produced in vitro,

(iii) Soluble products produced in vivo.

Enterotoxin(s). Substances producing a positive loop in the test rabbit model and derived from

(i) Organisms directly injected into the loop,

(ii) Soluble bacterial products produced in vitro,

(iii) Soluble bacterial products produced in vivo.

For convenience in the present discussion the existence of a single skin toxin and a single enterotoxin is envisaged, but this assumption will require further proof.

(i) Production of skin toxin.

In the present study, production of skin toxin from V. cholerae has been achieved in vitro by culturing the organisms in 5 per cent. Difco Bacto-peptone water, under the same growth conditions as used by De et al. (1962) for the preparation of cholera enterotoxin. Equally good yields were obtained from cultures in 1 per cent. peptone water. Skin toxin and enterotoxin are both produced when there is a large surface/volume ratio and both factors are developed in relatively young cultures. Neither are produced in poorly aerated cultures. The writer has confirmed that production of the skin reactive substance is greater in an aerated culture than in a static culture. The results of intradermal inoculations of filtrates of overnight aerated cultures of strains 12r and 569B in 5 per cent. Difco Bacto-peptone water have been compared with inoculations of the static culture filtrates of these organisms in the same medium and the presence of stronger skin reactive substances in the aerated cultures is clearly evident.

The production of skin toxin in cultures of the vibrio strains was first demonstrable after 4-6 hr incubation and

thereafter the concentration increased gradually with maximal production at 24-48 hr. The presence of skin reactive substances in these preparations was demonstrated by intracutaneous injection in guinea pigs and rabbits. Erythema, induration and a prolonged increase in capillary permeability were observed. Similar skin lesions were also noticed following intracutaneous injection of a filtrate of positive loop fluid. The skin reactions evoked by loop fluid and culture filtrates were compared by determining both dose response and time-course of induration and increasing capillary permeability in parallel tests on the same sets of guinea pigs. The time course of induration activity and the increase in capillary permeability produced by both loop fluid and culture filtrates in the same animals displayed the same sequence of events. Thus the skin responses evoked by loop fluid and culture filtrate suggest that the active principle in the 2 materials or the mechanism of their activity may be the same. We have demonstrated the presence of skin reactive substances in culture filtrates of classical cholera strains, and in culture filtrates of El Tor and NAG vibrios, and enteropathogenic Esch. coli.

(ii) Stability studies.

The effect of heat upon the skin reactivity of enterotoxin and skin toxin of V. cholerae have been compared in a series of experiments conducted in parallel. Both induration-evoking

factor and the capillary permeability factor of both materials are completely destroyed after exposure to heat at 56°C for 30 min. or at 100°C for 2-3 min., but they are stable at 37°C for at least 72 hr. The skin-indurating activities of the skin toxin and enterotoxin were tested over the pH range 4-9 and both materials were stable over this range of pH values. Dialysis of both materials against large volumes of distilled water resulted in no loss of skin reactivity. No difference was observed upon the skin activity on dilution of the toxins in the presence or absence of calcium ions.

The skin-reactive components of both skin toxin and enterotoxin in crude preparations appeared to be stable despite exposure to concentrations of formaldehyde up to 1 per cent. at 4°C; but they were significantly inactivated by exposure to 1.4 per cent. formaldehyde at 4°C, 18°C or 37°C. The toxins were not inactivated when they were oxidized by hydrogen peroxide nor when they were reduced with thioglycollic acid. The toxin retained its full activity after exposure to trypsin and chymotrypsin. It was completely inactivated by treatment with pancreatin. It appeared that lipase itself produced some degree of skin reaction. The toxin-lipase mixtures certainly showed reduced skin toxin activity so that there was partial or complete inactivation of skin toxin by lipase. The effect of phosphorylase was also studied and it was evident that it did not inactivate or enhance the cutaneous effects of skin toxin of V. cholerae.

(iii) Lethality studies.

The skin toxin was found to be relatively non-lethal for the 9-day chick embryo and the lethal effect of crude enterotoxin preparations was doubtful. No cytopathic effects were observed in tissue cell monolayers with HEp2 cells in culture with either of the toxins. The toxins were not lethal to mice on intraperitoneal or intravenous inoculations. The cutaneous effects of the skin toxin and enterotoxin were not inhibited by prior and sustained treatment of the animals with mepyramine maleate. This suggests that the toxin does not act through liberation of histamine. Both of the crude toxin preparations were found to haemolyse 1 per cent. sheep red cells. (but see p. 189).

(iv) Serological studies.

Antitoxic sera produced by immunizing rabbits with skin toxin were found to neutralize the cutaneous effects of homologous skin toxin and enterotoxin (loop fluid) completely. Inactivation of the skin toxin component of loop fluid by sera prepared against skin toxin derived from culture *in vitro* is further evidence that we may be dealing with the same factor in both of these materials. The normal sera of the animals taken before immunization were found to have no effect on these toxins. It was also observed that homologous anti-

bacterial serum having an agglutinating titre of 1 in 8,000 had no effect on the cutaneous activities of either of the toxins when the toxin-serum mixtures were tested on the skin of guinea pigs. This shows that the antibody against the skin toxin does not appear to be evoked by somatic vibrio antigens capable of evoking agglutinins. It was also observed that antitoxic serum prepared against one crude skin toxin preparation is equally effective in neutralizing skin toxin prepared from a heterologous strain. Further, by means of the agar well double-diffusion precipitation technique, it was possible to show that although the potent anti-bacterial serum produced a precipitate with antigen contained in the crude skin toxin, the precipitation band so obtained was not identical with the band produced between the toxin and the homologous antitoxic serum.

Agar gel immuno-diffusion reactions with a particular skin toxin and its homologous antitoxin, and against heterologous antitoxin prepared from a different skin toxin gave both bands of identity and non-identity, indicating serological overlapping between these antitoxic sera. Results of both animal experiments and agar gel diffusion studies suggested that the skin toxin may be converted to toxoid either by heating or when treated with formalin. These observations may find some application in the preparation of a harmless immunogen against the toxin which may prove beneficial; but it is necessary to use the toxoid to produce serum which is capable of neutralizing fresh skin toxin before we can say that we have

toxoided the original toxin without inactivating it. Limitations of time have precluded this final important step in the present study and it is intended to develop this aspect of the work as soon as possible.

Identification of skin toxin and enterotoxin by agar gel diffusion studies with homologous antitoxic serum resulted in the development of precipitin bands only with skin toxin; no such precipitin bands were observed with loop fluid (enterotoxin) although this was of comparable potency in producing unequivocal cutaneous effects. This was first thought to be a result of the presence of some inhibitory factor that prevented the enterotoxin from forming a precipitin band in double diffusion plates but results of further experiments excluded the presence of such an inhibitory factor. It was confirmed that the culture medium that was used for production of skin toxin does not produce any precipitin bands with the serum of a rabbit immunized with skin toxin. It was also observed that the normal sera of rabbits obtained before immunization with skin toxin do not produce precipitin bands. The precipitin bands that appeared between the skin toxin and antitoxin were thus apparently caused by a specific toxin-antitoxin reaction. As the enterotoxin does not give a precipitin band with homologous antitoxic serum whereas its cutaneous activity is completely neutralized by the same serum, it follows either that (i) skin toxin is different from enterotoxin

or (ii) the precipitin band is irrelevant and is not produced by the toxic component.

These findings are of importance but it should be borne in mind that immuno-diffusion in petri dishes does not produce a high degree of resolution. These developmental studies must now be repeated and expanded with the help of more critical procedures.

Adsorption studies with skin toxin showed that if some of the toxin was indeed adsorbed to activated charcoal the adsorbed toxin could not be eluted, but the findings did not exclude other possible reasons for the partial inactivation of skin toxin following exposure to charcoal. Finkelstein (1965) also observed that treatment of syncase choleragen with Kaolin or with activated charcoal removed the choleragenic activity. In the former case the endotoxin activity remained and could be assayed in the 11-day chick embryo, whereas charcoal adsorbed (or inactivated) both the choleragenicity and the endotoxin. When the supernates of the charcoal-treated and Kaolin-treated choleragens were combined, the choleragenic activity was not restored. These observations are of preliminary use in planning the further purification of the active materials.

It should be noted that the present work has demonstrated similarities between the skin-reactive components of skin toxin produced in vitro and loop fluid produced in vivo ("enterotoxin"). It is now necessary to compare and contrast these substances with respect to their choleragenic activities in experimental loops.

(v) Identification of the toxic factor.

Gallut (1965) pointed out that the antigenic structure of vibrios is complex and he was able to locate various antigens, demonstrable by classical serological methods, in cell wall, cytoplasm, cytoplasmic membrane and flagellum. He found difficulty in assigning a precise location to cholera "toxin" because a great number of preparative methods yield different products; moreover, most authors maintain that more than one toxic fraction exists in cholera toxin. Gallut noticed that cholera toxin prepared by autolysis of vibrios in saline and glucose medium is the same as that induced by the solubilization of vibrio cell walls. He therefore suggested that the toxin is located very superficially in V. cholerae. In support of this he pointed out that vibrios deprived of their cell walls, then washed and sterilized, are markedly less toxic than are intact vibrios of the same sample taken before autolysis and sterilized in the same way. These facts indicate that he was dealing with endotoxin.

In the present work, the skin reactive substances were present in the culture filtrate of V. cholerae as early as 4-6 hr following inoculation of a 5 per cent. peptone medium with the organism. As the skin toxic activity is demonstrable during the logarithmic period of growth it is more likely to be primarily a product of growth rather than a product of autolysis. Moreover, the soluble product was non-toxic to 11-day

chick embryos which are highly sensitive to endotoxic activity. The crude skin toxin preparation was also non-lethal to mice and it was strongly antigenic. These observations suggest that we are dealing with a soluble antigen that is probably an exoprotein and not endotoxin. However, further study is necessary to analyse the antigenic structure of the soluble products of V. cholerae and to characterize the skin toxin more precisely. In the present work the gel precipitation diffusion tests showed the existence of distinct precipitation lines that indicated the presence of several precipitating somatic antigens in the crude soluble products of V. cholerae used for the preparation of antitoxic sera. This is not unexpected because V. cholerae autolyses readily and somatic antigens will be present in the crude preparation of soluble products that was used for immunization of the animals.

The skin toxin and loop fluid (enterotoxin) which contains a component that is capable of producing skin lesions are both capable of producing positive loop lesions in normal rabbits. Both antibacterial and antitoxic sera were found to prevent the production of positive loop lesions in rabbits when the homologous vibrio strains, treated in vitro with either of these sera, were injected into the lumen of a loop preparation in a normal rabbit. This was not unexpected because, like the antibacterial serum, the antitoxic serum is also capable of agglutinating the homologous vibrio strains. The vibrio cells

agglutinated by either of these sera were presumably unable to multiply when injected into the lumen of the loop so that no toxin was produced to give rise to a positive loop.

The present work demonstrated that antitoxic serum is not only capable of neutralizing skin toxin derived from the homologous as well as heterologous strains but it is also capable of agglutinating both homologous and heterologous vibrio cells. On the other hand the antibacterial serum did not neutralize skin toxin. Thus the observation of Craig (1965) that his dermotoxin was not neutralized by antibacterial serum is confirmed.

The writer was also able to show that neither the skin toxin nor the living vibrio culture was able to produce positive loop lesions in rabbits that had been immunized with skin toxin. This may be because sufficient antibody was present in or near the lumen of the gut of the immunized rabbit to neutralize or agglutinate the challenge doses of toxin or the living vibrio cells respectively.

Ghosh (1965) was unable to produce positive loops by injecting living vibrio cells into loop preparations in rabbits that had been immunized with V. cholerae. This probably indicated either (i) that the antibacterial antibody present in the lumen of these vaccinated rabbits prevented the multiplication of the vibrio cells inoculated into the lumen of the gut and thus toxin could not be produced to give rise to positive loop lesions, or (ii) antitoxic antibody interfered

with the enterotoxic effect. The antigen used by Ghosh to produce antibacterial sera was saline suspension of live vibrios. The primary factor in the pathogenicity of V. cholerae appears to be the multiplication of the organism in the gut. There may then be released a toxin that causes the disease manifestations. From this it appears that to prevent the disease one would require only the presence of antibacterial antibodies to prevent infection; antitoxic antibodies would be unnecessary because no toxin would be produced if the vibrios are unable to multiply.

But it is the writer's experience and it was also mentioned by Felsenfeld (1965) that in cholera wards many of the beds are occupied by patients who have been recently vaccinated shortly before their cholera attack. Collee (personal communication) has pointed out that veterinary workers are at present exploring the possibility that failure of the usual protein-absorbing mechanism of new-born calves may render these animals particularly vulnerable to septicaemic disease and diarrhoea associated with Esch. coli. Unexplained failure of protein absorption may cause an antibody deficiency state to arise in the calf under natural conditions quite independently of deficiencies attributable to bad husbandry or absence of antibodies in ingested colostrum (see Penhale, 1965, p. 280). There may be a parallel between this observation and the fact that many of the victims of cholera are severely under-

nourished. However, Rosenberg et al.(1965), following extensive nutritional studies in cholera in East Pakistan, observed that "Although the hypothesis that dietary deficiency may predispose to cholera is suggested by the prevalence of malnutrition in foci of endemic cholera, (his) investigations provide no support for a causal relationship." The writer's personal observations in East Pakistan also suggest that dietary factors are not of primary importance in determining susceptibility to cholera.

From our experimental observations and from the work of others we now have evidence that a toxic factor is indeed produced by the vibrio growing in the intestine of the experimental animal or man or in appropriate broth cultures. This toxic factor occurs in preparations that produce profound changes in certain animal models and these changes mimic to a degree the physiological derangements of cholera. Benyajati (1965) demonstrated that administration of sterile syncase choleragen to human volunteers by means of a catheter and Crosby capsule into the small intestine elicited a diarrhoeal condition indistinguishable from naturally acquired cholera. The patients who manifested severe choleraic diarrhoea responded well to simple fluid and electrolyte replacement therapy, and did not exhibit any subsequent untoward effects or toxaemic manifestations. The observation that choleraic diarrhoea can be produced experimentally in animals and in man

by means of preformed toxins, leads to the speculation that perhaps natural food-borne "intoxication" may account for some bacteriologically-negative clinical cases of cholera.

Non-vibrio cholera-like diseases have been described by the workers in both Dacca and Calcutta. It will be unwise to assume that such diseases are unrelated to V. cholerae - especially as we know that certain cholera-genic toxins can produce physiological changes in the absence of the vibrio. It is thus possible that non-vibrio cholera-like disease may result from the ingestion of preformed toxin as in the case of staphylococcal food poisoning or botulism. The occurrence of cholera with equal frequency in many series among vaccinated and unvaccinated persons may be explained on the basis that a bacterial vaccine does not necessarily afford protection against one of the soluble products of the organism. Indeed, we are aware of the fact that immunization of animals and man with bacterial vaccines of V. cholerae does not yield neutralizing antibodies to the Craig skin toxin. However, such antibodies develop in patients on recovery from clinical cholera and also in animals immunized with soluble products of V. cholerae. This seems to suggest that the skin toxin is truly produced in the natural disease and it therefore seems worthwhile to pursue the antigen.

If one possessed a good concentration of antibody against cholera "toxins" it would not matter much if the vibrio

multiplied in the gut because the toxin would presumably be neutralized at the mucosal surface. This assumes that antibody would be effectively present at that site. There may then be 2 mechanisms by which it is possible to prevent experimental cholera immunologically, viz. by antibody action against the bacteria and by antibody against the toxin. Both antibodies would require to be effectively active in the gut lumen or at the mucosal surface. The heat-lability of the skin-reactive component of either skin toxin or enterotoxin and the neutralization of their toxic activities by antitoxic serum or formalin lead to the suspicion that a protein moiety is important. However, the toxin was not affected by treatment with trypsin, but was inactivated by pancreatin and lipase, implying the possible participation of polysaccharide and lipid components. The possibility that a trypsin-resistant protein or protein complex is involved cannot be dismissed on the evidence so far available. The possible role of the above enzymes in the intestinal tract of man and their relationship to susceptibility to cholera also merits further consideration.

Both the skin toxin and enterotoxin are soluble factors that are demonstrable in crude preparations derived from cultures of V. cholerae or from vibrio-induced rabbit gut loop fluid. They are equally active in producing effects in the skin or in the experimental loop. Studies of the time-course of induration activity and the increase in capillary permeability produced by both loop fluid and culture filtrates in the same

animals revealed the same sequence of events. Both of the toxins are non-lethal to mice and chick embryos and they are also non-toxic to epithelial cells in culture. They are equally inactivated by heat, stable over a wide range of pH, non-dialyzable, partially inactivated by exposure to formaldehyde, and not inactivated when they are oxidized or when they are reduced. Both of the crude toxin preparations are completely neutralized by antitoxic sera prepared against skin toxin. All of these observations suggest that the active principles in the 2 materials or the mechanism of production of their effects may be the same. However, these complex mixtures should now be submitted to detailed analysis so that the active principles may be precisely characterized. It should be noted that the in vitro-prepared skin toxin is actively haemolytic whereas loop fluid preparations with comparable cutaneous activity are only weakly haemolytic. The crude antigens also show differences in immuno-diffusion experiments. Physico-chemical procedures for the fractionation of such complex mixtures are now well developed and it should be possible to apply these techniques to the isolation of the skin-reactive component in each case.

If the skin-reactive component of the skin toxin and enterotoxin prove to be different, the disappointing conclusion will be that the skin toxin antigen will not necessarily give protection against enterotoxin. But if the

preparations contain the same skin-reactive principle and if this proves to be identical with enterotoxin, then skin toxin activity provides an easy procedure for assay. It can be further studied thoroughly *in vitro* and *in vivo*, both in regard to its chemical and biological properties together with the immunological reactions that it will provoke. The present work confirms that the activity of this toxin can be easily demonstrated and measured by the inflammatory reactions that it produces in the skin of guinea pigs and rabbits. It seems to be a good antigen. The writer hopes to apply some of the above findings in further attempts to define the toxic mechanism in cholera.

A SUMMARY OF THE

FINDINGS IN SECTION I

AND THE REFERENCES FOR

SECTIONS I AND II

ARE AT THE END OF

THIS THESIS.

Section II.

Introduction.

INTRODUCTION

Historical review of work on bacteriophage.

Twort in 1915 isolated a filtrable virus which produced a "glassy transformation" of micrococcal colonies during their growth on agar surface. Two years later d' Hérelle (1917) independently published his observations on a filtrable agent capable of transmissible lysis of growing cultures of enteric bacilli. These filter-passing, ultramicroscopic agents were designated as bacteriophage and the term became abbreviated to 'phage'.

After this discovery medical bacteriologists hoped to apply the bactericidal properties of the bacteriophage to the treatment of some infectious bacterial diseases. The study of the bacterial viruses thus rapidly became popular. Many of the leading bacteriologists following the first World War became involved and this soon led to great controversy as to whether phages were inanimate, autocatalytic agents or were self-reproducing organisms like viruses. Some of these controversies were discussed at a meeting in 1922 organized by the British Medical Association in Glasgow. A series of hypotheses had been discussed by d'Hérelle in 1926 and amongst them the "precursor" and the "virus" theories have survived to the present. According to the Precursor theory, bacteriophages are endogenous

substances existing in bacteria as precursors which spontaneously or after stimulation are transformed into characteristic lytic substances much as trypsinogen can be converted into trypsin. This theory had the support of workers like Bordet (1925), Northrop (1939), Krueger and Scribner (1939) and Felix (1953). In contrast, the Virus theory states that bacteriophages are autonomous microbes but obligate parasites of bacteria.

The current concept of bacteriophage is that it may exist in 3 stages - prophage, vegetative phage and mature phage. Outside the host bacterial cell, phages exist in mature form. In this form they are metabolically inert and very crudely resemble the spore state of bacteria. After adsorption to the host cell the contents of the phage particles are injected into the host cell, and new phage may begin to multiply. When it does, the multiplying intracellular stage of the phage particle is said to be vegetative phage. In this form it has almost unlimited reproductive capacity. Infection of bacteria with some phages may result in no perceptible change in the host cell and in that case the infecting phage is said to be temperate. The progeny of bacteria containing the component of this phage is known as prophage, the process is known as lysogenization and the bacteria are said to be lysogenic. This relationship has been termed symbiotic and is of great survival value to both phage and the host cell.

The early hopes of a medical application resulted in valuable work on bacteriophages during 1920-40. With the introduction of

chemotherapeutic agents the interest in the therapeutic applications of phage subsided. The revival of interest in bacteriophages is largely due to the stimulus provided by Delbrück. Since 1940 phages have served as the principal experimental objects for exploring the fundamental biological properties of viruses. The reason for this is obvious. The bacterial host of the phage can be handled in the laboratory and growth under controlled conditions is more easily achieved with bacteria than with the hosts of plant viruses and animal viruses.

The initial step in the detailed investigation of bacterial virus growth was first undertaken by Krueger (1931) and Schlesinger in 1932a (reviewed by Stent, 1964). In his second paper in this series Schlesinger (1932b, reviewed by Stent 1964) showed that phage adsorption is usually an irreversible process and the kinetics of the process imply that Brownian movement brings virus particles into random collisions with the bacterial surface. Delbrück (1940) showed that the physiological state of the bacteria affects the rate of adsorption. Stereoscopic electron micrographs produced by Anderson (1953) showed that the tailed bacteriophages attach to the host cell by the tips of their tails.

Burnet (1929) showed that 20 - 100 viruses suddenly appeared some 20 min. after a bacterial suspension was infected with a single phage particle. This observation of Burnet gave convincing support for d'Hérelle's conception that the infecting phage particle multiplies within the bacterium and that its progeny are

liberated upon lysis of the host cell. Finally this was demonstrated by Ellis and Delbrück (1939) in their one step growth experiment:-

The nature and kinetics of bacteriophage multiplication in the host cell are demonstrated in a one-step growth experiment for phage multiplication. The kinetics of virus multiplication within infected cells were first studied by Doermann (1952) by artificially disintegrating host cells at appropriate intervals in the latent period; the lysates were assayed to determine the mean number of mature phage particles present per infected host cell. It was found that no mature infectious phage particles are contained intracellularly until halfway through the latent period. Then the number of mature phage particles increases until the final crop of progeny is attained. This is released by spontaneous "lysis from within" at the end of the normal latent period. The mature phage particle or the infective particle is the typical extracellular stage which is assayed by its plaque-forming ability. The immature or vegetative phage particle is the intracellular stage undergoing multiplication. It is potentially capable of producing mature phage particles but it is not detectable by the plaque count method because it is non-infectious (Doermann, 1953).

Different methods were adopted by various workers for disrupting the infected bacteria to study the intracellular events that follow phage infection, e.g. sonic oscillation by Anderson and

Doermann (1952), chloroform by Séchaud and Kellenberger (1956), and streptomycin by Symonds (1957).

By high speed centrifugation of phage lysates, Schlesinger was the first to purify bacterial viruses and on chemical analysis these were found to contain equal proportions of protein and DNA (see preview by Stent, 1964). Afterwards, Anderson (1949, 1950) and Herriott (1951) showed that the viral DNA is contained within the protein coat of the head. Hershey and Chase (1952) performed an ingenious experiment by first labelling the DNA of the virus with radioactive phosphorus P32 and then the protein with radioactive sulphur S35 and they showed that the viral protein and DNA have independent functions in the infective process. Host bacteria were grown in a medium which contained either the radioactive isotope of sulphur S35 or that of phosophorus P32. These isotopes were incorporated by the bacteria and the constituents of the bacterial protoplasm became labelled with radioactive phosphorus or radioactive sulphur. Bacteria were not labelled with both isotopes because it is difficult to differentiate between them when they are present simultaneously. The labelled bacteria were then infected with phage. The phage reproduced within the bacteria and progeny viruses were released after lysis. These progeny were collected and it was found that they were labelled with the radio active isotopes. Analysis of the composition of the phage had revealed that they were composed primarily of protein and DNA. Phosphorus is one of the major constituents of DNA, but sulphur is absent. The protein on the other hand is devoid of phosphorus but

does contain sulphur. In the next step of the experiment, unlabelled bacteria were infected with the labelled phage and the distribution of the label was determined within the host bacterium. When the infection was brought about by S35 - labelled phage the host bacteria were unlabelled. The S35-labelled virus protein was found to be attached to the outside of the hosts in the coats of the infecting viruses. When P32-labelled phage was used, no label was found in the protein coats but it was found within the host bacteria. Therefore, it was the DNA that entered the infected bacteria while the protein remained attached on the outside. Thus the material injected into the bacterium by the virus is DNA, and it is this DNA that is necessary for the reproduction of genetically identical virus particles.

The current model of phage infection is that the phage tail makes contact with the cell wall of the sensitive bacterium; a phage enzyme is released and penetrates through the rigid layer of the cell wall. The products of this activity finally trigger contraction of the sheath leading to the penetration of the cell wall by the core and discharge of DNA into the cell. The protein cover of the phage thus behaves as a "micro-syringe" and appears to be relieved of any further function in the intracellular reproductive process after the DNA has safely entered the interior of the host cell. On gaining entrance into the bacterium, the phage DNA causes all normal bacterial growth to stop and directs the whole metabolic apparatus of the cell to produce viral DNA and

proteins. Some of the latter are used in making new phage heads and tails while others perform the enzymic function of phage DNA synthesis without becoming incorporated into the completed virus particles. Finally, about 20-30 minutes after infection, the newly synthesized phage protein and DNA are assembled into new phage particles and the bacteria undergo lysis liberating hundreds of mature particles. The point of particular interest here is that during the phage infection the material that is injected into the bacterial cell and which initiates the formation of new virus particles is the DNA. Putnam and Kozloff (1950) in their "transfer experiment" found that about half of the isotopically-labelled parental DNA atoms were transferred to the progeny phage particles. This was confirmed with improved experimental techniques by Maaløe and Watson (1951) and Watson and Maaløe (1953).

The discovery of bacterial transformation by Griffith in 1928 showed clearly that hereditary determinants could be transferred from one bacterium to another. It also laid the foundation for subsequent recognition of the hereditary material as DNA. It was also shown that the transformation of avirulent pneumococci to virulent ones could be effected in the test-tube (Dawson and Sia 1931) and that the transforming principle was present in the cell-free extracts of the virulent donor strain (Alloway, 1933). A systematic analysis of the chemical nature of transforming principle showed that it possessed all of the properities of DNA

(Avery, MacLeod and McCarty, 1944). Studies of both bacterial transformation and bacterial viruses consequently lead to the conclusion that DNA is the chemical basis of heredity.

Lysogeny. Very shortly after the discovery of bacteriophages, it was recognised by many workers (e.g. Bordet and Ciuca, 1921) that filtrates of bacterial cultures often contain bacteriophages that lyse other indicator strains of the same or related species. If a phage-containing filtrate is diluted serially and drops of each dilution are spotted on to a plate seeded with a sensitive strain of bacterium, one can observe at the higher phage concentrations zones of clearing, and at the lower phage concentrations discrete, small, zones of clearing or plaques. In general, there are 2 categories of bacterial strains of which the culture filtrates might frequently be found to contain phage. The first of these are the so-called "carrier-strains" in which the production of phage could be ascribed to a population equilibrium between phage-resistant and phage-sensitive cells, the latter being constantly infected by free phage particles (Jacob and Wollman, 1959). The second category is the group of lysogenic bacteria in which each cell can potentially produce phage. A strain can be considered lysogenic if, on repeated reisolation, its culture filtrates regularly form plaques when plated on appropriate sensitive indicator bacteria (Jacob and Wollman, 1959).

Lwoff (1953) defined a lysogenic bacterium as "a bacterium which possesses and transmits, which perpetuates, the power to

produce bacteriophage". The phage is not maintained as such within the bacterial cell but the genetic information necessary for phage production is carried in the form of non-infective units called prophage (Lwoff and Gutmann, 1950). In the prophage state, multiplication of the phage occurs without destruction of the bacterium and in co-ordination with division of the host. When phage genetic material is multiplying independently the phage is said to be in the vegetative state, which characteristically leads to the production of mature phage particles and lysis of the host cell. Lwoff and Gutmann (1950) showed that free phage particles contained in cultures of a lysogenic strain of Bacillus megaterium were due to lysis of a small number of the bacterial cells. Normally, lysogenic systems are very stable; transition from the prophage state to the vegetative state with the accompanying lysis of the cell and release of free phage particles occurs only rarely. In some lysogenic systems, however, the amount of free phage can be greatly increased by exposure to inducing agents such as ultraviolet (UV) irradiation, ionising radiations, or nitrogen mustards (Lwoff 1953). This process is known as induction. Following induction, bacterial growth proceeds for a time corresponding to 1 or 2 divisions, then mass lysis occurs and phage may be released by almost every bacterium (Lwoff, Siminovitch, and Kjeldgaard, 1950). Only certain lysogenic systems are inducible, and in general inducibility is a property of the prophage (Jacob and Wollman, 1959). Physiological conditions in culture affect the response to induction, e.g. starvation before UV

irradiation greatly reduces the phage yield obtained (Jacob, 1952).

Release of phage by lysogenic bacteria is detected using a sensitive indicator strain whose cells may respond in a least 2 ways to infection by the phage. The phage may establish a new lysogenic system in a proportion of the sensitive cells, this process being called lysogenization, and the phage responsible being known as temperate phage. Alternatively, the phage may enter the vegetative state upon infecting the indicator cells causing lysis of the bacteria and release of phage particles which in turn may either lysogenize or lyse further cells of the indicator. The relative frequencies with which the 2 responses occur depends on the conditions of infection and the genetic constitution of the phage (Jacob and Wollman, 1959) and determines the appearance of zones or plaques observed on a lawn of indicator bacteria. If there is 100 per cent. lysogenization, all of the cells infected with phage will remain viable, and no zone will be detected; if there is 100 per cent. lysis, i.e. if the phage is virulent for the indicator strain, a zone of complete clearing will be observed. Turbid zones of partial clearing will be observed if some of the cells are lysogenized and some are lysed.

Enumeration of Phage Particles

Quantitative estimation of bacteriophages depends upon employing a simple and accurate means of enumeration of their

presence in a phage suspension. Various methods have been used for counting phage particles, the most common involving plaque counts on nutrient agar plates seeded with sensitive bacteria. The modern plaque assay procedure is a modification of d'Hérelle's technique introduced by Gratia (1936) and by Hershey, Kalmanson and Bronfenbrenner (1943). The viable phage particles are counted by assay which consists of seeding a few milliliters of melted semi-solid nutrient agar at 45°C with about 10^7 host bacteria and mixing this with an appropriate dilution of the test phage suspension. This phage-culture mixture is then poured over the surface of a sterile nutrient agar plate. When the upper soft agar layer has solidified, the plate is incubated at 37°C overnight. During incubation the uninfected bacteria multiply to form a thick turbid lawn of bacterial growth on the agar surface of the plate. Each infected bacterium produces a crop of progeny phages after a short time. The progeny of each parent phage then infect neighbouring bacteria which in turn are lysed. This process of phage reproduction and bacterial lysis continues in each focus of infection until brought to a halt by a decline in the bacterial metabolism on which phage multiplication depends. The result is a visible, circumscribed area of clearing in the confluent bacterial growth, known as a plaque. The final diameter of the plaques depends on the phage type, the bacterial host strain, and the exact conditions of plating and incubation but it is generally of the order of a few millimeters. Since the bacteria

in the inoculum are present in gross excess, each of the phage particles will infect a single bacterium and this ultimately gives rise to a plaque.

One-step growth experiment with phage

In 1939 Ellis and Delbrück devised their one - step growth experiment to study the kinetics of phage multiplication in a bacterial population. It is known that, on infection, the DNA of the phage particles enters the bacterial cells and these cells then synthesize materials needed for phage production. After a certain time, mature phage particles form in the infected cells. The production of mature phage proceeds at a constant rate until eventually the infected cells lyse, liberating the phage particles. The time between infection and lysis is the latent period which is characteristic for a particular phage and bacterial host strain. The first half of the latent period, during which no mature phages are present in the infected cell, is called the eclipse period.

In the one-step growth experiment a young culture of sensitive bacteria is infected with a dilution of phage suspension and incubated for a few minutes to allow most of the phage particles to adhere to the bacteria. Residual free phage particles are neutralized by the addition of antiserum; the mixture is then highly diluted in warm broth and maintained at 37°C. Samples are thereafter removed at different times and immediately assayed by

the agar layer method for the number of plaque-forming units they contain. The plates are examined after overnight incubation at 37°C.

The plaque count remains constant for some time after infection; this is related to the number of infected cells present in the samples. The onset of lysis in the cells, which defines the end of the latent period, is then heralded by a sudden increase in the number of plaques found on the plates. This number increases for a further period, sometimes called the rise period, until all of the infected cells have lysed. Later, a constant number of plaques is found on the plates corresponding to the total number of phages liberated by all of the infected cells and thus a plateau is reached. No further phage multiplication occurs after this stage, since progeny phage and residual uninfected bacteria in the mixture have been separated from each other by high dilution of the phage-culture mixture made shortly after the initial infection.

Free phage particles are absent from the sample during the latent period because of the addition of antiphage serum and because of the high dilution in broth. Thus the plaques that are formed from the samples taken during the latent period arise solely from the infected bacteria. The phage particles that emerge from each of these infected bacteria on plating are localized on the plate and give rise to only a single plaque. On the other hand, samples plated at the plateau stage contain free progeny phage

particles each of which generates a plaque. Thus the plaque count during the latent period gives the average number of infected bacteria per sample, while the plaque count at the plateau gives the average number of progeny phage particles released by these infected bacteria. The ratio $\frac{\text{Plaque titre at plateau}}{\text{Plaque titre during latent period}}$ indicates the average number of phage particles liberated by each of the infected bacteria. It is called the burst size.

Both the latent period and the burst size may very widely depending upon the strain of phage and bacterium used and on the exact physiological conditions under which phage growth is allowed to proceed. The latent period is longer at lower temperatures but shorter at higher temperatures of incubation for a particular strain of phage and bacterium (Ellis and Delbrück, 1939).

The nutritional and physiological state of the host strain can also affect the burst size as well as the latent period. In a poor growth medium or in an old bacterial culture the latent period is usually longer and the burst size is smaller than in cultures growing rapidly in a favourable medium (Delbrück, 1940, and Hedén, 1951). The latent period and burst size also vary widely for different phage strains and also for various host bacteria.

The experimental findings of a one-step growth experiment with cholera bacteriophage are recorded in the Results section of this thesis.

Inactivation of phage by heat.

In 1926 d'Hérelle observed that many phages were inactivated by heating at 75°C for 30 min. but some phages survived heating at 70°C. Heat-inactivation of phage is presumed to result from protein denaturation and takes place in accordance with kinetics of the first order (Adams, 1959). Inactivation of phage is accompanied by liberation of nucleic acid into the solution. This results in production of empty ghost cells of phage which are unable to adsorb to bacteria (Lark and Adams, 1953).

There are great variations in the degree of heat resistance of bacteriophages active against different species of bacteria. The chemical composition of the medium in which the phage is propagated and tested also affects the heat susceptibility of a phage (Nanavuty, 1930; Burnet and McKie, 1930; Gratia, 1940). There is variation in the composition of different lots of non-synthetic medium and the results of heat-susceptibility tests of a phage may not be precisely reproducible unless one works with a synthetic medium. A coli-phage was inactivated 10 times faster in saline than in broth (Nanavuty, 1930). It was observed by Burnet and McKie (1930) that many phages were far more susceptible to heat-inactivation in 0.1 N solutions of sodium or potassium salts than in broth.

Genotypic and phenotypic heat-resistant mutants occur in wild type phage stocks at various frequencies and the rate of heat-inactivation of all of the phage particles in such a stock is not

uniform. Mutants of coli-phage T5 are about 1000 times more heat-resistant in 0.1N Nacl than is the parental wild type (Lark and Adams, 1953).

In addition to characteristics relating to morphology, serology and host range, the thermal death point of a bacteriophage is of use in its characterization and identification.

Inactivation of phage with anti-phage serum.

In 1921 Bordet and Ciuca first showed that anti-phage antibodies capable of neutralizing the infectivity of homologous phage particles can be developed in the serum of rabbits inoculated with phage lysates. It is evident that different phage lysates carry different antigens; the antibodies developed by immunization of rabbits with one kind of phage usually do not neutralize the infectivity of another kind of phage. In some cases an antiserum containing antibodies developed against one strain does neutralize the phage particles of another strain. Phage strains that cross-react in this manner carry common antigens and are therefore said to be serologically related. Serological cross-reaction tests provided the first valid method for establishing whether different types of bacterial viruses are really generically related (Adams and Wade, 1955; Burnet, 1933, 1934). Thus the T-even strains, T2, T4 and T6, among the 7 T phages acting on Esch. Coli were first found to be closely related to each other and unrelated to the other members of the series. Neutralization of phage by anti-

phage antibody has, therefore, been an extremely useful tool in bacterial virus experimentation because the procedure allows identification and classification of the viruses into groups in which the antigenic relationships are correlated with morphological and biological resemblances (Delbrück, 1946).

The number of infective phage particles decreases continuously with time when a homologous antiserum prepared in a rabbit is mixed with the phage suspension. The kinetics of this inactivation process can be followed by diluting samples of the phage-antiserum mixture at various times after the start of the reaction and plating samples on sensitive indicator bacteria for plaque assay by the agar layer method. Since the dilution of the mixture effectively stops further neutralization and since the reaction of the phage with its neutralizing antibody is usually irreversible (Hershey, 1943), the number of plaques formed indicates the number of phages in the mixture that are not yet neutralized by the serum at the moment of dilution. In many cases, neutralization follows an exponential course, and the logarithm of the fraction of the surviving phage that remains active after exposure to antiserum is proportional to the concentration of the antibody and the time of contact with it. This can be expressed by the formulae:

$$P/Po = e^{-Ktc} \quad ;$$

$$\text{or } \log e \frac{Po}{P} = Ktc \quad ;$$

$$\text{or } \log 10 \frac{Po}{P} = 0.43\ Ktc \quad ;$$

$$\text{or } K = 2.3\ D/t.\ \log 10 \frac{Po}{P}$$

Where Po is the initial phage concentration; P is the phage remaining active after time t min. contact with a concentration c (or dilution $^1/D$) of the antiserum; and K is a constant (fractional rate of inactivation) which characterizes the rate of inactivation of a given phage by a given antiserum.

The exact mechanism of the neutralization of phage particles by the antiphage antibodies is not fully understood. Studies have shown that the antibody molecules can attach themselves to virus particles without necessarily causing loss of infectivity as demonstrated by the fact that treatment of the virus-antibody complex with papain results in destruction of antibody and recovery of infectivity of the virus (Kalmanson and Bronfenbrenner, 1943). Inactivation of the phage particles results only from the fixation of an antiphage antibody to a "critical site" on the virus. This critical site for phage T2 is in the tail of the phage (Lanni and Lanni, 1953).

The logarithmic inactivation kinetics described by the above equation are usually valid only for the neutralization of 90-99 per cent. of the phage population. The remaining 10-1 per cent. is neutralized at a much slower rate (Andrews and Elford, 1933; Delbrück, 1945). Thus the inactivation curves are not always exponential. There may be (i) an initial lag, or (ii) a decreasing slope after 99 per cent. of the phage has been inactivated.

Besides its use in phage neutralization experiments, antiphage-antibody can also be employed for agglutination or precipitation

of the homologous phage. If a concentrated phage suspension is mixed with a strong antiphage antiserum a visible precipitate is formed containing both phage particles and antibody molecules and the complex slowly settles to the bottom of the tube (Burnet, 1933). Chemical analysis of such serological precipitates provided one of the early methods for determining the composition of the virus particles (Hershey, Kalmanson and Bronfenbrenner, 1943).

Antigenic relationships in cholera phage groups I, II, III and IV

Bordet and Ciuca (1921) observed that injection of rabbits with phage lysates stimulates the production of phage-neutralizing antibody. It was not possible to produce antiphage serum by immunizing rabbits with the host bacteria. In 1922 Otto and Winker (cited by Adams, 1959) used an adsorption test with host bacteria to demonstrate that phage is antigenically distinct from the host bacteria. In an extensive study, Lanni and Lanni (1953) observed the existence of two distinct antigens in phage T2. One of the antigens was localized in the phage tail and reacted with the neutralizing antibody. The second precipitating and complement-fixing antigen appeared to be localized in the head. The two antigens were confined to the surface structures of the phage. The internal contents of the phage liberated by osmotic shock gave no sign of serological activity (Hershey and Chase, 1952, Hershey, 1955). Rountree (1952) observed the existence of

2 distinct antigens in staphylococcal phage 3A. Only 1 of the antigens reacted with neutralizing antibody. Likewise Fodor and Adams (1955) demonstrated 2 antigens in phage T5, 1 of which reacted with neutralizing antibody.

The neutralization of infectivity of a phage by the anti serum of a second phage indicates a close biological relationship between the 2 phages providing certain sources of error are eliminated. The antigenic specificity of bacteriophages forms the most important criterion in their classification. The rate of neutralization/effected by an antiphage serum is greatest with the homologous phage. In the case of different types of phages that overlap serologically, the rates of neutralization differ. The host ranges of mutants of a phage are serologically identical (Luria, 1945, Craigie and Yen, 1938b). The antigenic specificity of cholera phages of types A, B, and C, were studied by Asheshow, Asheshov, Khan, Lahiri and Chatterje (1933). These phages were found to be serologically distinct. Antigenic relationships in cholera phages were also studied by Mukerjee (1962) and these are investigated in the present work.

Phage-mediated transduction.

The phages are involved as carrying agents or vectors in mechanisms of transduction in bacteria. The simplest concept of the role of phage in transduction is that certain strains of temperate phages are able to carry a piece of genetic material from a donor bacterium on which the phages have multiplied to a

recipient bacterium which they infect (Zinder and Lederberg, 1952; Zinder, 1953). Among the recipient cells surviving the infection, some have acquired new genetic properties originating from the donor bacterium. The occurrence of transduction has been reported in Esch. coli (phages lambda, P1 and 363: Morse, 1954; Lennox, 1955; Jacob, 1955); Shigella (phage P_1: Lennox, 1955; Adams and Luria, 1958), Pseudomonas (Loutit, 1958; Holloway and Monk, 1960), Staphylococcus (Morse, 1959); Proteus (Coetzee and Sacks, 1960); and B. subtilis (Thorne, 1961).

In many instances transduction is not specific and any character of the donor may be transmitted. It appears as if the genetic material of the donor was disrupted during phage multiplication and that this permitted the segments of the genetic material to be incorporated by chance into occasional phage particles. Non-specific transduction was observed in Salmonella (Zinder and Lederberg, 1952). Linked characters may be transduced together (Stocker, Zinder, and Lederberg, 1953; Lennox, 1955) and such a possibility was used for genetic analysis of small chromosomal segments of bacteria (Demerec and Demerec, 1956). Among the characters that can thus be transduced from donor to recipient bacteria is the lysogenic character itself. A vector phage particle may carry, in addition to its own genetic material, a piece of bacterial chromosome carrying one or more unrelated prophages (Jacob, 1955).

In addition to this mode of transduction, which is generalized and non-specific, another mode of transduction characteristically

specific and restricted to certain genetic characters of the donor has been described by Morse, Lederberg and Lederberg (1956) in phage lambda. In this case only a limited segment of genetic material of a donor bacterium can be transferred to a recipient.

Besides phage-mediated transfer of genes, the bacteriophage itself may play a part in determining the phenotype of bacteria. Thus, as result of lysogenization with certain phages, changes may be observed in properities of the host. Such phage-mediated acquisition of new traits, whose connection with prophage is often much less obvious than capacity for phage production or immunity, is called conversion (Barksdale, 1959 Groman, 1953). An example of phage conversion was encountered by Freeman in 1951, who found that if certain strains of Corynebacterium diphtheriae that fail to produce diphtheria toxin are treated with a preparation of phage derived from virulent toxigenic bacilli of the same species, a proportion of the survivors acquire the hereditary ability to synthesize toxin. They also acquire immunity to lytic infection by phage (Freeman, 1951; Freeman and Morse, 1952). Further study of this phenomenon by Groman (1953, 1955) and by Barksdale and Pappenheimer (1954) showed that the capacity of the corynebacterium to produce diphtheria toxin was conferred by the presence of a specific phage. In other words, conversion of the non-toxigenic strain to toxigenicity was not due to selection by phage of a minority of toxigenic survivors, but to the establishment of lysogeny. Another instance of phage conversion was observed by Japanese workers (Iseki and Sakai, 1953; Uetake, Nakagawa and Akiba, 1955 ; Terada, Tomii and Kurosaka, 1956) who noticed that the presence

of certain antigenic structures on the surface of salmonella organisms is the consequence of lysogenization with particular phage types. In the "Prophage typing of El Tor vibrios", Takeya and Shimodori (1963) observed that lysogeny was associated with the virulence of the organisms (El Tor strains). It is reported that lysogeny is more common in El Tor strains than in classical cholera vibrios (Newman and Eisenstark, 1964).

Phage can thus be regarded as an important factor in bacterial ecology. Our aim in the present study is to determine whether it is possible to transmit factors associated with virulence of V. cholerae to other vibrios either by transduction or by phage conversion as has been observed in C. diphtherae or in Salmonella. To study the probable existence of similar phenomena operating in vibrios, it was necessary to isolate temperate bacteriophages and their host lysogenic strains from a number of vibrio strains obtained from different sources. The techniques adopted for detection of lysogenic strains and temperate phages will be described in detail.

Isolation and characterization of biochemical mutants

Mutants that grow under conditions that do not support growth of the wild type organism are easy to select. Thus phage-resistant or drug-resistant mutants are easily isolated from large populations of cells sensitive to these agents. They can be selected by replica-plating the sensitive cells on to nutrient agar containing the antibacterial agent so that the sensitive cells are killed or their growth is suppressed. Resistant mutants are left to multiply and produce colonies. Similarly, prototrophic mutants are selected from cultures of auxotrophic strains by plating washed cultures on minimal medium. Auxotrophic mutants cannot be isolated in this way because they are unable to grow on the minimal medium which suffices for the growth of the wild type. Auxotrophs need some additional substance or occasionally a mixture of 2 or more substances, before growth can occur. The growth requirement may be for a vitamin, an amino acid, or a nucleic acid base or nucleoside. Auxotrophic mutants evidently have lost the capacity to synthesize for themselves the substances that they are found to require.

The initial step in the isolation of biochemical mutants is to treat wild type strains with some mutagenic agent so as to increase the proportion of mutants among the survivors. Ultra-violet (UV) light is most commonly used as a mutagenic agent, but chemical mutagens, e.g. manganese chloride and acriflavine, have also been used for the isolation of biochemical mutants. In a typical procedure, washed bacterial suspensions may be exposed to a dose of

UV radiation sufficient to kill 99-99.9 per cent. of the original population. The irradiated bacteria are suspended in complete medium, e.g. nutrient broth, and incubated. The small proportion of auxotrophic mutants is then isolated either by a delayed enrichment method, or by a limited enrichment method, or preferably by replica-plating (Hayes, 1964).

In the delayed enrichment method, the culture containing the mutants is diluted and plated on minimal agar so that isolated colonies may appear after incubation; a small volume of molten minimal agar is then poured over the spread surface and allowed to set so as to sandwich the bacteria between the 2 layers of minimal agar. On incubation, only the non-mutant, prototrophic bacteria give rise to colonies whose positions are marked and thereafter a layer of nutrient agar is poured over the minimal agar surface, and the plate is re-incubated. The nutrient diffuses into the minimal agar, allowing the auxotrophic mutants to produce colonies which are subsequently identified by their delayed appearance.

In the limited enrichment method, the irradiated culture is spread on a minimal agar plate containing a limiting concentration of nutrient so as to recognize the presumptively auxotrophic bacteria directly by the small size of their colonies.

An ingenious method for isolating rare auxotrophs was introduced by Davis (1948) and Lederberg (1950). It is based on the mode of action of penicillin which kills only growing bacteria but is not toxic to non-growing cells. Thus when a

suspension of cells is made in liquid minimal medium containing penicillin, the non-auxotrophic organisms nearly all grow and are killed. The occasional auxotrophic cells on the other hand, are unable to grow and tend to survive. Thereafter, the penicillin may either be inactivated by penicillinase or the surviving cells may be centrifuged from the medium, washed free of penicillin, and subcultured on plates of solidified minimal medium. Various growth factors are then spotted singly or in groups on to the plates. After incubation, only the auxotrophs responding to the particular supplements will grow.

Though penicillin is an ideal selective agent for sterilizing non-mutant populations in minimal medium, experiments with large numbers of bacterial species failed to yield any mutants among the survivors of irradiated populations exposed to the action of penicillin in minimal medium. However, success was obtained when an extra stage of cultivation of the irradiated bacteria was allowed before they were exposed to penicillin in minimal media (Davis, 1949). This modified penicillin technique was also employed in the present work for isolation of biochemical mutants of V. cholerae.

Genetic Studies with V. cholerae

A mechanism of genetic recombination resulting from conjugation was discovered by Lederberg (1947) in Escherichia coli. Bacterial transduction, another means whereby genetic recombination occurs in bacteria, was demonstrated by Zinder and Lederberg (1952)

in S.typhimurium (see p. 210). It was shown that bacteriophage could serve as vectors for genetic material which could be transduced from cell to cell with the result that the transduced cell exhibited certain genetic characters of the donor cell on which the phage had been propagated. Analogous studies have been carried out in a variety of bacteria including V. cholerae with a view in the latter case to studying the nature of variations that may result and to determine their possible bearing on the epidemiology of cholera.

Antigenic mutations are known to occur in V.cholerae (Shrivastava and White, 1947; Bhaskaran and Gorrill, 1957). The nutritional requirements of various vibrio strains were investigated by Bhaskaran and Rowley (1956). A screening procedure was adopted by Bhaskaran (1958) to select strains that might be suitable for genetic recombination studies in V.cholerae. This procedure involved cultivating a streptomycin-resistant methionine-requiring strain with several purine-requiring streptomycin-sensitive strains to determine if recombinants arose that were streptomycin-resistant and independent of purine and methionine. Secondly, a stable non-motile streptomycin-resistant V.cholerae strain was similarly cultivated with several other streptomycin-sensitive motile strains of V. cholerae to see if any streptomycin-resistant motile recombinants could be isolated. Bhaskaran succeeded in this screening procedure in selecting a strain (strain 129) that was consistently capable of giving rise to recombinants with 2 potential partners (121 and C4). Control

experiments revealed conclusively that true genetic transfer occurred.

This genetic transfer was thought at first to be mediated by a phage (phage 129) because plaque-like clearings were produced by strain 129 on its partner strain 121, but the concept was proved to be erroneous when all attempts to isolate the phage were unsuccessful. The clearings were considered to be due to a bacteriocine-like agent (Bhaskaran, 1959). It was shown later that any strain that acquired bacteriocinogenic property from strain 129 was itself capable of transferring the character to other strains and to undergo genetic recombination with them. Thus it was obvious that the bacteriocinogenic factor (designated as P factor) conferred fertility to vibrio cells and this enabled them to conjugate. It thus seemed analogous to the F (fertility) factor of E.coli. Later studies showed that crosses between V.cholerae strains proved fertile only when one of the mating type strain was P^+ (possessing P factor) and the other was P^- (devoid of P factor); $P^+ \times P^+$ crosses were less fertile, and $P^- \times P^-$ crosses were completely sterile. P^+ cells appeared to act as donors and P^- cells as recipients, but linkage between donor markers was rarely found among the recombinants. Thus, the chromosomal fragments transferred by the donors were probably very small (Bhaskaran, 1960; Bhaskaran and Iyre, 1961). By analysis of the recombinants derived from crosses between 2 mutants of V. cholerae which differed from one another in 7 genetic factors, Bhaskaran (1964) concluded that in a majority of the recombinants the contribution of the P^+ parent was confined to single genetic

factors. He noted exceptions in the case of linked factors that segregated together in a small percentage of the recombinants.

Applications of phage procedures in practical microbiology

It has been shown that there are phages active against most bacteria, each having a marked degree of specificity for its host species.

This has been made use of in subclassification of several groups of bacteria. The typing of bacteria by specific bacteriophage and the precise tracing of carriers and the lines of spread of infectious diseases by this technique has been one of the most important advances in epidemiology in recent years. Craigie and Yen (1938a, 1938b) obtained different salmonella phage preparations by adapting a type II Vi typhoid phage on different strains of Salmonella typhi. These adapted phages not only became specific for the strains of S.typhi on which they were propagated but they also behaved in an identical manner with strains that were epidemiologically related. The practical value of this example of phage-typing has been proved beyond any reasonable doubt in practice and it is now recognized as a valuable aid to the survey and control of typhoid fever. Similar schemes are being utilized in mapping the spread of enteric fevers caused by S.paratyphi A (Banker, 1955) and S.paratyphi B (Felix and Callow 1951). Phage-typing is also of practical use in tracing infections caused by Staphylococcus aureus, Corynebacterium diphtheriae, and V.cholerae (Mukerjee, 1963b).

Phages can be distinguished by means of their host range; the morphology of particles as revealed by electron micrographs; the morphology of plaques that they produce; their biochemical and immunological characters; and their susceptibility to various environmental factors.

Phage typing of V.cholerae.

d'Hérelle first observed the presence of bacteriophage in the stools of patients with cholera in 1920. The historical development of studies on cholera phage has been reviewed in a monograph on cholera by Pollitzer (1959).

Since 1927 investigations carried out in India on cholera bacteriophage yielded valuable information on bacteriophages in general and cholera phages in particular. Most of these studies had as their aim the use of phage for therapeutic purposes. Thirteen types of cholera phage were recognized. Types A, B, and C were described by Asheshov (1930); types D, E and F by Pasricha, De Monte and Gupta, (1932a, 1932b); types G, H, and J by Morison (1933) and type K was discovered by Pasricha (1933). Phage L was isolated by Anderson (1935), and Phage M by Pasricha, De Monte and Gupta, (1936). Phage II of White was reclassified by Pasricha, Lahiri and De Monte, in 1941, as phage N. The classification of the cholera phages was based on their reciprocal action on the phage-resistant secondary growths of the vibrios. The differences in the sensitivity of cholera vibrios to phages were observed by Asheshov et al (1933). The strains of cholera vibrios collected

from different parts of India were classified into 4 types by these workers who used a type A phage which after adaptation could acquire virulence for all of the types of vibrios.

No systematic studies for the purposes of epidemiological investigation had yet been made on the typing of cholera vibrios with cholera bacteriophage when the Expert Committee on Cholera of the World Health Organization in its report in 1952 (W.H.O. Report, 1952) recommended that vibrio strains be studied "from the point of view of their epidemiological significance, advantages being taken of phage-typing for the purpose". Accordingly the W.H.O. International Reference Centre for phage-typing of cholera vibrios was established in Calcutta and a scheme of phage-typing was developed by Dr. S. Mukerjee. The old laboratory collections of cholera phages belonging to standard types B, C, D, E, F, G, H, M and R were used in the preliminary experiments. The results were unsatisfactory so freshly isolated local strains were used and these were classified into 4 distinct phage groups on the basis of their lytic patterns produced with vibrio strains. This classification was further confirmed by other biological characteristics of the phages including plaque morphology, thermal death points, generation times and neutralization by antiphage sera (Mukerjee, 1961a, b, c; 1962). By use of these 4 groups of cholera phages a typing scheme for identification of V. cholerae strains has been developed by Mukerjee (1963b). Five phage types of V.cholerae have thereby been identified

[Mukerjee, Guha and Guha Roy, (1957); Mukerjee et al. (1959); Mukerjee, Guha Roy and Rudra (1960, 1963a, 1963b); Mukerjee (1963b)]. Type 2 V.cholerae could further be classified into 3 sub-types by phage adaptation. Type 1 has been found to consist of 3 sub-types demonstrable by their susceptibility to lysis by 2 new phages isolated from lysogenic strains of cholera vibrios in 1964. Strain identification of V.cholerae by phage-typing has provided much useful information about the epidemiology of cholera. The existence of at least 4 different endemic foci harbouring different phage-types of V.cholerae has thus been discovered in India. Epidemiological lines of spread of cholera from Calcutta to neighbouring districts have been confirmed by phage-typing data which suggest exchange of infection between these areas. It has been observed that marked variations in the incidence of phage-types and sero-types of V.cholerae may take place in an area within a short period (Mukerjee, Guha Roy and Rudra, 1963a).

Phage-typing of V.eltor.

Interest in the strain identification of V.eltor for epidemiological purposes has been stimulated by the pandemic spread of cholera El Tor in South East Asian Countries since 1961. Development of a phage-typing scheme for the El Tor vibrios encountered considerable difficulties. Phages used for typing of V.cholerae were found to be unsuitable for the purpose. A number of schemes suggested by different workers (Nicolle, Gallut and Le Minor, 1960. Nicolle et al. 1962; TaKeya and Shimodori, 1963) were developed but none of them provided a method of strain identification of practical

epidemiological value. By use of a series of phages isolated from lysogenic strains of El Tor vibrios, Mukerjee (1964) devised a typing scheme for V.eltor and 5 phage-types of the strains have been identified. He also found that phage-types of V.eltor of one country often differed from those of neighbouring countries. The possibility of utilizing the difference in phage susceptibility patterns of V.cholerae and V.eltor strains for differentiating the 2 vibrios of O-group I has been considered by Mukerjee (1960, 1963a), Mukerjee and Guha Roy (1961), and Newman (1960).

The susceptibility of a vast number of V.cholerae and V.eltor strains isolated in different parts of the world at different times has been studied by Mukerjee (1964) with group IV cholera phage at its critical test dilution. He found that this phage was consistently lytic for all strains of V.cholerae but for none of the V.eltor strains. The phage susceptibility test has been used in a number of laboratories including those of Felsenfeld (1963) Moor (1963) and TaKeya and Shimodori (1963) who have confirmed its value. The test has so far proved to be the most dependable of the available methods for the differentiation of V.cholerae and V.eltor

Identification of non-agglutinable (NAG) vibrios.

It was observed by Mukerjee (1964) that the NAG vibrios showed much less susceptibility to cholera bacteriophages than V.cholerae. The group II phage is lytic to none of the NAG strains and the other 3 groups of phages are lytic for only a small proportion of these strains. Thus this lytic pattern may be utilized for the differentiation of V.cholerae from NAG vibrios.

Identification of S-R dissociation of V.cholerae strains.

The group II cholera phage was found to have specific lytic affinity for smooth elements of a culture of V.cholerae (Mukerjee, 1959). The detection of degrees of S-R dissociation of strains of V.cholerae is thus possible by noting their lysability by group II phage.

Therapeutic and prophylactic uses of cholera phage.

Earlier studies by workers during the 2 decades from 1928 into the therapeutic and prophylactic uses of cholera bacteriophage gave conflicting results and ultimately the use of phage for these purposes was abandoned. A team of Russian workers (Sayamov, 1963) reported encouraging results with their specially prepared cholera phages in cholera epidemics in East Pakistan and Afghanistan, both for prophylactic and therapeutic purposes. For mass prophylaxis the phage was administered orally with simultaneous injection of a single dose of cholera vaccine to the entire population of the epidemic area. For treatment, a single dose of phage was given intravenously with saline or intramuscularly followed by an oral dose of phage every day. The phage used was prepared by propagation of cholera vibrios through alternate passages in vivo in the small intestine of guinea pigs and in vitro in bile. Old collections of phages prepared by propagation in broth culture of V.cholerae were incapable of parasitizing cholera vibrios in vivo.

Dutta and Panse (1963) observed that the fatal diarrhoea caused by V.cholerae in infant rabbits could be prevented by oral admini-

stration of cholera phages. The gut inflammatory reactions due to V. cholerae in the ligated loop of small intestine of rabbits were found to be prevented by administration of cholera phage (Mukerjee and Ghosh, 1961). Cholera phage was detected in adequate concentration in the intestinal tracts of rabbits within a few minutes of its parenteral administration by Mukerjee and Ghosh (1962). The administration of cholera phage to cholera patients in Calcutta hospitals in 1962 and 1963 did not afford clear evidence of the curative value of cholera phage (Mukerjee, Rudra and Guha Roy, 1963).

Aims of the present study.

The above facts indicate that phage studies in cholera have been of considerable importance. Although the use of phage preparations in the treatment of cholera is now in doubt, further studies on cholera phages still promise to be of value in elucidating the epidemiology of the disease and in solving the problems posed by V. eltor. The present work was therefore carried out to study the cholera bacteriophages in greater detail and to examine the possibility of the genetic transfer of virulence factors from pathogenic to non-pathogenic vibrios.

Materials and Methods.

Source and characterization of vibrio strains

A strain was accepted as vibrio when it fulfilled the following criteria; (1) Gram negative curved or "comma-shaped" rod with rounded or slightly pointed ends; (2) actively motile; (3) single long terminal flagellum (confirmed in a few cases by E-M observation); (4) grew on ordinary media; free growth occurred at pH 9.0 in peptone water and on nutrient agar, producing translucent, moist greenish colonies 1-2 mm. in diameter in 18-20 hr. at 37°C; (5) oxidase positive; (6) fermented glucose without producing gas; (7) did not ferment dulcite; (8) did not attack cellulose; (9) reduced tellurite and (10) liquefied gelatin.

Vibrio cholerae had the following additional characteristics; (1) fermented sucrose and mannose, but not arabinose (Heiberg, 1934); (2) agglutinated with Group O-1 antiserum (Gardner and Venkatraman, 1935); (3) did not lyse sheep or goat red blood cells; and (4) was suceptible to Group IV cholera phages at Routine Test Dilution (Mukerjee, 1961).

Vibrio eltor. This resembles V. cholerae but (1) is resistant to group IV cholera phage; (2) is not inhibited by polymyxin B (Gan and Tjia, 1963); and (3) may be classified as haemolytic or non-haemolytic according to the effect of a culture on sheep red cells.

Non-agglutinable vibrios (NAG). Vibrios that do not agglutinate with Group O-1 antiserum are classified according to Heiberg's sugar reaction. Some of them resemble the cholera and El Tor vibrios in biochemical properties. They are resistant to Group IV cholera phage. In addition to the above-mentioned constant characters the

more variable diagnostic features of the vibrio strains used in the present study are shown in table 42.

Table 42.

Details of vibrios studied in the present work.

Strain	Serotype	Locality of Isolation	Source	Year of Isolation	CR*	VP**	HL***
V. cholerae							
12r	Ogawa	East Pakistan	Case	1961	+	-	-
583	Inaba	"	"	"	-	-	++
Vo	Ogawa	"	"	"	-	-	-
Vi	Inaba	"	"	"	+	-	-
834	Ogawa	"	"	1963	+	-	-
569B	Inaba	Calcutta	"	1943	+	-	-
V154	Ogawa	"	"	1960	+	-	-
PR508	"	"	"	"	+	-	-
PR1212	"	"	"	"	+	-	-
352	Hikojima	"	"	"	+	+	-
BH29	Ogawa	"	"	"	+	-	-
BH202	Inaba	"	"	"	+	-	-
601	Inaba	Bombay	"	1961	+	-	-
3A	Ogawa	"	"	1962	+	-	-
1843	Inaba	Baroda	"	1964	+	-	-
1844	"	"	"	"	+	-	-
B2	"	Batavia	"	1927	-	-	-
8021	Ogawa	?	?	1962	+	-	-
7270	Hikojima	?	?	"	+	++	-
7254	Inaba	?	?	"	+	-	-
60	Hikojima	?	?	"	+	+	-
C116	Ogawa	Chunking	Case	1945	+	-	-
M7	Ogawa	Madura	?	1964	+	-	-
M8	"	"	"	"	+	-	-
M9	Inaba	"	"	"	+	-	-
M10	"	"	"	"	+	-	-
M11	"	"	"	"	+	-	-
M12	"	"	"	"	+	-	-
B37	Ogawa	Bangalore	"	1964	+	-	-

Table 42 (contd.)

Strain	Serotype	Locality of Isolation	Source	Year of Isolation	CR*	VP**	HL***
V. eltor							
H_1	Ogawa	Hong Kong	Case	1963	+	++	++
H_2	"	"	"	"	+	++	++
H_3	"	"	"	"	+	++	++
H_4	Inaba	"	"	"	+	++	++
H_5	Ogawa	"	Night soil	"	+	++	+
H_6	"	"	"	"	+	++	+
H_7	Inaba	"	"	"	+	++	++
H_8	Ogawa	"	"	"	+	++	++
10255	"	"	Case	"	+	++	++
10256	"	"	"	"	+	++	++
K001	"	Korea	"	"	+	++	-
34	"	Phillip-ines	"	?	+	++	+
388nh	"	"	"	1962	+	++	-
DH1961	"	Thailand	"	1961	+	++	-
U13	"	"	"	1960	+	++	-
126	Hikojima	Indonesia	"	?	+	++	++
N2B	Ogawa	New Guinea	"	1962	+	++	-
NG149	Inaba	"	"	"	+	++	-
NG107	Hikojima	"	"	1963	+	++	-
D67	"	El Tor	Carrier	1930	+	++	++
D20	"	"	"	1930	+	++	++
DL133	Inaba	"	"	1933	+	++	++
B19a	Ogawa	Burma	Case	1963	+	++	++
M45	"	Malaya	"	"	+	++	++
P18149	Hikojima	Phillip-ines	?	"	+	+	-
P18326	"	"	"	"	+	+	-
P18136	"	"	"	"	+	+	-
P17978	"	"	"	"	+	+	-
P16718	Ogawa	"	"	"	+	+	-
P16466	"	"	"	"	+	+	+
P16759	"	"	"	"	+	+	-
M - 3734	Hikojima	Malacca	"	"	+	++	-
M - 335	"	"	"	"	+	++	-
M - 385	"	"	"	"	+	+	-
T - 3613	Inaba	Taiwan	"	1962	+	++	+
T - 3601	"	Phillip-ines	"	"	+	++	+

Table 42 (contd.)

Strain	Heiberg	Locality of Isolation	Source	Year of Isolation	CR*	VP**	HL***
NAG							
965	I	East Pakistan	Case	1962	+	+	++
658	II	"	"	"	+	-	++
172	V	"	"	"	-	-	++
454	III	"	"	"	+	-	++
H_9	I	Hong Kong	"	1963	+	++	-
H_{10}	III	"	"	"	+	+	-
H_{11}	I	"	"	"	+	-	-
H_{12}	III	"	"	"	-	-	-
W343	II	?	?	?	+	+	+
W255	I	"	"	"	-	-	+
586	II	"	"	"	+	-	+

CR* = Cholera red reaction ;

VP** = Voges-Proskauer reaction;

HL*** = haemolysis;

+ = weak positive;

++ = strong positive;

? = information doubtful or not known.

Assay of phage by the agar layer method

The agar layer method for plating bacterial viruses was developed by Gratia (1936) and independently by Hershey, Kalmanson, and Bronfenbrenner (1943). The method is now in general use.

Procedure. A measured amount of the phage lysate and a drop of concentrated suspension of the host bacteria are mixed in about 2-2.5 ml. of warm molten 0.6-0.7 per cent. agar. The mixture is poured over the surface of an ordinary nutrient agar plate and allowed to harden to form a thin layer. After the upper layer has solidified, the plate is incubated at 37°C. The bacteria grow to produce a multitude of tiny superficial colonies in the upper layer, and are nourished by the lower layer. They form an opaque lawn in which plaques are easily seen as clear "holes".

The details of the procedure are as follows. The soft 0.6-0.7 per cent. agar is melted, cooled to 46°C and transferred with a warmed pipette in 2.5 ml. amounts to warmed test tubes which are held at 46°C in a water bath. The host bacterial suspension is prepared by washing the surface growth of an overnight agar slant of the organism with 5 ml. of nutrient broth. One drop of this suspension is added to each of the tubes of soft agar and thereafter a measured quantity of the suspension of phage dilution is added to the soft agar - culture mixture and the entire contents of the tube are poured immediately over the surface of an agar plate. The plate is rocked gently to mix the bacteria and phage particles and also to spread the mixture over the surface. Both the base agar and soft agar layers should be allowed to harden in petri dishes resting on a levelled surface so as to ensure uniform distribution

of the plaques on the surface of the plate.

By this method the host bacteria and the phage particles are more uniformly distributed over the surface of the plate than by the spreading technique. Phage samples up to 1 ml. in volume can be plated per petri dish. Because there is rapid diffusion of the phage particles in the soft agar layer the plaque size is larger than that given by the spreading method and hence variation in plaque morphology may be more readily studied.

Replica plating

The replica plating technique was devised by Lederberg and Lederberg (1952). This method permits the transfer or replica plating of bacterial growth from an initial plate to corresponding sites on a series of other plates. The technique is useful for many purposes including (i) the rapid detection of biochemical mutants, (ii) determination of spectra of antibiotic sensitivity, and (iii) the scoring of large numbers of colonies of recombinant bacteria, in genetic analysis, for inheritance of various characters.

A sterile square of velveteen is placed, nap up, on a cylindrical support of wood or metal having a diameter slightly less than that of a petri plate. A rubber band is used to hold the fabric firmly in place. The bacterial colonies on the initial agar plate are then transferred to the velveteen by inverting this "master" plate on to the fabric and pressing gently. The imprinted fabric can now serve as the master pattern for the inoculation of

other plates. Each thread of the pile fabric acts as an inoculating needle for sterile agar plates that are subsequently inverted on to the velveteen. As many as 6 to 8 replicas may be printed from a single pad. About 10-30 per cent. of the bacteria of the initial plate are transferred to the velveteen and about the same proportion of these is deposited on the surface of the replica plate (Lederberg and Lederberg, 1952). The resulting growth on the replica plates will be at corresponding sites on the initial plates since members of each clone that developed on the initial plate are distributed to the new plates without disturbance of their spatial relationships. Lederberg and Lederberg (1952) used this technique for detection of phage resistant mutants of E. coli. The growth from a phage-susceptible population which had developed on plain nutrient agar was imprinted on velveteen and serial replicas were then transferred to several plates containing nutrient agar plus phage. Colonies consisting of phage-resistant cells were found to develop on identical sites on each replica plate. This indicated that the resistant cells transferred to the phage plates were derived from small clones of resistant mutants already present at corresponding sites on the initial plain agar plate.

Methods of detection of lysogenic strains

In order to demonstrate lysogeny in El Tor strains the following methods were used:

(1) El Tor strains were grown together in nutrient broth. At times varying from 24 hr to 3 days portions of these broth cultures were sterilized by heating in the water bath at 56°C for ½-1 hr. The remainder of each culture was sterilized with a few drops of chloroform which was removed by bubbling sterile air through the culture 24 hr after its addition. These heat-inactivated and chloroform-sterilized broth cultures of the El Tor strains would contain phage particles if the strains were lysogenic because in a culture of lysogenic bacteria a small proportion of the population undergoes spontaneous lysis with concomitant release of infectious phage particles whose presence may be detected by spotting on lawns of sensitive bacteria. The treated broth cultures were therefore spotted undiluted and after serial tenfold dilutions in broth on lawns made separately from young cultures of various strains as shown in table 43 (see Results).

(2) The above heat-inactivated or chloroform-sterilized broth-cultures containing the potential phage, were passaged in young nutrient broth cultures of their respective suspected indicator strains in order to enhance the concentration of phage; thereafter they were treated as before for detection of phage.

(3) For detection of lysogeny the methods of Clarke (1964) and Papavassiliou (1960) were also tried. Both methods involved stabbing the prospective lysogenic strains into a soft overlay plate

seeded with strains sensitive to the potential phages that were expected to be isolated from these lysogenic strains. In a positive case, zones of complete or partial clearing would form around the stabs on incubation of the plates at 37°C for 24 hr. In doubtful cases, further passages of the suspected areas were made by picking the respective spot together with the underlying agar and incubating in nutrient broth at 37°C overnight. This passage was expected to raise the concentration of the phage that was being sought.

(4) Test for lysogeny was also done on nutrient agar plates inoculated by stabbing with prospective lysogenic strains grown in nutrient broth at times varying from 24 hr to 3 days. After incubating overnight at 37°C, the plates were sterilized over chloroform vapour and thereafter they were seeded separately with the young broth cultures of the same strains in soft semi-solid agar poured on to the plates. In a positive case small areas of partial or complete clearing would appear around the stabs after incubation.

(5) Strains of V. cholerae and V. eltor were grown separately on nutrient agar slants for 24 hr at 37°C. Cells from each of the slants were suspended in 9 ml. of sterile 0.85 per cent. sodium chloride. These suspensions were then exposed to doses of UV irradiation for 1-3 min. at a distance of 20 cm. from the source on sterile petri dishes kept constantly shaking. After irradiation, the contents of the dishes were transferred to sterile test tubes and 1 ml. of X10 broth was added to each tube;

the reconstituted broth cultures were incubated at 37°C for 24 hr in the dark to prevent uncontrolled photoreactivation. The source of UV light was enclosed in a cabinet and the whole procedure of irradiation was carried out with a yellow filter fitted. After incubation for 24 hr the cultures were centrifuged at 1,200 g for 10 min. and the supernatants were sterilized by chloroform and spot tested as before for detection of phage on lawns prepared separately from different vibrio strains.

(6) Vibrio strains suspended in distilled water were disintegrated with a Mullard ultrasonic disintegrator. The bacterial suspension was put into a small tube, pre-cooled in ice, and kept in iced water during the disintegration process which proceeded for 5, 10 and 15 min.; aliquots of the treated suspension were added to 10-ml. amounts of nutrient broth and incubated overnight at 37°C. These were then filtered through membrane filters. The filtrates were spot tested for the presence of phage on lawns made from young cultures of vibrio strains.

Methods for isolation of biochemical mutants using chemical mutagens.

Manganese chloride. Aliquots (10 ml.) of overnight broth cultures of the strain under test were centrifuged. The supernatant was discarded and 5 ml. of a 0.4 per cent solution of $MnCl_2$ at 37°C was added to the deposit and mixed well. This mixture was then incubated at 37°C in the water bath for 1 hr. Thereafter the

cells were deposited by centrifugation and the $MnCl_2$ was discarded. The deposit was then mixed well in 5 ml. nutrient broth containing graded concentrations of penicillin ranging from 500-3000 units per ml. of the media and incubated overnight at 37°C. Serial tenfold dilutions were made from these and 0.1-ml. volumes were spread on nutrient agar plates. After overnight incubation, the colonies from the plates were replicated on plates containing minimal media. After 72 hr incubation at 37°C the master and the replica plates were compared for isolation of mutants.

Acriflavine. Strains of vibrio under test were grown at 37°C overnight in nutrient broth containing acriflavine 1:60,000. Serial tenfold dilutions were then made in nutrient broth and 0.1-ml. volumes were spread on nutrient agar plates. After overnight incubation at 37°C, the colonies were replicated on minimal media and the plates were examined for evidence of mutants as before.

Methods for electron microscopy of cholera bacteriophage.

The specimen grids.

Collodion films on copper grids, 3.05 mm. in diameter, were coated with carbon in vacuo (Bradley, 1960). These films were then used for holding the specimen.

The physical state of the surface of a support film affects the spreading of the negative staining material. Brenner and Horne (1959) observed that phosphotungstate did not spread well on carbon

films contaminated with oil molecules. It was therefore necessary to de-grease the film by dipping the grid into chloroform immediately before use.

Phosphotungstic acid preparation. For negative staining, a 2 per cent. aqueous solution of phosphotungstic acid was made up and adjusted to pH 7.2 using normal KOH (Brenner and Horne, 1959).

Ammonium acetate solution. This was 0.1 M ammonium acetate in distilled water; the pH was adjusted to 7.0.

Preparation of the specimen. High-titre phage suspension (about 10^{10} PFU/ml) in T_1N_1 medium was filtered through an Oxoid membrane filter to remove the bacterial debris. The filtrate containing the phage particles was centrifuged at 135,000 g for 1 hr in a Spinco ultracentrifuge and the supernatent fluid was discarded. The pellet was re-suspended in 0.3 ml. of 0.1 M neutral ammonium acetate solution. One drop of this phage suspension was taken on a clean microscope slide. A similar drop of 2 per cent. phosphotungstic acid solution was added and the drops were mixed well together. A freshly de-greased support film was then touched on to the surface of the mixture. The excess liquid was removed from the grid with filter paper so as to leave a thin film covering the grid. After drying near a 60-watt table lamp the specimen was ready for examination in the electron microscope.

Electron microscopy. An A.E.I. E.M.6. electron microscope was used. The accelerating voltage was 50 KV providing an electron beam with a wavelength of 0.05Å. Before taking an electron micrograph of the image, astigmatism was corrected. The image was received on 3¼ x 3¼ in. plates.

Techniques for making preparations that reveal stages in bacterial lysis and bacteriophage proliferation

A suspension of cholera phage and the susceptible growing vibrio strain were mixed in liquid culture medium and incubated at 37°C in the water bath. Samples were taken from this phage-culture mixture at different time intervals; they were immediately chilled in iced water and sedimented by centrifugation at 2000 g for 15 min. The sediments were washed by successive recentrifugation and resuspension in 1 per cent. formalin in saline and then 1 per cent. formalin in distilled water. Finally the deposits were resuspended in small volumes of distilled water. This procedure has the advantage of yielding a clean preparation free from nutrient and from metabolic products that tend to obscure fine details of the relation between bacteria and developing phage particles (Wyckoff, 1949). The final suspensions were serially diluted in distilled water. A minute drop from each dilution was placed on copper grids coated with collodion and carbon in 2 layers and allowed to dry in a dessicator. Up to 6 grids were mounted on a holder and shadowed with gold palladium alloy (60:40) at an angle

of 15°-18°. The specimens were then ready for examination in the electron microscope.

A control specimen without addition of phage was also performed to exclude artefacts.

Results.

RESULTS

Screening tests for lysogenic strains.

The detection of lysogenic strains was attempted with El Tor vibrios as lysogeny is more common in El Tor strains than in classical cholera vibrios. Various methods were adopted (see Methods). The presence of the temperate phage was looked for in heat-inactivated or chloroform-sterilized broth cultures of each of a large number of El Tor vibrios (31) on lawns of a phage-sensitive indicator strain of *V. cholerae*. The experimental observations are summarized in the following tables. The results were consistently negative with the exception of tests that involved *V. cholerae* strain 508 as an indicator strain.

Table 43.

Results of various methods used for the detection of lysogenic strains.

Lawn made with strain	Dilution of heat-inactivated and chloroform-sterilized broth cultures*							
	Heat- inactivated				Chloroform-sterilized			
	N	10^{-1}	10^{-2}	10^{-3}	N	10^{-1}	10^{-2}	10^{-3}
H_1	-	-	-	-	-	-	-	-
H_2	-	-	-	-	-	-	-	-
H_3	-	-	-	-	-	-	-	-
H_4	-	-	-	-	-	-	-	-
H_5	-	-	-	-	-	-	-	-
H_6	-	-	-	-	-	-	-	-
H_7	-	-	-	-	-	-	-	-
H_8	-	-	-	-	-	-	-	-
10255	-	-	-	-	-	-	-	-
10256	-	-	-	-	-	-	-	-

- = No lysogeny.

* = Broth cultures of strains H_1, H_2, H_3, H_4, H_5, H_6, H_7, H_8, 10255 and 10256 grown for times varying from 24 hr to 3 days at 37° C.

Table 43. (contd.)

Lawn made with strain	Dilution of heat-inactivated* and chloroform-sterilised broth cultures at times varying from 24 hr to 3 days							
	Heat-inactivated				Chloroform-sterilised			
	N	10^{-1}	10^{-2}	10^{-3}	N	10^{-1}	10^{-2}	10^{-3}
K001	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-
388nh	-	-	-	-	-	-	-	-
DB 1961	-	-	-	-	-	-	-	-
U 13	-	-	-	-	-	-	-	-
126	-	-	-	-	-	-	-	-
N2B	-	-	-	-	-	-	-	-
NG149	-	-	-	-	-	-	-	-
NG107	-	-	-	-	-	-	-	-
D67	-	-	-	-	-	-	-	-
D20	-	-	-	-	-	-	-	-
DL133	-	-	-	-	-	-	-	-
B19a	-	-	-	-	-	-	-	-
M45	-	-	-	-	-	-	-	-

- = No lysogeny

* = Broth cultures of strains K001, 34, 388nh, DB1961, U13, 126, N2B, NG149, NG107, D67, D20, DL133, B19a and M45 grown together at times varying from 24 hr to 3 days at 37°C.

Some of the above broth cultures gave slightly equivocal results; on further passage with the prospective sensitive strain, there was no evidence of the existence of phage particles.

TABLE 44.

Results of spot test of chloroform-sterilised 24 hr broth cultures.

Broth cultures of strains	Lawns made with young cultures of strains													
	12r	569B	834	V 154	508	PR 1212	352	601	583	8021	7270	7254	V_0	V_1
H_1	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_2	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_3	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_4	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_5	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_6	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_7	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_8	-	-	-	-	+	-	-	-	-	-	-	-	-	-
126	-	-	-	-	+	-	-	-	-	-	-	-	-	-
10255	-	-	-	-	+	-	-	-	-	-	-	-	-	-
10256	-	-	-	-	+	-	-	-	-	-	-	-	-	-
34	-	-	-	-	+	-	-	-	-	-	-	-	-	-
NG149	-	-	-	-	+	-	-	-	-	-	-	-	-	-
NG107	-	-	-	-	+	-	-	-	-	-	-	-	-	-
D67	-	-	-	-	+	-	-	-	-	-	-	-	-	-
D20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
388nh	-	-	-	-	+	-	-	-	-	-	-	-	-	-
N2B	-	-	-	-	+	-	-	-	-	-	-	-	-	-
M-3734	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M-3735	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M-385	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M-C-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P16466	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P16718	-	-	-	-	+	-	-	-	-	-	-	-	-	-
T3613	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T3601	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P18149	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P18136	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P16759	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P18326	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P17978	-	-	-	-	+	-	-	-	-	-	-	-	-	-

- = No plaque

+ = Discrete plaque.

Table 44 (contd.)

Broth cultures of strain	Lawns made with young cultures of strains							
	BH29	BH202	34	1843	1844	B2	60	C116
H_1	-	-	-	-	-	-	-	-
H_2	-	-	-	-	-	-	-	-
H_3	-	-	-	-	-	-	-	-
H_4	-	-	-	-	-	-	-	-
H_5	-	-	-	-	-	-	-	-
H_6	-	-	-	-	-	-	-	-
H_7	-	-	-	-	-	-	-	-
H_8	-	-	-	-	-	-	-	-
126	-	-	-	-	-	-	-	-
10255	-	-	-	-	-	-	-	-
10256	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-
NG149	-	-	-	-	-	-	-	-
NG107	-	-	-	-	-	-	-	-
D67	-	-	-	-	-	-	-	-
D20	-	-	-	-	-	-	-	-
388nh	-	-	-	-	-	-	-	-
N2B	-	-	-	-	-	-	-	-
M-3734	-	-	-	-	-	-	-	-
M-3735	-	-	-	-	-	-	-	-
M385	-	-	-	-	-	-	-	-
M-C-13	-	-	-	-	-	-	-	-
P16466	-	-	-	-	-	-	-	-
P16718	-	-	-	-	-	-	-	-
T3613	-	-	-	-	-	-	-	-
T3601	-	-	-	-	-	-	-	-
P18149	-	-	-	-	-	-	-	-
P18136	-	-	-	-	-	-	-	-
P16759	-	-	-	-	-	-	-	-
P18326	-	-	-	-	-	-	-	-
P17978	-	-	-	-	-	-	-	-
Control *V.cholerae* 508	-	-	-	-	-	-	-	-

- = no plaque formation

Thus, of 31 El Tor strains tested, 25 gave evidence of lysogenicity in tests with V. cholerae strain 508 as indicator. The chloroform-sterilized broth cultures of strains that produced discrete plaques on lawns of strain 508 as shown in Table 44 also produced similar plaques only with strain 508 when other methods of detection of lysogeny were tried (see Methods). Control tests with drops of chloroform-sterilized cultures of V. cholerae strain 508 against all of the potential indicator cultures confirmed that strain 508 was not itself lysogenic.

Attempts to increase yield of phage.

Attempts to increase the yield of phage from various El Tor vibrios by ultraviolet irradiation or ultrasonic disintegration of the 17 lysogenic strains listed in Table 45 were unsuccessful. An increase in number manifested by production of semiconfluent lysis in place of discrete plaque formation at the same dilution was observed in some cases, but no significantly enhanced yield was obtained.

Before further work was attempted, experience in the handling of cholera phages was obtained by performing the following studies with known phages.

Table 45

Lysogenic strains used in attempts to raise the plaque titre by UV irradiation or ultrasonic disintegration

Lysogenic strain	Susceptible indicator strain
H_1	508
H_2	"
H_3	"
H_4	"
H_5	"
H_6	"
H_7	"
H_8	"
126	"
10255	"
10256	"
34	"
NG149	"
NG107	"
D67	"
388nh	"
N2B	"

The One-step Growth Experiment

Procedure - The details of a typical one-step growth experiment for cholera bacteriophage are as follows:-

Reagents:-

(1) Stock cholera bacteriophage group III diluted in nutrient broth to a concentration of 1×10^7 particles/ml.

(2) Susceptible young culture of V. cholerae strain 154 grown in broth to a concentration of 10^7 cells/ml. approximately.

(3) Antiphage group III serum diluted 1:10 in broth.

(4) Nutrient broth for dilution of the infected culture.

(5) Nutrient agar plates and tubes containing 3 ml. each of 0.7 per cent. melted agar in nutrient broth.

The reagents are accurately measured in test tubes as stated in the protocol and brought to 37°C before starting the experiment so as to eliminate the effect of temperature variation on the bacterial metabolism.

Protocol:-

Time in min.	Tube	Procedure
0	(1) Adsorption	0.1 ml. of phage group III (1 x 107 particles/ml.) is added to 0.9 ml. of host strain 154.
8	(2) Serum	0.1 ml. of (1) is added to 0.9 ml. of 1:10 dilution of anti-phage 3 serum.
18	(3) Dilution	0.1 ml. of (2) is added to 9.9 ml. of broth.

The dilution tube (3) is kept in a water bath at 37°C. Samples of 0.1 ml. are removed at various time intervals and plated by agar layer method against the indicator strain 154 as shown in table 46.

Table 46.

Plaque counts at different times after adsorption of group III cholera bacteriophage to V. cholerae strain 154

Time in min.	Further dilution of dilution tube (3) in broth	Inoculum per plate	Mean plaque count	Total plaque count per ml.
20	Undiluted	0.1 ml.	97	97×10^5
25	"	"	102	102×10^5
30	"	"	101.5	101.5×10^5
35	1:10	"	47.5	475×10^5
45	1:10	"	198.5	1985×10^5
55	1:100	"	34	3400×10^5
65	"	"	43.5	4350×10^5
75	"	"	27	2700×10^5
85	"	"	26	2600×10^5
95	"	"	15	1500×10^5
105	"	"	6	600×10^5

The one-step growth curve for group III cholera bacteriophage was obtained by plotting the plaque counts against various times in min. as shown in fig. 31.

Following the same method as with group III cholera bacteriophage, the plaque counts at different times after adsorption of group I cholera phage to V. cholerae strain 154 are shown in table 47 and the one-step

growth curve is drawn by plotting the plaque counts against time in min. as shown in fig. 32.

Table 47.

Plaque counts at different times after adsorption of group I cholera bacteriophage to V. cholerae, strain 154.

Time (min.)	Total plaque count/ml.
15	21×10^7
20	36×10^7
25	43×10^7
30	36×10^7
35	24×10^7
40	27×10^7
45	56×10^7
50	95×10^7
55	201×10^7
60	354×10^7
75	374×10^7
90	89×10^7

Fig. 31. The one-step growth curve of group III cholera phage

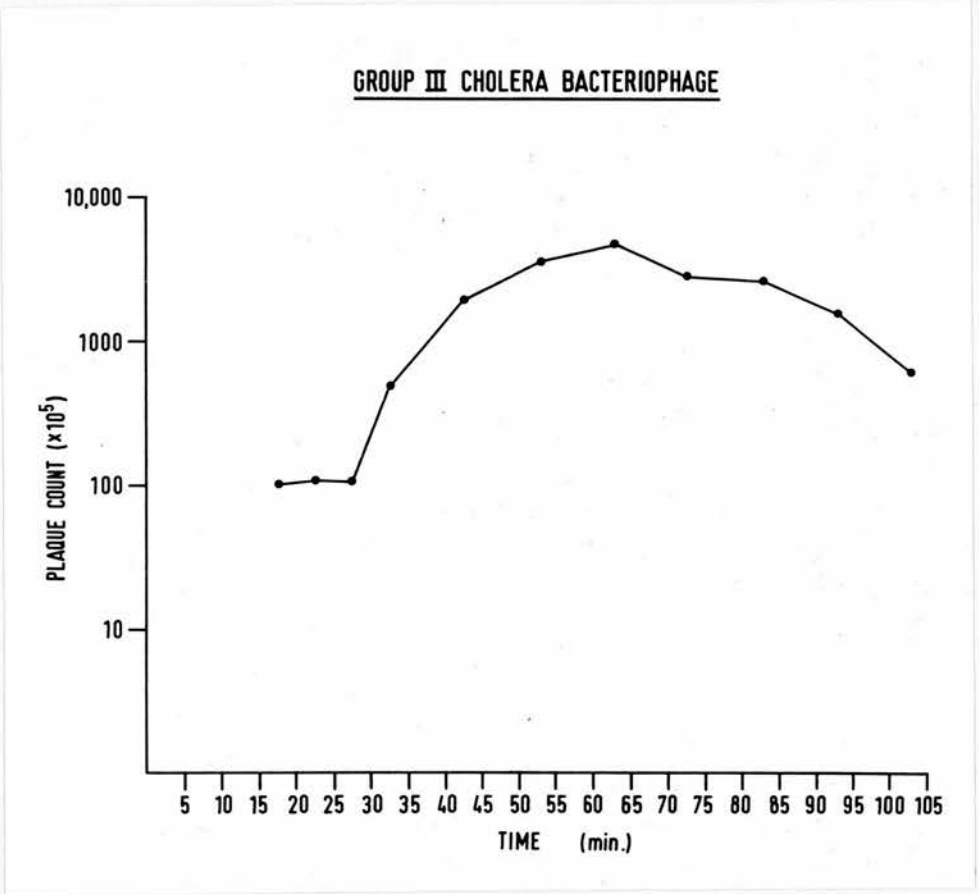
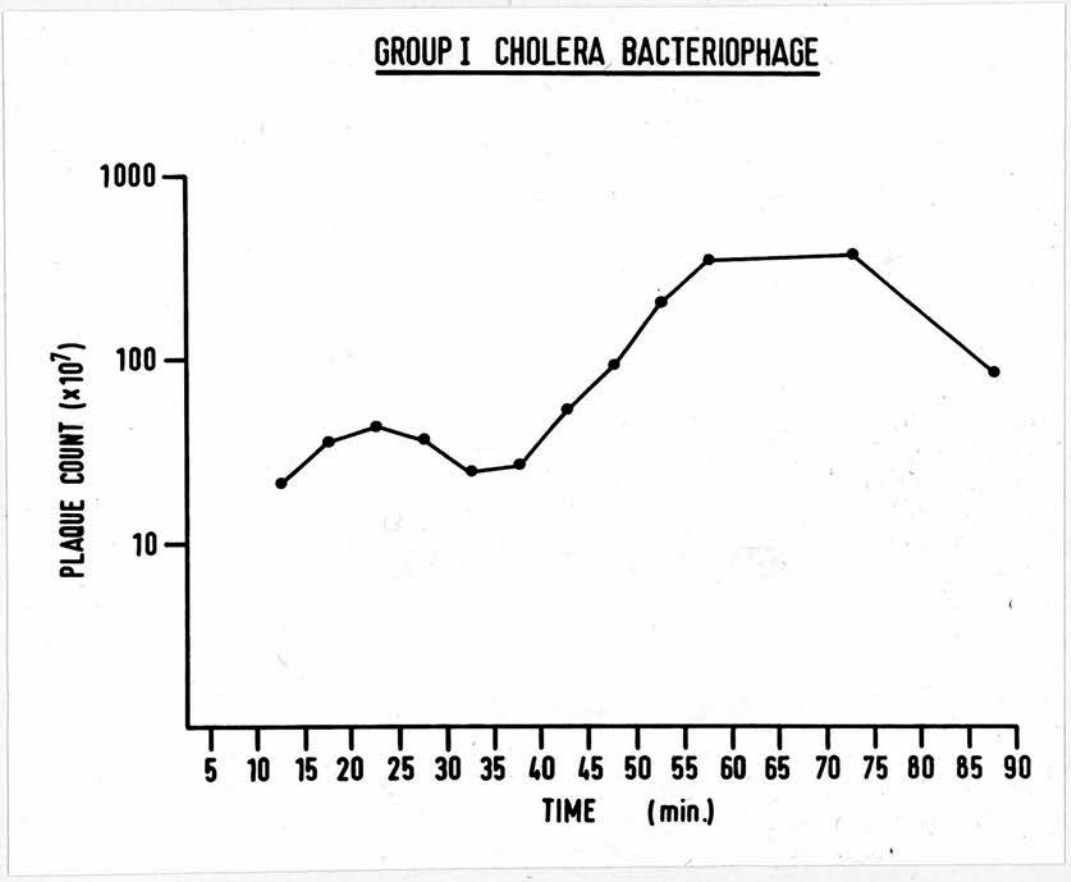

GROUP III CHOLERA BACTERIOPHAGE
10,000
1000
100
10
PLAQUE COUNT (x10⁵)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
TIME (min.)

Fig 32. The one-step growth curve of group I cholera phage


GROUP I CHOLERA BACTERIOPHAGE
1000
100
10
PLAQUE COUNT (x10⁷)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
TIME (min.)

Proportionality of phage concentration to plaque count

The result of an experiment in which equal volumes of increasing concentrations of a phage suspension were plated on agar plates is shown in fig 33. It is evident from the graph that each twofold increase in concentration of phage produced a corresponding two-fold increase in the average number of plaques per plate. Thus by counting the number of plaques produced, the number of plaque-forming units (PFU) in the original phage suspension can be calculated. This is known as the plaque titre of the phage suspension under test.

To determine the infective titre of a phage suspension, the number of plaques formed on the assay plate is multiplied by the dilution factor. For example, if 0.1 ml. of a 10^{-5} fold dilution of a phage lysate produced an average of 15 plaques per plate, one would calculate a titre of $15 \times 10 \times 10^5 = 1.5 \times 10^7$ infective units per ml. of the phage suspension.

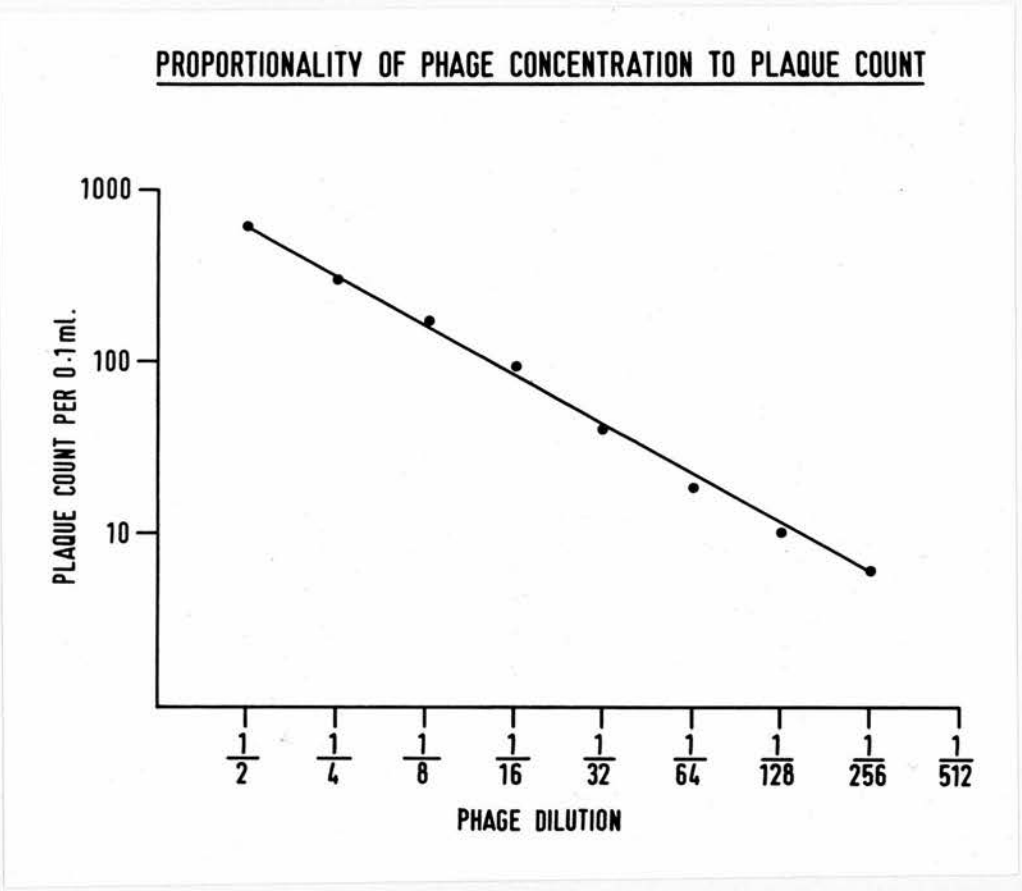
The proportionality of phage concentration to plaque count was observed with phage group I and indicator strain 154. The phage suspension was diluted in nutrient broth to contain 5×10^4 PFU/ml. approximately. From this suspension, successive twofold dilutions were made in nutrient broth. A 0.1-ml. volume from each dilution was plated in duplicate by the agar layer method against 0.2 ml. of the young culture of indicator strain 154. The plates were incubated at 37°C overnight. The plaques produced in each of the plates are shown in table 48 and the data are presented graphically in fig. 33.

Table 48.

Proportionality of group I phage concentration to plaque count.

	No. of plaques produced in mixtures of a standard bacterial concentration (*V. cholerae* strain 154) and phage suspension diluted 1 in .							
	2	4	8	16	32	64	128	256
Expt. 1	692	310	191	103	31	16	8	7
Expt. 2	541	290	164	94	52	22	13	6
Mean	616.5	300	177.5	98.5	41.5	19	10.5	6.5

Fig. 33


PROPORTIONALITY OF PHAGE CONCENTRATION TO PLAQUE COUNT
1000
100
10
PLAQUE COUNT PER 0·1 ml.
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
PHAGE DILUTION

Heat-inactivation experiments and stability studies

The heat susceptibility of the 4 groups of cholera phages were studied under identical conditions. Each of the phages under test was put in thin-walled small glass tubes and heated in a thermostatically controlled water bath. After being kept in the water bath at the desired temperature, 0.1-ml. samples were removed and plated with a sensitive strain of V. cholerae by the agar layer method. Readings were taken after incubating the plates overnight at 37°C. The tests were done in duplicate and the results are shown in table 49.

The stability of group I and group II phages at 37°C for a period of 20 days was also studied and the results are shown in table 50.

Table 49.

Effect of heating at different temperatures for 30 min. on cholera phages.

Phage of group	Character of plaques after exposure to temperatures of								Range of Thermal death point °C
	37°C	55°C	58°C	60°C	64°C	68°C	71°C	74°C	
I	cl	cl	cl	cl	>scl	++++	6	-	64-71
	cl	cl	cl	cl	>scl	++++	10	-	
II	cl	cl	cl	cl	cl	scl	26	-	68-71
	cl	cl	cl	cl	cl	scl	34	-	
III	cl	cl	>scl	scl	scl	++++	-	-	58-68
	cl	cl	>scl	scl	scl	++++	-	-	
IV	cl	cl	cl	cl	>scl	60	-	-	64-68
	cl	cl	cl	cl	>scl	45	-	-	

cl = confluent lysis; scl = semiconfluent lysis.

Table 50.

Stability of group I cholera phage at 37°C

Days of incubation at 37°C	Mean plaque count and character. Dilution of Phage in T_1N_1 medium.				Titre after each incubation	Original titre
	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
1	scl	++++	48	4	4.8×10^9	9.0×10^9
2	scl	++++	32	3	3.2×10^9	
4	scl	+++	10	-	1.0×10^9	
8	scl	+++	10	1	1.0×10^9	
10	scl	84	6	-	8.4×10^8	
20	scl	50	4	-	5.0×10^8	

- = no plaques; +++, ++++ = increasing numbers but uncountable;

scl = semiconfluent lysis.

Table 50 (contd.)

Stability of group II cholera phage at 37°C

Days of incubation at 37°C	Mean plaque count and character				Titre after each incubation	Original titre
	Dilutions of phage in T_1N_1 medium					
	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
1	scl	+++	32	3	3.2×10^9	4.5×10^9
2	scl	+++	20	1	2.0×10^9	
4	scl	+++	9	-	9.0×10^8	
8	scl	75	5	-	7.5×10^8	
10	++++	50	-	-	5.0×10^8	
20	++++	49	-	-	4.9×10^8	

- = No plaques;

+++, ++++ = increasing numbers but uncountable;

scl = semiconfluent-lysis.

Inactivation of cholera organisms and cholera phage by ultraviolet irradiation

Killing effect of UV light on V.cholerae

A suspension matching International Opacity Tube No. 4 was made in Ringer solution (¼ strength) with an overnight nutrient agar slant culture of a vibrio strain. Aliquots (3 ml) of the suspension were taken in sterile petri plates and irradiated with constant shaking at a distance of 60 cm. from an ultra-violet light (UV) source for 15, 30, 45, 100 and 200 sec. respectively. Viable counts before and after irradiation of the samples were done by the method of Miles and Misra (1938). The results were as follows:

Viable count before irradiation	1.33×10^8 / ml.
Viable count after irradiation for 15 sec.	3.0×10^6 / ml.
Viable count after irradiation for 30 sec.	8.75×10^5 / ml.
Viable count after irradiation for 45 sec.	7.5×10^4 / ml.

N.B. After 100 and 200 sec. irradiation, no colonies were cultured from 0.1-ml. volumes of dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} used for viable counts.

By calculation, the killing effects of the UV irradiation for the above time periods are as follows:-

Per cent of killing after 15 sec. irradiation = 97.74

" " " 30 sec. " = 99.34

" " " 45 sec. " = 99.94

Inactiviation of cholera bacteriophage by UV light

This was studied with group 1 cholera bacteriophage. A volume of 4 ml. of the phage suspension was taken in a sterile petri dish and irradiated with constant shaking at a distance of 60 cm. from an ultra-violet light (UV) source for ½, 1½, 3½, 7½, 11½ and 16 min. respectively. The table 51 and the fig. 34, summarize the results of an experiment in which the surviving fractions of the phage particles were assayed by the agar layer method using the indicator strain 154.

Table 51

Inactiviation of cholera phage by ultra-violet irradiation

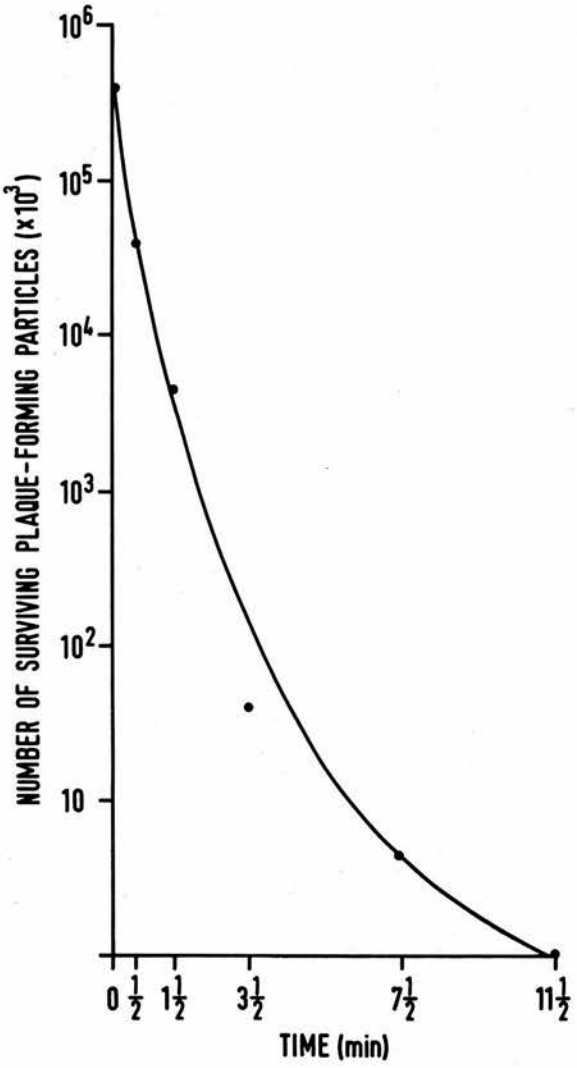
Period irradiated in min.	Dilution of the phage after irradiation in nutrient broth						Plaque count/ml.
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
½	CL	Scl	+++	40	6	-	4×10^{7}
1½	Scl	+++	47	5	-	-	4.7×10^{6}
3½	41	1	-	-	-	-	4.1×10^{4}
7½	4.5	1	-	-	-	-	4.5×10^{3}
11½	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
Control 0	CL	CL	Scl	+++	40	3.5	4×10^{8}

CL = confluent lysis ; Scl = semiconfluent lysis ; +++ = uncountable number of plaques.

It is evident from the results that cholera bacteriophage particles were completely inactivated by irradiation with UV light for a period exceeding 7½ min. but less than 11½ min.

Fig. 34

EFFECT OF TIME OF EXPOSURE ON INACTIVATION OF CHOLERA PHAGE (GROUP I) BY UV IRRADIATION.



Effects of drugs on cholera phages

Explanatory note: Combined therapy with phage and antibiotics is sometimes attempted. The writer therefore investigated the effects of chemotherapeutic agents on cholera phages.

The effects of penicillin, streptomycin sulphate and sulphamezathine on cholera phage of groups I and IV were studied. Different concentrations of these drugs were mixed with constant volumes of phage suspension each having a plaque titre of 10^7 particles per ml. and the mixtures were kept overnight at room temperature. A 0.1-ml. volume of both undiluted and diluted phage-drug mixtures was plated by the agar layer method on to the phage-sensitive antibiotic-resistant indicator strain 154. The plates were then incubated at 37°C for 18-24 hr. The plaques were counted and the effect of the particular drug on each of the phage suspensions was determined by comparing the plaque count of the test sample with the count obtained from the corresponding control. The actual procedures followed for each of the drugs and the results obtained are summarized, as follows:-

Penicillin. A 0.5-ml. volume of the phage suspension was added to 5000 units of penicillin contained in 0.5 ml. of sterile nutrient broth. The number of plaques that developed after overnight incubation of the test and control plates are shown in table 52.

Table 52

The effect of penicillin on cholera phage

Phage Group	Test (penicillin present)		Control (penicillin absent)	
	Mean plaque count at dilutions		Mean plaque count at dilutions	
	Undiluted	10^{-4}	Undiluted	10^{-4}
I	Cl	102	Cl	104
IV	Cl	71	Cl	100

Cl = confluent lysis

Streptomycin sulphate. A 0.5-ml. volume of the phage suspension was added to each of the solutions containing 100, 50, 25 and 12.5 mg. of streptomycin sulphate per 0.5 ml. of sterile nutrient broth. The plaque counts developing in plates incorporating these mixtures after overnight incubation are recorded in table 53.

Table 53.

The effect of streptomycin sulphate on cholera phage

Phage Group	Test			Control	
	Streptomycin sulphate conc./ml. of mixture	Mean plaque count at dilutions		Mean plaque count at dilutions	
		Undiluted	10^{-4}	Undiluted	10^{-4}
I	100	Cl	95	Cl	106
	50	Cl	104		
	25	Cl	106		
	12.5	Cl	105		
IV	100	Cl	88.5	Cl	100
	50	Cl	90		
	25	Cl	98		
	12.5	Cl	100		

Cl = confluent lysis

Sulphamezathine. A 0.5-ml. volume of phage suspension was added to a solution of 166 mg. of sulphamezathine solution contained in 0.5 ml. of sterile nutrient broth and the mixture was kept at room temperature overnight. The numbers of plaques that developed after plating the mixture on to a sulphonamide-resistant indicator by the agar layer method are shown in table 54.

Table 54.

The effect of sulphamezathine on cholera phage

Phage Group	Test		Control	
	Mean plaque count at dilutions		Mean plaque count at dilutions	
	Undiluted	10^{-4}	Undiluted	10^{-4}
I	Cl	104	Cl	106
IV	SCl	1.5	Cl	100

Cl = confluent lysis; SCl = semi confluent lysis

From the above results the effects of the various drugs are summarized in table 55.

Table 55

The effects of drugs on cholera bacteriophage

Name of drug	Concentration per ml.	Effect on phage (per cent inhibition)	
		Phage Group I	Phage Group IV
Penicillin	2500 units	Nil	29
Streptomycin sulphate	50 mg.	10.3	11.5
Sulpha-mezathine	83 mg.	Nil	98.5

These results seem to be important and several repetitions of the experiment produced similar results.

The effect of pH on group II and group III cholera phages

The effect of pH on cholera bacteriophage was determined by mixing equal volumes of phage lysates with T_1N_1 broth at pH values varying from 4.5 to 9.5. The phage-broth mixtures were incubated at 37°C overnight and thereafter 0.1 ml. from each mixture was plated by the agar layer method on to sensitive V. cholerae strain 154. After overnight incubation at 37°C the character of the plaques was observed in each plate and the stability of the phage was determined. The results with 2 of the cholera phages are shown in table 56.

Table 56

The effect of pH on cholera phage

Phage	General character of plaques after exposure to pH of									
	4.5	5	5.5	6	6.5	7.2	8	8.5	9	9.5
Group II	DP	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	DP
Group III	DP	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	DP

DP = Discrete plaque Cl = Confluent lysis

These results show that the character of plaques is not influenced by prior exposure of phage to conditions in the pH range 5 - 9.

Production of high-titre serum in rabbits using phage with adjuvant

An emulsion was made with 1 ml. of cholera phage suspension containing 2.5 x 10^9 particles/ml. with 1 ml. of a mineral oil/ Arlacel A mixture as suggested by Dr. W.J. Herbert. Rabbits were immunised with a single subcutaneous dose of 1 ml. of this emulsion. From the third week after injection test bleedings were made from each of the rabbits. It was observed that in the presence of the adjuvant a single 0.5-ml. volume of the phage suspension incorporated in the emulsion was quite enough to produce high-titre serum. Cholera phages of Group I and Group II have thus been used with adjuvant for the production of antiphage sera (see p. 277 for details of comparative experiments without adjuvant). A dilution of 1 in 2048 of antiphage Group I serum and a dilution of 1 in 512 of the antiphage Group II serum produced with adjuvant neutralised about 97 per cent. and 98 per cent. of the homologous phages respectively.

Neutralisation of phage group IV by antiphage group IV serum

Method:-

(1) Twofold serial dilutions of the antiserum in nutrient broth up to 1:1024 were held in the water bath at 37°C for 5 min. prior to "time 0"

(2) At "time 0": Series (A) 1 ml. of phage stock ($1x10^7$ PFU/ml.) was added to each of the dilutions of the antiserum.

Series (B) 1 ml. of the same phage stock was added to 1 ml. of the nutrient broth and at once assayed by diluting 10^{-4} in nutrient broth and plating 0.1 ml. by the agar layer method against the indicator strain 154.

(3) At 20 min: (A) All the mixtures of antiserum and phage were diluted 10^{-2} in nutrient broth and a volume of 0.1 ml. from each dilution was plated by the agar layer method against the same indicator strain 154.

Series (B) The phage suspension was again diluted and assayed as at time 0.

(4) All the plates were incubated overnight at 37°C. The resulting plaques were counted and the log. percentages of the surviving phage particles were plotted against the time in minutes. The dilution of the antiserum that inactivated approximately 99 per cent. of the phage in 25 min. was then calculated from the graph and was found to be in the region of 1 in 256 (see fig. 35 and table 57).

Table 57.

Results of neutralization of phage group IV by antiphage group IV serum

Assay of phage and phage-serum mixture.	Mean number of plaques	Po*	log.Po	log% survivors
Phage group IV	66	1.32×10^7	7.1206	2
Phage group IV and serum dilution		P**	log P	
1:4	Nil	Nil	Nil	Nil
1:8	"	"	"	"
1:16	"	"	"	"
1:32	3.5	7×10^3	3.8451	$\bar{2}$.7245
1:64	15.5	3.1×10^4	4.4914	$\bar{1}$.3708
1:128	8.5	1.7×10^4	4.2304	$\bar{1}$.1098
1:256	52.5	1.05×10^5	5.0212	$\bar{1}$.9006
1:512	194	3.88×10^5	5.5888	0.4682
1:1024	538	1.08×10^6	6.0334	0.9128

* Po = initial phage concentration

** P = phage remaining after 25 min. contact with the serum dilution.

$$\% \text{ survivors} = \frac{P}{Po} \frac{100}{1}$$

$$= (\log P - \log Po) + 2$$

Key to plot the graph

$\bar{2}.7245 = -2.0000$
$+0.7245$
$- 1.2755$

Using graph paper and taking 10 small divisions of the graph = 0.2, we have $-1.2755 = -63.75$ small divisions

Similarly

$\bar{1}.3708 = -1.0000 + 0.3708 = -.6292 = -31.46$ small divisions

$\bar{1}.9006 = -1.0000 + 0.9006 = 0.0994 = -4.97$ small divisions

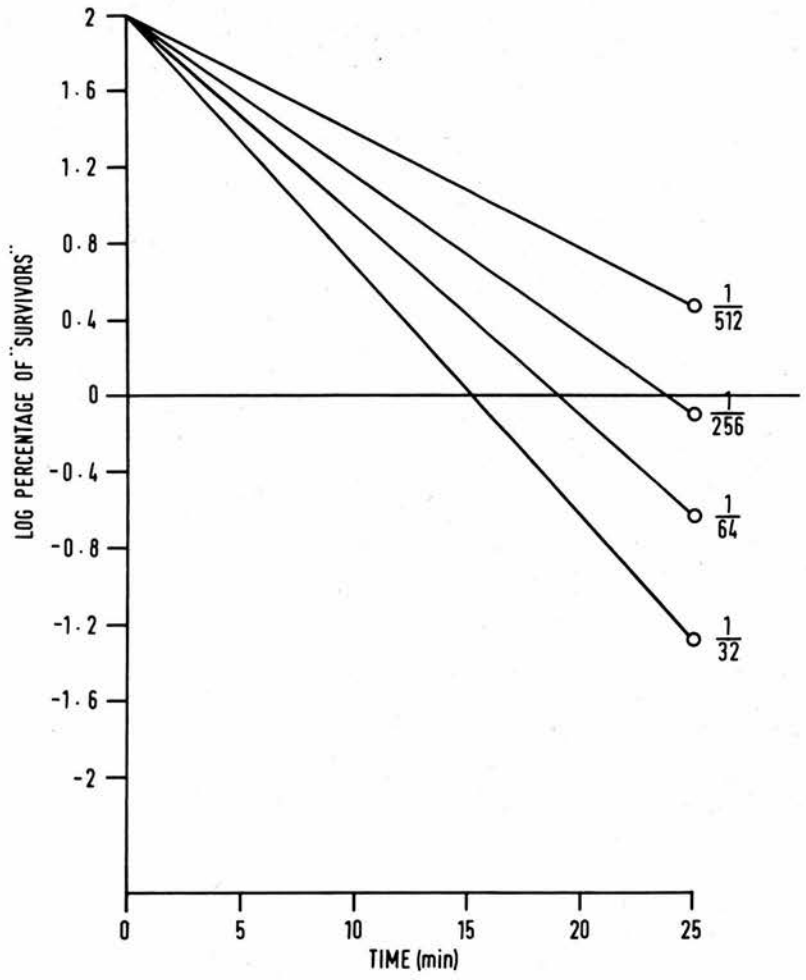
$0.4682 = 23.4$ small divisions

The dilution of the antiserum that inactivated approx. 99 per cent. of the phage was mixed with phage stock held at 37°C and samples were removed at 5, 10, 15, 20, 25 and 30 min. Dilution of each of the above samples up to 1 in 100 in broth was sufficient to dilute the antiserum beyond its effective concentration. A 0.1-ml. volume of each of the diluted samples taken at the above time intervals was plated by the agar layer method with the indicator strain 154 and the plates were incubated overnight at 37°C. A control phage assay was also included in the test and plated similarly after diluting 10^{-4} in broth. Plaques were counted in all of the plates and the numbers are recorded in the table. The details of the experiment are as follows:

Fig. 35

The log percentage of "survivors", i.e. residual non-inactivated plaque-forming particles, is plotted against time of exposure to the antiserum.

THE RATE OF INACTIVATION OF CHOLERA PHAGE (GROUP IV) PARTICLES BY VARYING DILUTIONS OF HOMOLOGOUS ANTI—SERUM *



From the previous experiment it was known that a dilution of 1 in 256 of antiphage group IV serum neutralized approximately 99 per cent. of phage group IV. To 1 ml. of this 1 in 256 dilution of the serum, 1 ml. of a suspension of phage group IV containing $1x10^7$ plaque forming units per ml. was added. A control was incorporated in the test by replacing serum with broth.

Both the test and control mixtures were kept in the water bath at 37°C. At intervals of 5 min. each of the test samples was diluted 10^{-2} in broth and 0.1 ml. was plated by the agar layer method. The control sample was diluted 10^{-4} in broth and a volume of 0.1 ml. was plated similarly. All of the plates were incubated at 37°C overnight. The plaques were counted and the numbers were as shown in table 58.

The mathematical calculations based on these data made it possible to draw regression lines that reveal the kinetics of phage neutralization in each case (fig. 36).

Table 58.

Data obtained and mathematical calculation involved in assay of phage antiserum.

Time in min. t	Total Plaque Count X	log of Plaque Count.	log% Survivors x	$(X - \bar{X})$	$(t - \bar{t})^2$	$(t - \bar{t})(X - \bar{X})$
t=0 (control)	1.106×10^7	7.0253	2.0000	1.5832	$(-16.66)^2 = 277.56$	$-16.66 \times 1.5832 = -26.3761$
t=10	3.0×10^5	5.4771	0.4518	0.0350	$(-6.66)^2 = 44.36$	$-6.66 \times 0.0350 = -0.2331$
t=15	2.13×10^5	5.3284	0.3031	-0.1137	$(-1.66)^2 = 2.76$	$(-1.66)(-0.1137) = 0.18874$
t=20	1.16×10^5	5.0645	0.1948	-0.2220	$(3.34)^2 = 11.16$	$(3.34)(-0.2220) = -0.74148$
t=25	8.5×10^4	4.9294	-0.0959	-0.5127	$(8.34)^2 = 69.56$	$(8.34)(-0.5127) = -4.2759$
t=30	4.7×10^4	4.6721	-0.3532	-0.7700	$(13.34)^2 = 177.96$	$(13.34)(-0.7700) = -10.2718$
100 $\bar{t} = \frac{100}{6} = 16.66$ (mean)		2.5006 $\rightarrow$ $\bar{X} = \frac{2.5006}{6} = 0.4168$ (mean)			$\Sigma 583.36$	$\Sigma -41.70964$

The equations $(X - \bar{X}) = \beta(t - \bar{t})$ (1) Where β is the required slope

or $X = \beta(t - \bar{t}) + \bar{X}$

and $$\beta = \frac{\Sigma (t - \bar{t})(X - \bar{X})}{\Sigma (t - \bar{t})^2} \quad (2)$$

Substituting the values from the above table we have from equation (2)

$$\beta = \frac{-41.70964}{583.36} = -0.0715$$

Again taking the values from the above table,

$$X = \beta (t - \bar{t}) + \bar{X}$$

Value of X at t_o $= -0.0715 (-16.66) + 0.4168$
$= 1.191 + 0.4168$
$= 1.6078$

Similarly:

Value of X at t 10 = -0.0715 (-6.66)+0.4168

= 0.4762+0.4168

= 0.893

Value of X at t 15 = -0.0715 (-1.66)+0.4168

= 0.1187+0.4168

= 0.5355

Value of X at t 20 = -0.0715x3.34+0.4168

= -0.2388+0.4168

= 0.178

Value of X at t 25 = -0.0715x8.34+0.4168

= -0.596+0.4168

= -0.1795

Value of X at t 30 = -0.0715x13.34+0.4168

= -0.954+0.4168

= -0.537

Now, the log percentages of the surviving phage particles at different times are plotted as shown in fig. 36 and the Regression line is obtained by plotting the different values of X against time in the same graph. Thus we have log Po = 1.61 at time t = 0 min. and log P = 0 at time t = 22.5 min.

Hence, from the equation

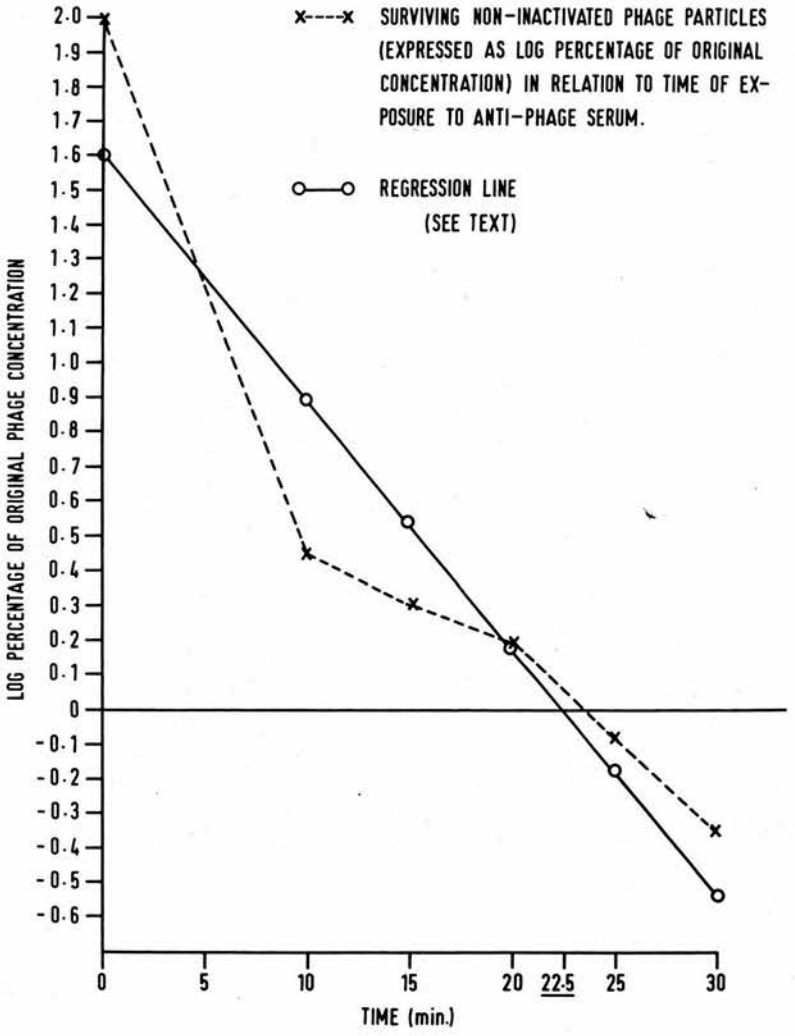
$$K = 2.3 \frac{D}{t} \times \log 10 \frac{Po}{P}$$

$$K = 2.3 \frac{D}{t} (1.61)$$

$$= 2.3 \frac{256}{22.5} \times 1.61$$

i.e. K = 42.13.

Fig. 36.


x----x SURVIVING NON-INACTIVATED PHAGE PARTICLES (EXPRESSED AS LOG PERCENTAGE OF ORIGINAL CONCENTRATION) IN RELATION TO TIME OF EXPOSURE TO ANTI-PHAGE SERUM.
o—o REGRESSION LINE (SEE TEXT)
LOG PERCENTAGE OF ORIGINAL PHAGE CONCENTRATION
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
-0·1
-0·2
-0·3
-0·4
-0·5
-0·6
0
5
10
15
20
22·5
25
30
TIME (min.)

The neutralization of group I, group II and group III cholera bacteriophages with the respective antiphage serum was also studied and the results are shown in table 59.

Table 59.

Results of neutralization of phage particles with homologous antiphage serum

Assay of phage and phage-serum mixtures	Mean number of plaques	Total plaque count	% of phage surviving	% of phage inactivated
Phage group I	21.5	4.3×10^6	100	Nil
Phage group I and antiphage group I serum				
diluted 1 in 256	-	-		
1 in 512	-	-		
1 in 1024	4	8×10^3	0.18	99.82
1 in 2048	51	1.02×10^5	2.37	97.63
Phage group II	74	1.48×10^7	100	Nil
Phage group II and antiphage group II serum				
Diluted 1 in 256	11.5	2.3×10^4	0.16	99.84
1 in 512	89	1.78×10^5	1.2	98.8
Phage group III	39	7.8×10^6	100	Nil
Phage group III and antiphage group III serum				
diluted 1 in 64	5	1×10^4	0.13	99.87
1 in 128	103.5	2.07×10^5	2.65	97.35

Assay of the normal sera of rabbits before starting immunization with cholera phage lysates

A volume of 1 ml. of a phage suspension containing 10^7 plaque-forming particles per ml. was added to 1 ml. of the undiluted normal serum of the rabbit to be immunized with the same phage. The mixture was held in the water bath at 37°C. A control test of the phage lysate was also incorporated by replacing the serum with broth. At 30 min. intervals, samples from each of the phage - serum mixtures and controls were withdrawn and diluted to 10^{-4} in broth and a volume of 0.1 ml. was plated by the agar layer method against the indicator strain 154. All the plates were incubated at 37°C overnight; plaques were counted and the numbers are shown in table 60.

Table 60.

Effect of normal sera on cholera bacteriophage

Phage-serum mixture	No. of plaques resulting from plating phage-serum mixture at time intervals of (min.)			No. of plaques produced by phage suspension control at time intervals of (min.)		
	0	30	60	0	30	60
Phage group I and anti-group I phage serum	20	23	21	21	22	20
Phage group II and anti-group II phage serum	76	71	75	74	73	75
Phage group III and anti-group III phage serum	37	41	40	39	38	40

It is evident from the results shown in table 60 that all of the normal sera under test were devoid of any antiphage activity.

Serological studies

Attempts to produce high-titre antiphage sera in rabbits.

A high-titre antiserum is produced against a bacterial virus in essentially the same manner as that employed in the preparation of an antibacterial serum.

The phage stocks used for immunization of rabbits in the present work were produced in nutrient broth and filtered through Oxoid membrane filters to remove the bacterial debris. All of the animals were bled before starting immunization so that any phage-neutralizing activity of normal sera would have been detected. Two subcutaneous injections of 4 ml. each were given weekly for 3 weeks and a test bleeding was done 5 days after the last injection. The low titre of the first test sera obliged us to continue immunization and we gave a further course of subcutaneous injection of 4 ml. weekly for 3 weeks with a second test bleeding 1 week after the last injection. Titres were still disappointingly low and the animals were again inoculated with the respective phage for another 2 weeks. Instead of injecting the same amount of phage in 1 place injections were made subcutaneously in 4 different places simultaneously for 2 weeks and a test bleeding was done 3 days after the last injection. Even this did not produce a sufficiently high-titre serum in any of the rabbits. The last course was therefore repeated and the rabbits were finally bled 4 days thereafter (see table 61). The blood was allowed to clot at room temperature, and then placed in the refrigerator overnight. The serum was separated by centrifugation and stored in sterile screw-capped vials in the deep-freeze. All of these operations were performed aseptically.

Table 61

Immunization schedule for the production of high-titre antiserum to cholera phage in rabbits

Phage Antigen	Titre	Description of the rabbits.				Date of normal bleeding (0)	Date of immunizing dose *						First test bleeding (1)	Date of immunizing dose +		
		colour	weight	Sex	Age		1st dose	2nd dose	3rd dose	4th dose	5th dose	6th dose		7th dose	8th dose	9th dose
Group IV	3.8×10^8	Grey	9½lb.	F	6 months	25.11.64	25.11.64	28.11.64	2.12.64	5.12.64	9.12.64	12.12.64	17.12.64	19.12.64	26.12.64	2.1.65
Group IV	3.8×10^8	Black	4¾"	M	5 "	25.11.64	25.11.64	28.11.64	2.12.64	5.12.64	9.12.64	12.12.64	17.12.64	19.12.64	26.12.64	2.1.65
Group III	2×10^9	White	5½ "	M	5 "	25.11.64	25.11.64	28.11.64	2.12.64	5.12.64	9.12.64	12.12.64	17.12.64	19.12.64	26.12.64	2.1.65
Group III	2×10^9	White and Brown	5½ "	M	5 "	25.11.64	25.11.64	28.11.64	2.12.64	5.12.64	9.12.64	12.12.64	17.12.64	19.12.64	26.12.64	2.1.65

* = 2 doses per week, each 4 ml. subcutaneously

\+ = q dose of 4 ml. per week subcutaneously

• = 1 divided dose of 4 ml. per week subcutaneously

X = 1 final divided dose of 4 ml. subcutaneously.

	Second test bleeding (2)	Date of immunizing dose. 10th dose	Date of immunizing dose. 11th dose	Third test bleeding (3)	X 12th dose	Final bleeding (4)	Reciprocal of phage neutralizing titre obtained at bleeding number 0	1	2	3	4
	9.1.65	12.1.65	19.1.65	22.1.65	26.1.65	30.1.65	-	10	100	500	1024
	9.1.65	12.1.65	19.1.65	22.1.65	26.1.65	30.1.65	-	10	100	400	512
	9.1.65	12.1.65	19.1.65	22.1.65	26.1.65	30.1.65	-	10	100	400	512
	9.1.65	12.1.65	19.1.65	22.1.65	26.1.65	30.1.65	-	10	100	400	512

Studies of antigenic relationships between the 4 cholera phage groups I, II, III and IV

To determine the antigenic relationships in these phages, each of the phage stocks were diluted in nutrient broth to a concentration of 10^7 PFU/ml. The homologous sera were also diluted. Thus the antiphage group I and antiphage group II sera were diluted to 1/50 and those of phage group III and phage group IV to 1/25. One volume of serum dilution was added to a similar volume of each of the phage dilutions. The phage-serum mixture was incubated at 37°C for 20 min. in a water bath. A control was incorporated by replacing sera with broth. After 20 min. incubation, the test samples were diluted to 10^{-2} and the controls 10^{-4} in broth. A 0.1-ml. volume of each of the test and control samples was plated by the agar layer method against the indicator strain 154. All of the plates were incubated at 37°C overnight. The results are shown in table 62.

Table 62

Antigenic relationships between the 4 groups of cholera phages

Antiserum against phage group	Phage group tested	Mean No. of Plaques after incubation		Inhibition
		Test	Control	
I	I	-	103.5	+
	II	Scl	108.5	-
	III	2	106	+
	IV	Scl	115	-
II	I	-	103.5	+
	II	-	108.5	+
	III	1.5	106	+
	IV	Scl	115	-
III	I	-	103.5	+
	II	Scl	108.5	-
	III	3	106	+
	IV	Scl	115	-
IV	I	Scl	103.5	-
	II	Scl	108.5	-
	III	Scl	106	-
	IV	1	115	+

Scl = Semi Confluent Lysis.

Electron microscopy of some cholera phages

Various aspects of the study of cholera bacteriophage have interested many workers during recent years, but proper attention has not been given to structural morphology. The ultrastructure of some of the cholera phages had been observed by electron microscopy in the present study before Vieu, Nicolle and Gallut (1965) and Takeya et al. (1965) published their limited findings on the electron microscopy of cholera phages.

Explanatory note: The "negative contrast" method for electron microscopy of bacteriophage particles by Brenner et al. (1959) has been followed in this study. It consists of embedding an electron-transparent object in a structureless electron-dense material, potassium phosphotungstate, which introduces contrast by negative staining. This technique has already been recognised by Hall (1955) and Huxley (1956).

It is well known that bacterial phages contain deoxyribonucleic acid which is contained in the protein coat of its head. The phosphotungstate does not stain either the protein or the nucleic acid of the phage particle directly, and these remain relatively electron-transparent objects in an electron-dense surrounding of phosphotungstate. If there are any cavities within the phage head these will be replaced by the phosphotungstate. Thus the contrast provided by an empty head is different from one that is full of DNA.

In addition to providing high contrast, excellent preservation of external form is also obtained by application of this technique (Brenner et al., 1959).

The different cholera phages studied in the present work are detailed in table 63.

Table 63

Cholera bacteriophages studied in the electron microscope

Cholera phage	Propagating strain of V. cholerae	Source
326	1212	Pakistan-SEATO Cholera Research Laboratory
175	154	"
510	601	"
group I	154	"
" II	154	"
" III	154	"
" IV	154	"
16718	16718	Captain R.S. Swanson
16466	16466	"

Stocks for electron microscpy of these phages were prepared on the respective sensitive strains of V. cholerae grown in T_1N_1 medium.

The following table of measurement conversions is included at this point for convenience in analyzing the elctron micrographs.

Measurement in electron microscopy

Particle size

Equivalent values for 1 mm. on screen/print at various magnifications:

6,000x	=	1,666Å	=	166.6 mμ	=	0.17 μ
8,000x	=	1,250Å	=	125.0 "	=	0.125 μ
10,000x	=	1,000Å	=	100.0 "	=	0.100 μ
13,000x	=	769Å	=	80.0 "	=	0.08 μ
16,000x	=	625Å	=	62.5 "	=	0.06 μ
20,000x	=	500Å	=	50.0 "	=	0.05 μ
30,000x	=	333Å	=	33.3 "	=	0.03 μ
40,000x	=	250Å	=	25.0 "	=	0.025 μ
50,000x	=	200Å	=	20.0 "	=	0.020 μ
80,000x	=	125Å	=	12.5 "	=	0.012 μ
100,000x	=	100Å	=	10.0 "	=	0.010 μ
180,000x	=	56Å	=	5.6 "	=	0.006 μ
200,000x	=	50Å	=	5.0 "	=	0.005 μ
300,000x	=	33Å	=	3.3 "	=	0.0033 μ
333,000x	=	30Å	=	3.0 "	=	0.003 μ
360,000x	=	28Å	=	2.8 "	=	0.0028 μ

The electron micrographs of the phages showed the following morphological details:

(A) Standard cholera phages of Mukerjee's groups I - IV.

Phage I. This looks like phage III but it is of variable size measuring from 858Å x 792Å to 1155Å x 1056Å. The tail is 297Å long and 165Å broad (see fig. 37).

Phage II. The head is 627Å x 627Å and the tail appears to be 264Å x 99Å (see fig. 38).

Phage III. This looks like coli phage T3 and Brucella phage (Bradley and Kay, 1960); it also resembles Pseudomonas phages 20 and Pc (Bradley 1963). It has an almost circular head measuring 660Å x 627Å. The tail is short and wedge-shaped and is 198Å long (see fig. 39).

Phage IV. This phage has a regular hexagonal head like staphylococcus phages 7, P42D, and 581, with a tail like that of staphylococcus phages P71, 187, 52, 72 and P52A (Bradley, 1963). It also resembles coli phage T5 and typhoid phage 1 (Bradley and Kay, 1960). The head measures 980Å x 952Å and the tail is 2240Å long and 112Å broad (see fig. 40).

(B) Miscellaneous cholera phages

Phage 326. This phage was isolated from water at Pakistan - SEATO Cholera Research Laboratory, Dacca. The head of this phage is a 6-sided regular polyhedron measuring 1,600Å x 1,250Å.

The tail is 2,150Å long, 100Å broad and has a contractile sheath measuring 1,050Å x 250Å with longitudinal striations. There is a constriction of the tail at its junction with the head, and there is a central hollow tubular core. The diameter of the core is 50Å (see fig. 41).

This phage thus has many features in common with typhoid phages 3T, C_4, 11F, 66F and 2 (Bradley and Kay, 1960) and coli phage T2 (Brenner et al., 1959). It also looks like the Pseudomonas phage 12 S (Bradley, 1963). It has a long tail but it does not resemble standard group IV phage or phages I, II or III.

Phage 16718. This phage was received from NAMRUN ; it was isolated from stool filtrates during the 1963 El Tor epidemic in the Philippines. It looks like a phage of group III. The head is 693A x 594A and the tail is 231A long (see fig. 42.).

Phages 16466, 175 and 510. Phage 16466 was isolated during the 1963 El Tor epidemic in the Philippines. Phages 175 and 510 were isolated in Dacca. These 3 phages resemble Pseudomonas phage 12B (Bradley, 1963). They are similar in appearance, but they differ in size (see figs. 43, 44, 45).

Fig. 37. Electron micrograph of group I cholera phage particles demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.

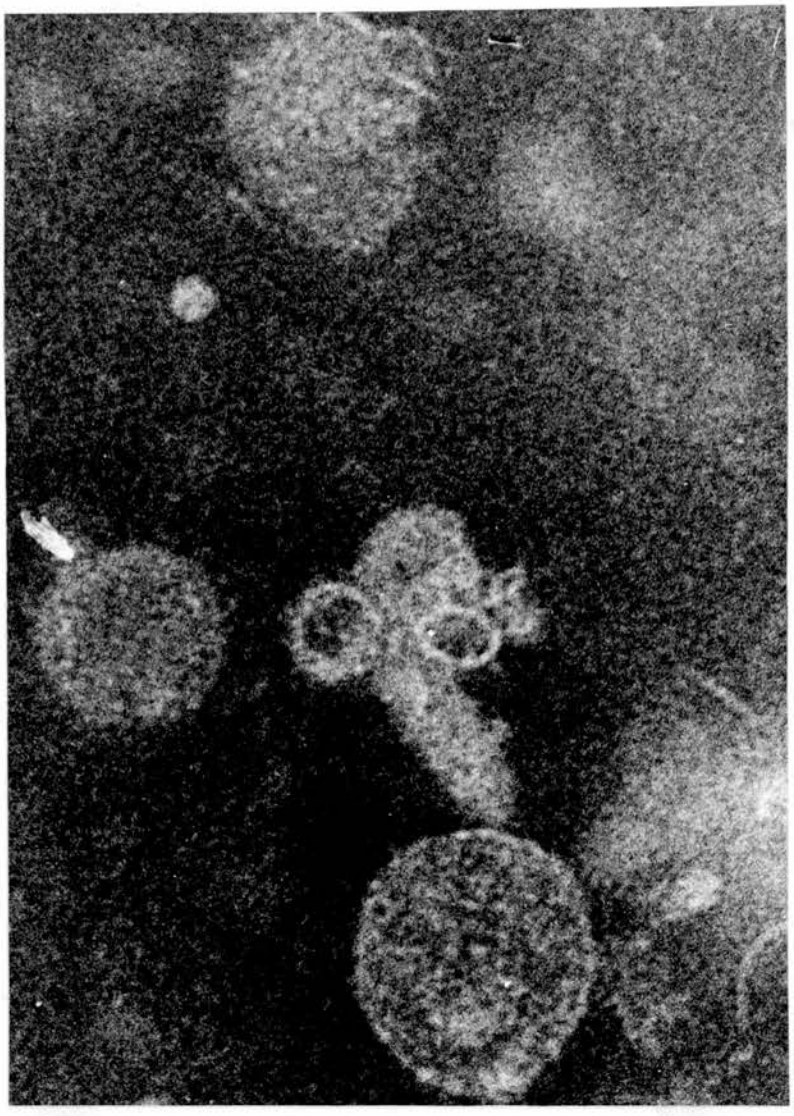


Fig. 38. Electron micrograph of group II cholera phage demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.

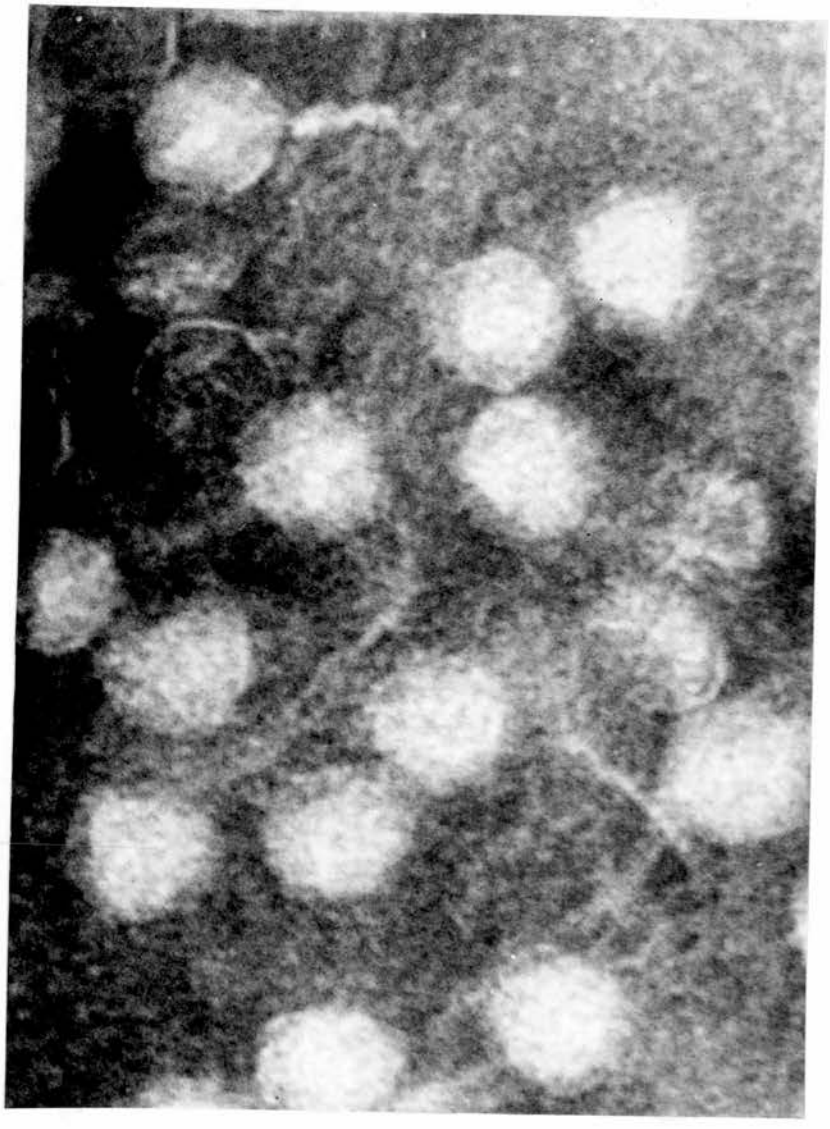


Fig. 39. Electron micrograph of group III cholera phage demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000

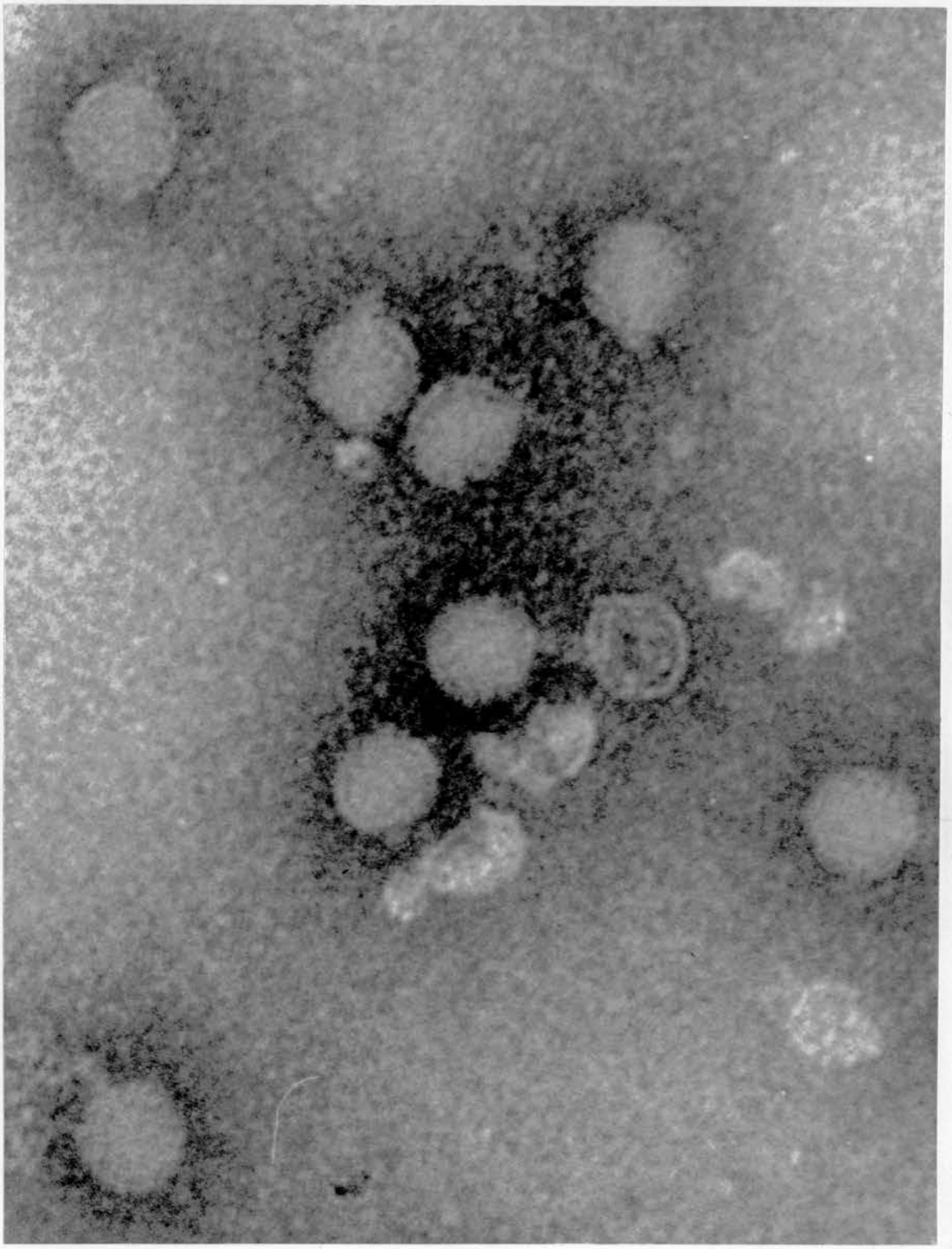


Fig. 40. Electron micrograph of group IV cholera phage demonstrated by negative staining with phosphotungstate at pH 7.0 . x 360,000.

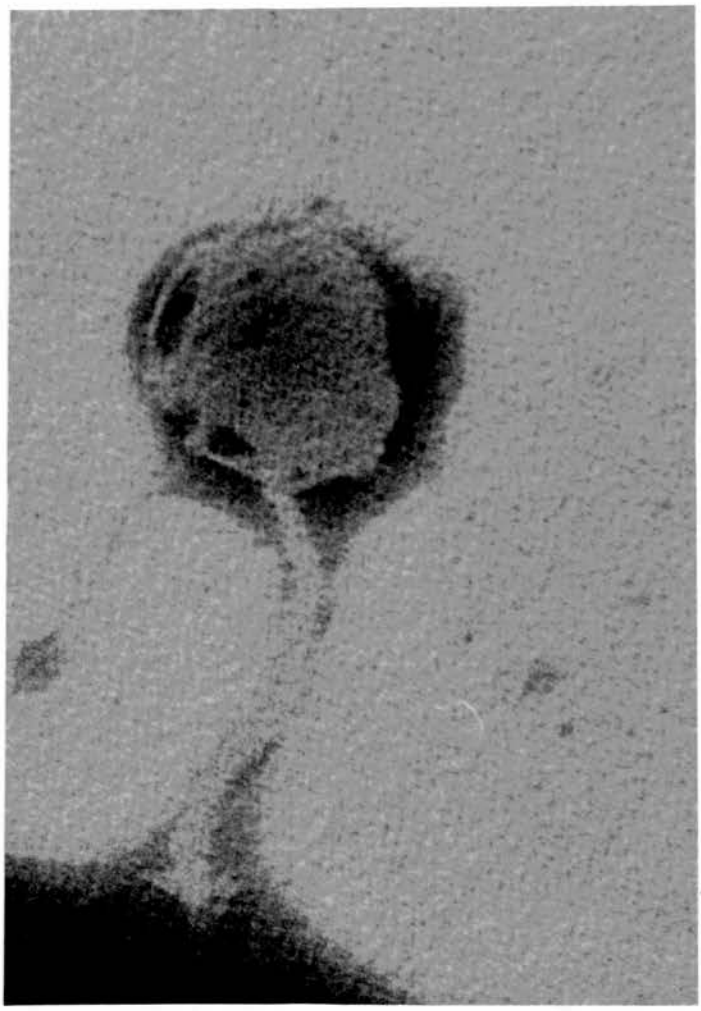
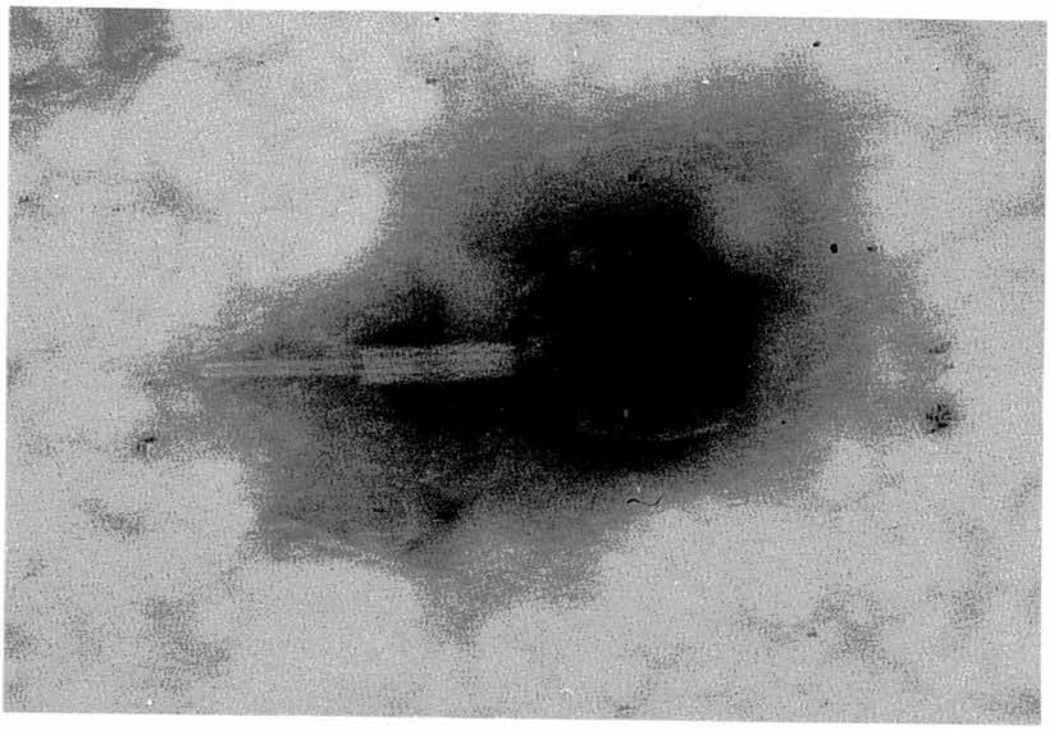


Fig. 41. Electron micrograph of cholera bacteriophage 326 with contracted tail sheath demonstrated by negative staining with phosphotungstate at pH 7.0 . x 200,000.



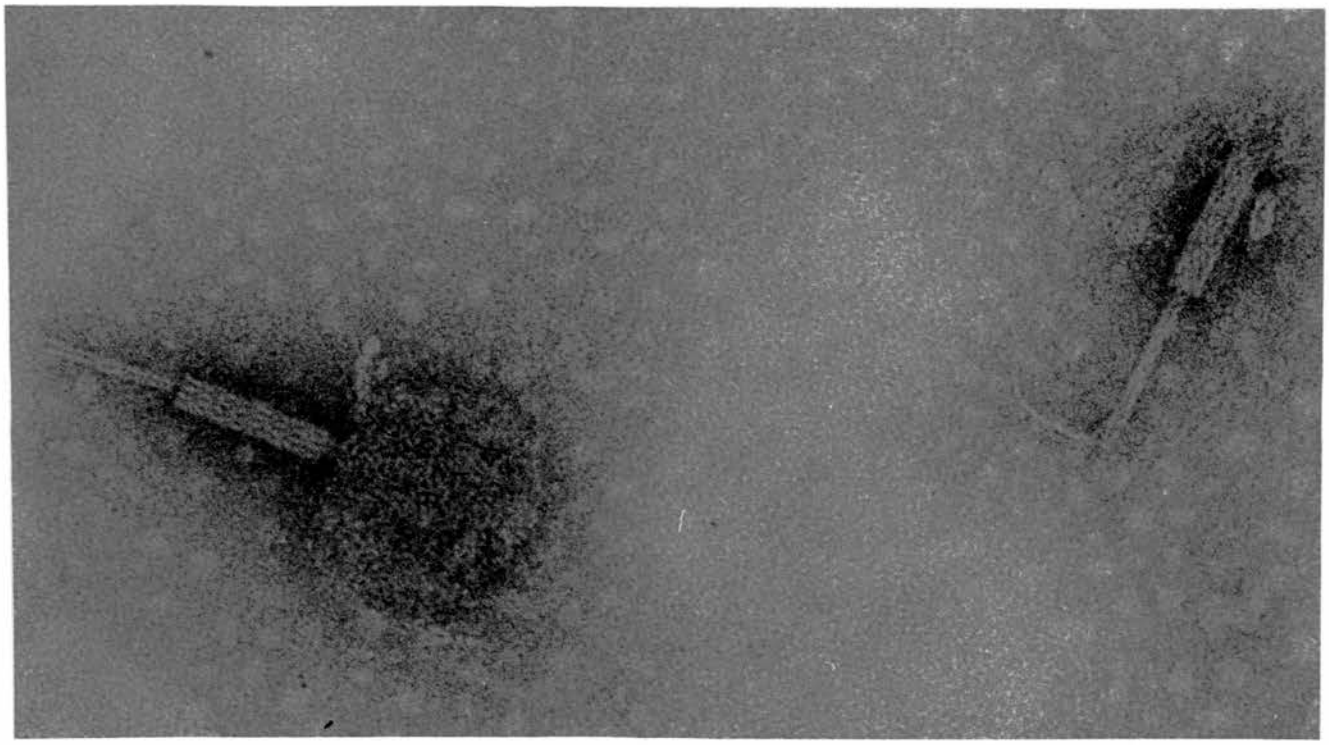


Fig. 42. Electron micrograph of particles of phage 16718 demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.

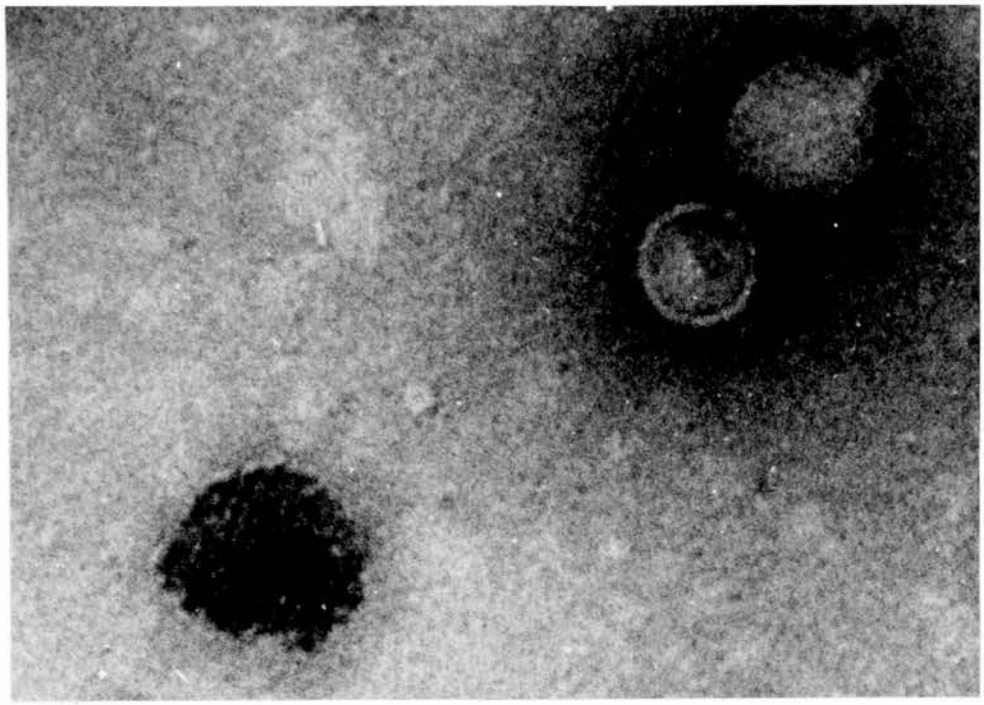


Fig. 43. Electron micrograph of particles of phage 16466 demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.

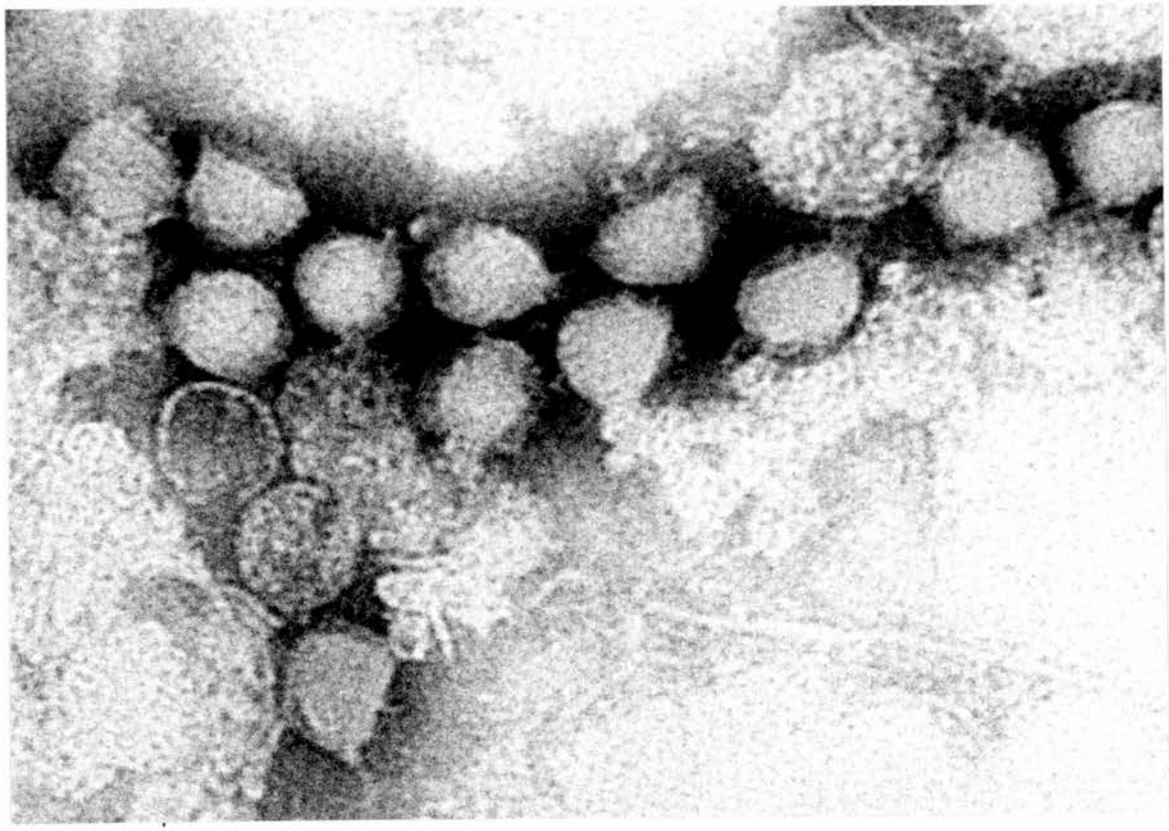


Fig. 44. Electron micrograph of particles of phage 175 demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.

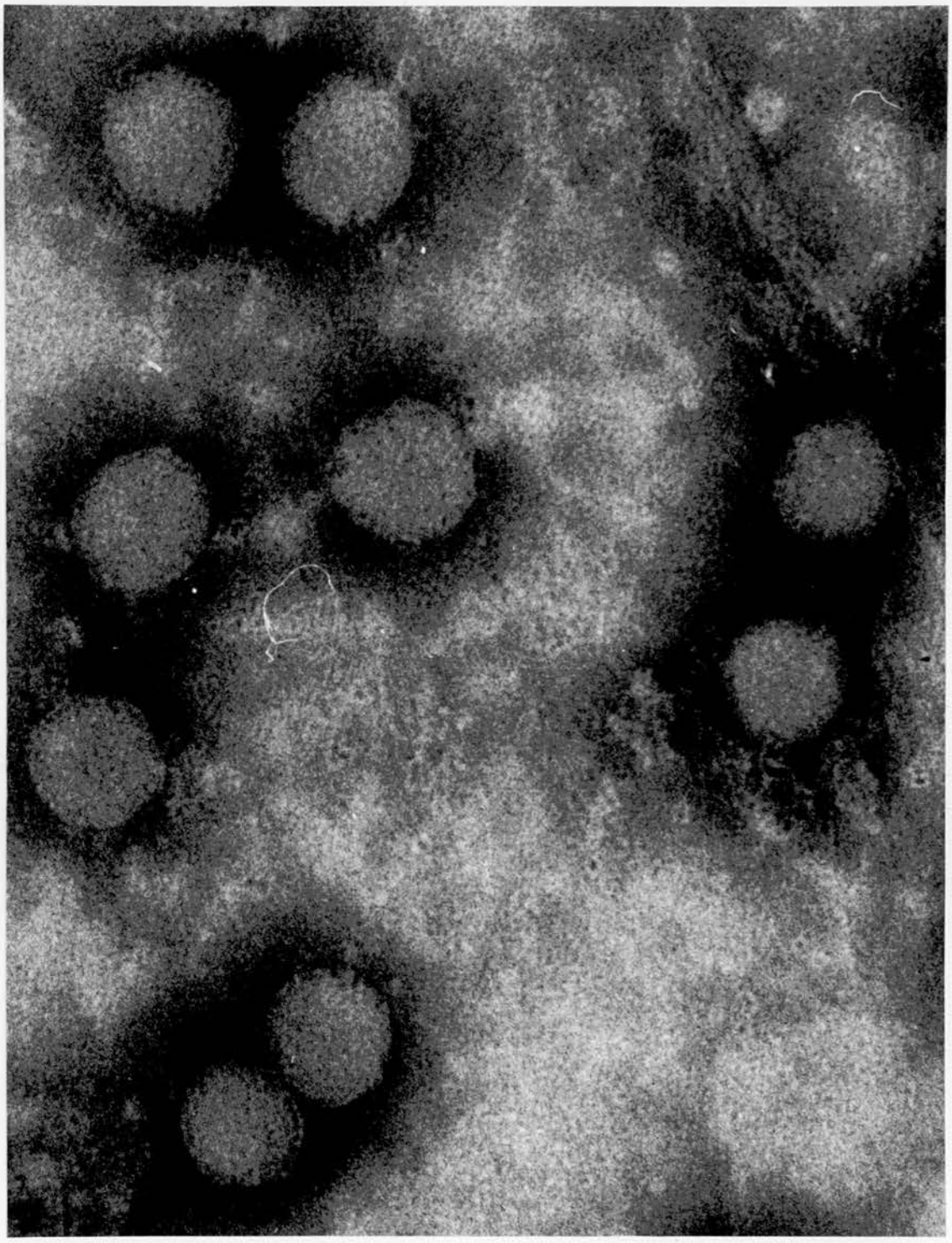
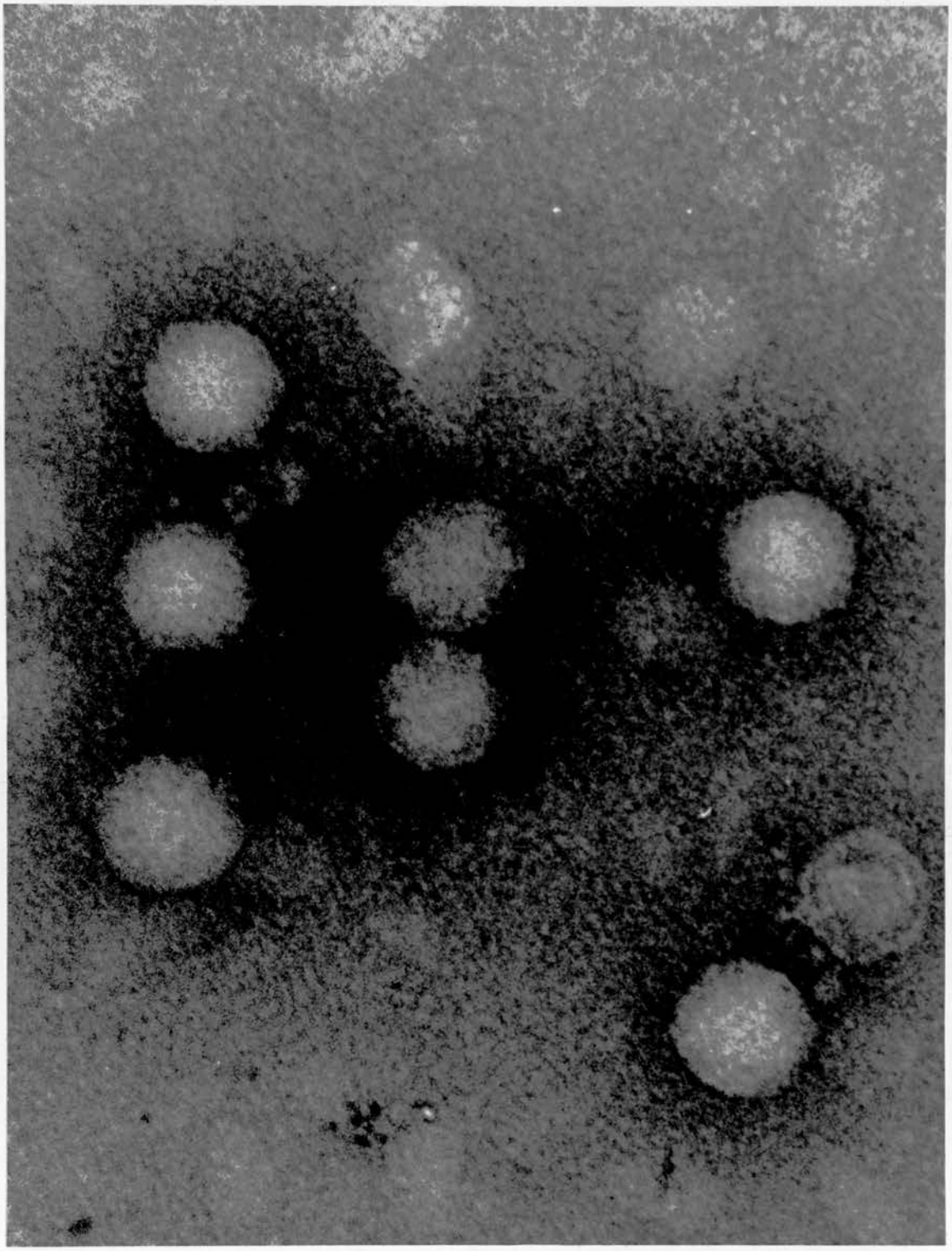


Fig. 45. Electron micrograph of particles of phage 510 demonstrated by negative staining with phosphotungstate at pH 7.0 . x 300,000.



The results of bacterial lysis and bacteriophage proliferation in a phage-culture mixture

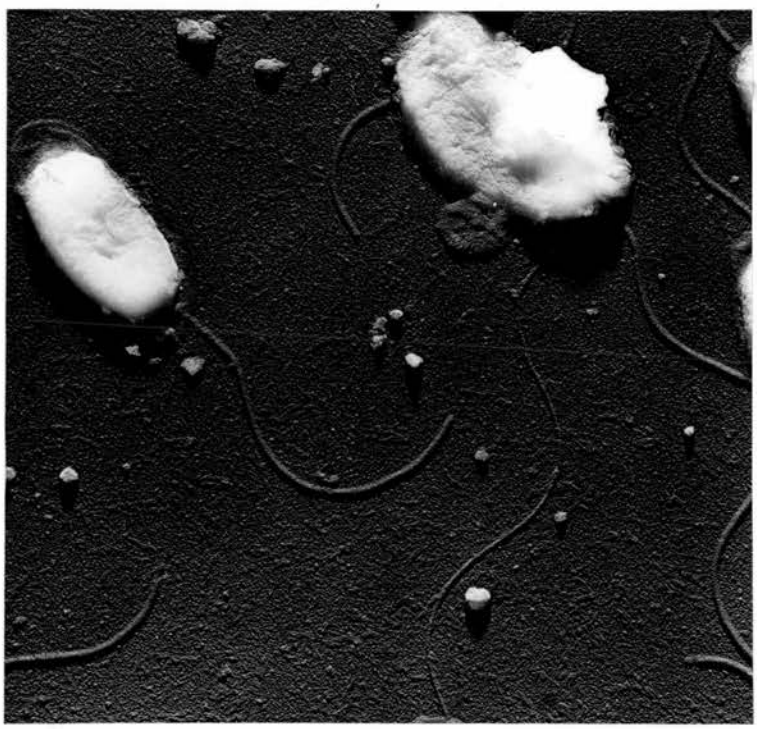
The stages of bacterial lysis and bacteriophage proliferation in a mixture of cholera phage (510) and susceptible growing vibrio strain (601) have been shown in the following microphotographs taken at various time intervals after addition of phage to the culture. The experimental procedures involved in the study have already been described (see Methods and Materials). The microphotographs taken with different grids show that complete lysis of the bacteria had taken place at 180 min. after addition of phage to the culture (see figs. 46, 47, 48, 49 and 50).

Fig. 46A. Electron micrograph prepared from a culture of *V. cholerae* 10 min. after the addition of phage suspension.

-Gold-palladium shadowed preparation
x 20,000.

Fig. 46B. Electron micrograph of control specimen without phage.

-Gold-palladium shadowed preparation
x 20,000.



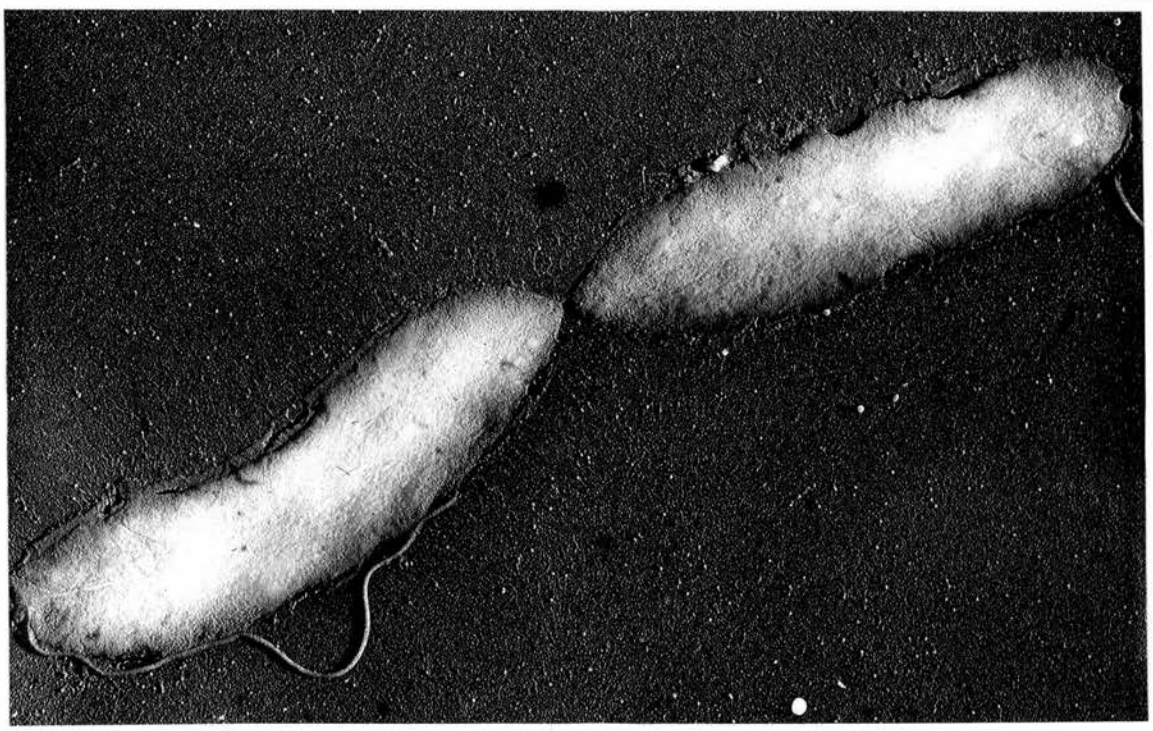
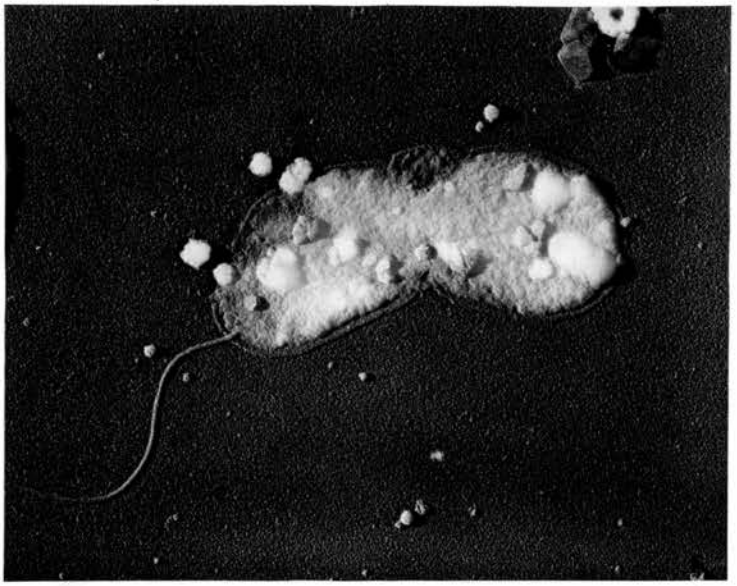


Fig. 47A. Electron micrograph after 30 min. of addition of phage to the culture. Gold-palladium shadowed preparation x 20,000

Fig. 47B. Electron micrograph of control specimen. Gold-palladium shadowed preparation x 20,000



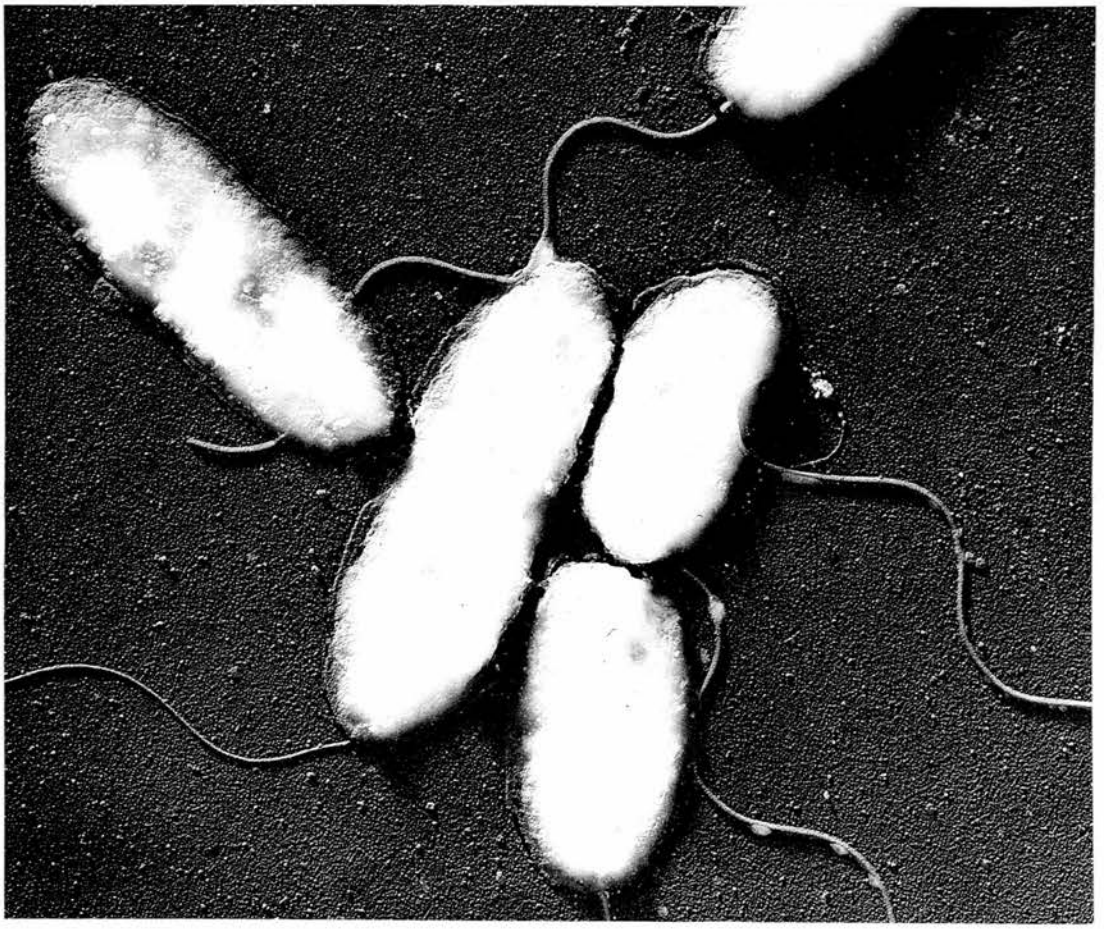
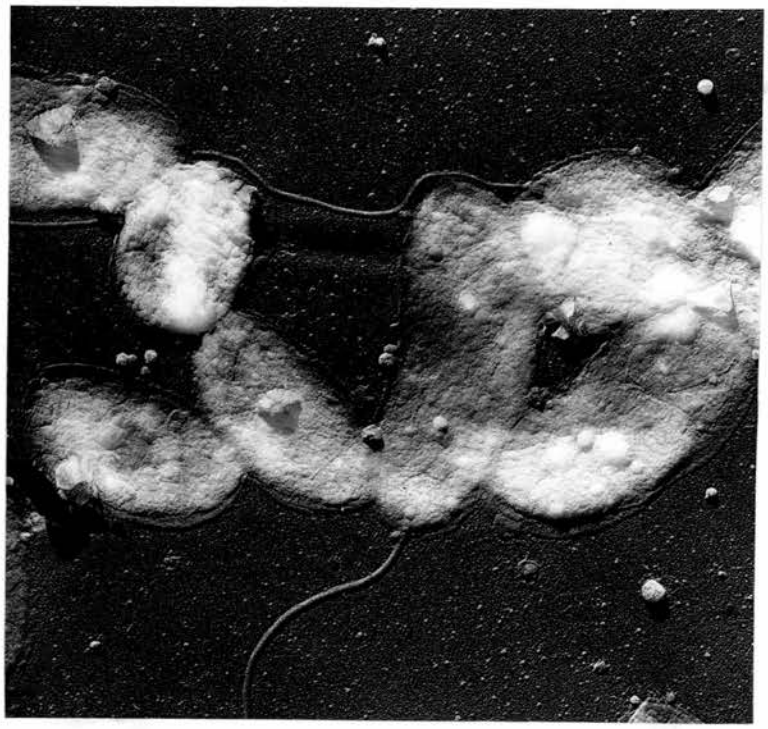


Fig. 48A. Electron micrograph after 60 min. of addition of phage to the culture.

Gold-palladium
shadowed preparation
x 20,000

Fig. 48B. Electron micrograph of control specimen.

Gold-palladium
shadowed preparation
x 20,000.



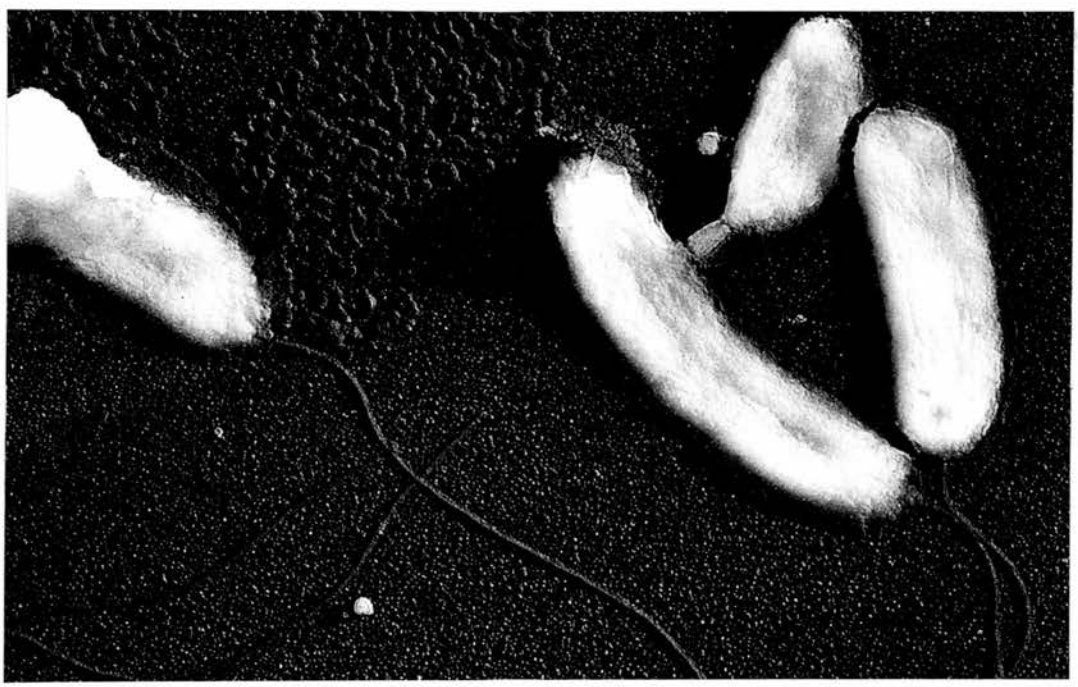
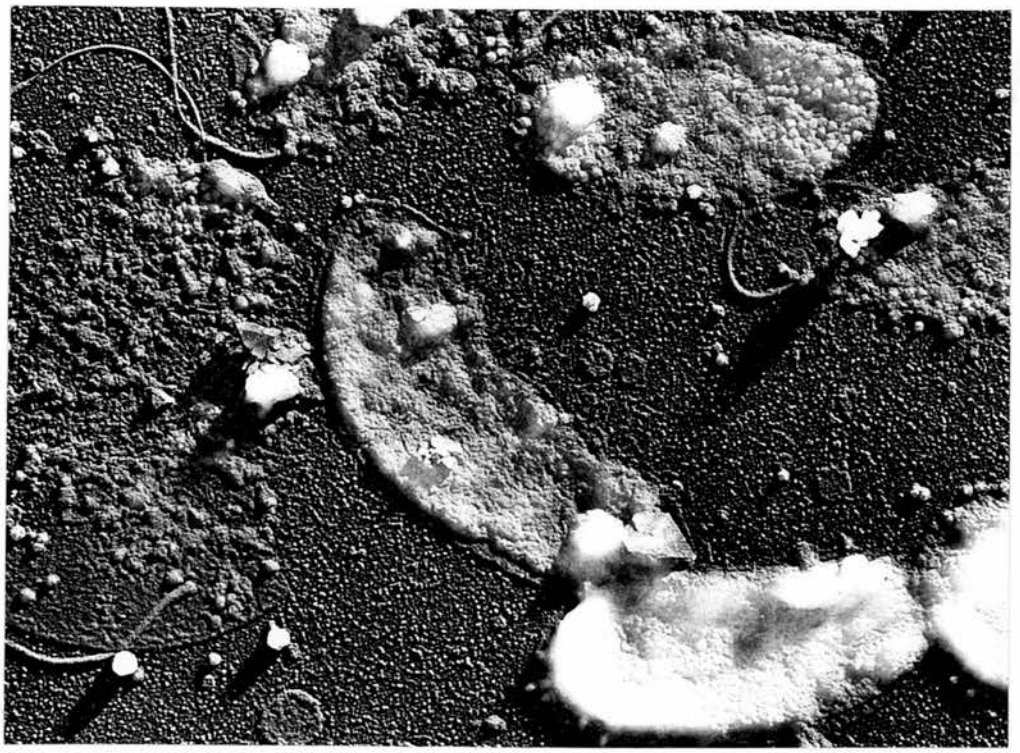


Fig. 49A. Electron micrograph after 120 min. of addition of phage to the culture.

Gold-palladium
shadowed preparation
x 20,000.

Fig. 49B. Electron micrograph of control specimen.

Gold-palladium
shadowed preparation
x 20,000.



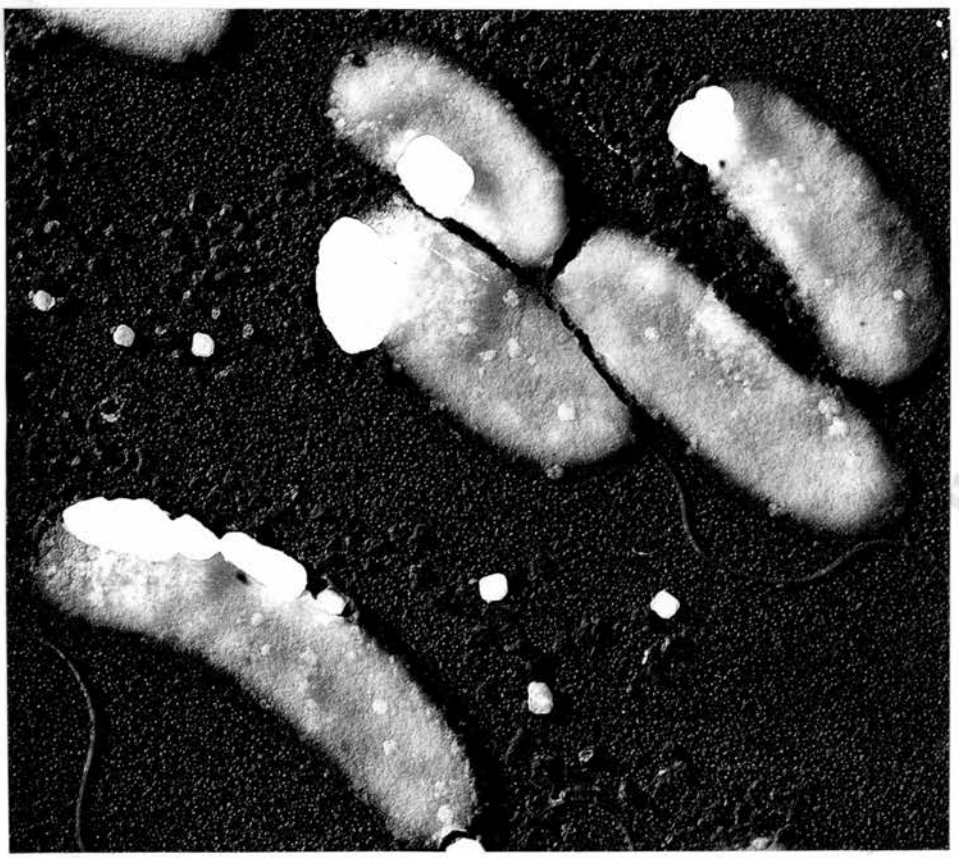
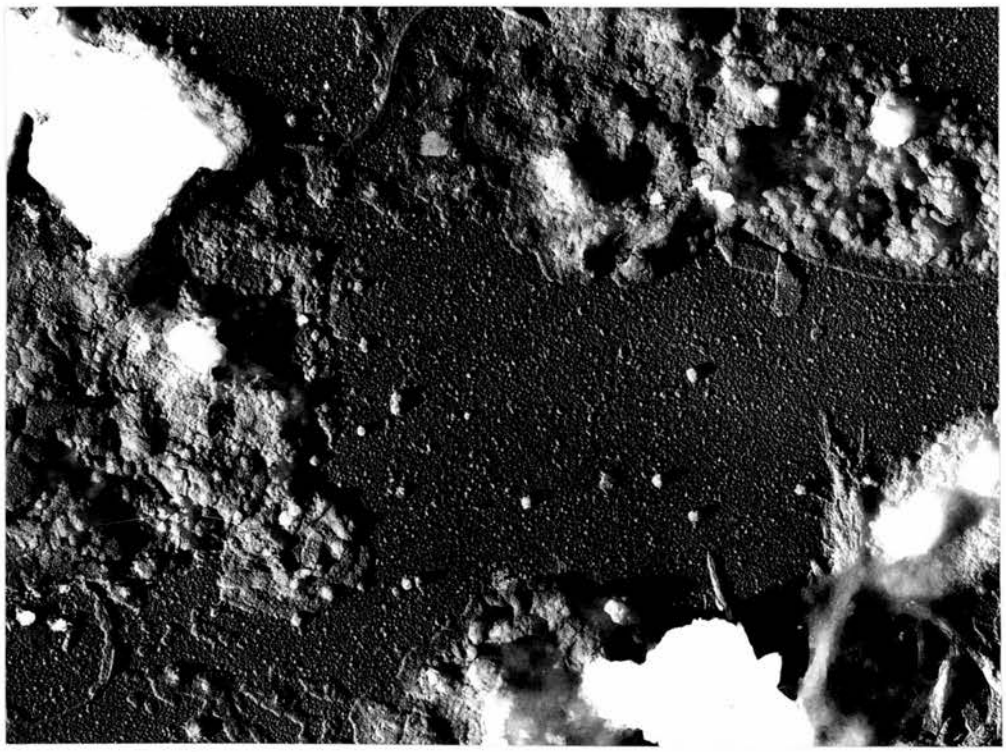


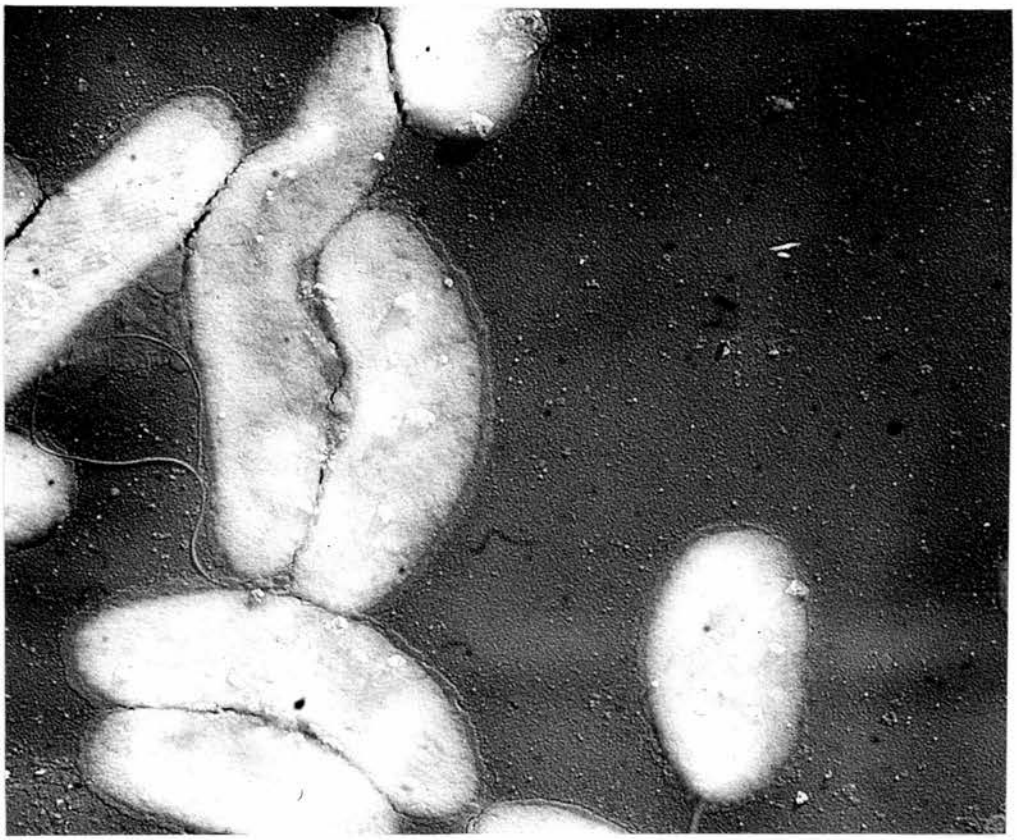
Fig. 50A. Electron micrograph after 180 min. of addition of phage to the culture.

Gold-palladium shadowed preparation x 20,000.

Fig. 50B. Electron micrograph of control specimen.

Gold-palladium shadowed preparation x 20,000.





Results obtained following different methods for isolation of biochemical mutants

The principles and techniques involved in the isolation of biochemical mutants have been described in the Introduction and in the Methods sections. Following those principles and techniques a number of vibrio strains were tested for isolation of such mutants (see table 44). A summary of the tests and their results is as follows:-

(i) Cells from 48-hr broth cultures of strains were sedimented separately by centrifugation and washed twice in sterile normal saline and finally re-suspended in sterile saline. A 0.1-ml. volume of each of the suspensions was inoculated into 10-ml. volumes of minimal liquid medium containing graded concentrations of penicillin ranging in doubling concentrations from 500 units to 3,000 units per ml. of the medium. The inoculated media were incubated overnight at 37°C. At the end of this period, 0.01-ml. and 0.1-ml. aliquots from each of the tubes were spread on to nutrient agar plates. After overnight incubation, discrete colonies were observed in the plates seeded with inoculum taken from tubes containing high concentrations of penicillin. The potential mutant colonies thus obtained on the plates were subcultured at random in minimal liquid medium. All of these subcultures were found to have grown after 24-48 hr incubation at 37°C. Some of the plates with discrete colonies were also subjected to replica plating. Identical growths in master and replica plates were observed in all such plates. Increase of the duration of

penicillin contact with the cultures was also tried with no better result.

(ii-a) Cells from overnight broth cultures of the strains were sedimented separately by centrifugation and re-suspended in sterile saline. The saline suspensions of each of the strains were UV-irradiated for 45 sec. at a distance of 60 cm. from the UV source and then added to 10-ml amounts of nutrient broth. After 24 hr incubation, the cells from each of the strains were again sedimented by centrifugation, washed twice in saline and re-suspended in saline matching International Opacity Tube No. 5. A 0.1-ml. volume of each treated suspension was added to minimal liquid medium containing graded concentrations of penicillin from 500 units to 3,000 units per ml. of the medium and incubated overnight at 37°C. A loopful from each of the tubes was then streaked on to nutrient agar plates. After overnight incubation, plates having isolated colonies were subjected to replica plating on minimal agar. Identical colonies were observed in all of the master and replica plates excepting 1 where 3 colonies were not found on the replica plate though their presence in the corresponding master plate was clear. These potential mutant colonies were used separately to prepare lawns on minimal agar plates. Different growth factors (see table 64) were then spotted on to specified areas of the lawns and the plates were incubated at 37°C for 72 hr. No growth resulted on the supplemented or on unsupplemented areas of these lawns.

Table 64

Growth factors used for isolation of biochemical mutants.

Growth factors	Concentration/ml
Tyrosine	0.01-1 mg/ml
Phenylalanine	"
Isoleucine	"
L-cystine	"
L-arginine	"
Histidine	"
Tryptophan	"
L-lysine	"
Threonine	"
Valine	"
Inosine	"
Adenosine	"
Uracil	"
Adenine	"
Guanine	"
Xanthine	"
Hypoxanthine	"
Vitamin B_{12}	50 µg/ml
Aneurine hydrochloride	1 mg/ml
D-pantothenic acid	1 mg/ml
Glutamic acid	0.01-1 mg/ml
Nicotinamide	1 mg/ml
Guanosine	0.01-1 mg/ml
Cytosine	0.01-1 mg/ml
Biotin	1 µg/ml
Methionine	0.01-1 mg/ml
Leucine	"
Asparagine	"
Proline	"
Aspartic acid	"

(ii-b). Suspensions matching Opacity Tube No. 4 were made in ¼-strength Ringer solution from overnight nutrient agar slants of vibrio strains and they were then subjected to the following treatments successively:

Irradiation. A 3-ml aliquot of each of the suspensions was irradiated for 45 sec. at a distance of 60 cm. from the UV source.

Intermediate cultivation. A 0.5-ml. volume of each irradiated sample was inoculated into a 2-ml. volume of minimal liquid medium and incubated at 37°C for 6 hr.

Penicillin treatment. After 6 hr incubation, 0.1 ml of each of the samples was added to 2-ml. volumes of minimal liquid medium containing penicillin (500 and 1000 units/ml.) and the mixtures were incubated at 37°C overnight.

Plating auxotrophs. An adequate amount of penicillinase (1 ml. inactivates 100,000 units of penicillin) was added to neutralize the penicillin in the above mixture; 0.3-ml. and 0.1-ml. volumes from each tube were spread on nutrient agar plates and incubated for 24 hr.

Testing auxotrophs. The colonies that grew on the above plates were potential mutants. These were then replica-plated as before

and lawns of suspected mutant colonies were made on minimal media. Growth factors in groups as shown in table 65 were spotted on specified areas of the lawns. No growth was observed on either of the supplemented or unsupplemented areas of the lawns with the exception of those in which group I growth factors were applied on lawns made from suspected mutants of strains H_1 and H_6.

These mutants were identified as cystine-requiring.

Table 65.

The growth factors used in groups for isolation of biochemical mutants.

Group	Growth factors
I	Lysine, Arginine, Methionine, Cystine.
II	Leucine, Isoleucine, Valine.
III	Phenylaline, Tryptophane, Tyrosine.
IV	Histidine, Threonine, Glutamic Acid, Proline, Aspartic Acid.
V	Adenine, Guanine, Xanthine, Hypoxanthine, Cytosine, Uracil.
VI	Nicotinamide, Pantothenic acid, Aneurine hydrochloride, Vit. B_{12}

No improvement on these results was obtained by supplementing the minimal media for intermediate cultivation of the irradiated strains prior to penicillin treatment with 20 per cent. sucrose plus 0.2 per cent. casein or asparagin and DL-methionine.

Production of biochemical mutants from strains as shown in table 44 was tried using $MnCl_2$ (see Methods). The potential mutants thus obtained were used to prepare lawns on minimal media; growth factors (see tables, 64 and 65) were spotted on specified areas of the lawns. No mutants were observed after prolonged incubation of the plates for 72 hr at 37°C.

Isolation of biochemical mutants from vibrio strains was also attempted after treating the organisms with acriflavine (see Methods). The vibrio strains detailed in table 44 were grown separately in nutrient broth containing acriflavine at a concentration of 1:60,000 for 24 hr at 37°C. Serial tenfold dilutions in nutrient broth were then made from each of the cultures and 0.1-ml. volumes of each dilution were plated on nutrient agar plates and incubated at 37°C for 24 hr. The plates were then replica-plated on minimal agar plates. By comparing the master and replica plates, potential mutant colonies were identified. They were spread on minimal media and growth factors (see Tables 64 and 65) were spotted on to the seeded plates. The potential mutants did not grow. No differences between the supplemented and unsupplemented areas of the lawns were observed.

Genetic recombination cross experiments between V. cholerae and V. eltor

(1) Cross experiments were performed with streptomycin-sensitive lysogenic El Tor strains and the streptomycin-resistant non-lysogenic phage-sensitive V. cholerae sub strain SR508. The results showed that a mixture of the streptomycin-resistant strain (SR508) and any of the streptomycin-sensitive lysogenic El Tor strains H_1, H_6 or NG 149 produced a limited growth of colonies on nutrient agar plates containing streptomycin at a concentration of 50 µg per ml. of media. However it was evident that V. cholerae strain 508 itself did not persist on these plates because the survivors obtained from the different crosses were resistant to group IV phage. Classical V. cholerae is always suspectible but all El Tor strains are typically resistant to this phage (Mukerjee, 1963a). It was also observed that in the control plates containing a similar concentration of streptomycin, none of the El Tor strains appeared whereas SR508 grew well when the strains were inoculated individually on to these plates. The reason for the non-growth of the streptomycin-resistant strain SR508 on streptomycin medium when it was inoculated along with an El Tor strain is not clear. Perhaps the El Tor strain liberated phage in sufficient amount to destroy the phage-sensitive strain SR508, but experiments to confirm this did not reveal such complete destruction as would be necessary for absolute exclusion of SR508. It was quite clear that the inoculation of plates with the above mixture yielded only colonies of streptomycin-resistant variants with characters of El Tor strains. It is likely

Control tests confirmed that the El Tor strains used in these experiments were highly sensitive to streptomycin and resistant variants did not arise spontaneously after such a plating procedure. It is likely that the variants produced in the cross experiments were recombinants. The results are summarized in the following tables (66 and 67).

Table 66

Susceptibility of various strains to group IV phage

Lawn produced from strain derived from:	Result in presence of phage diluted:			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Survivor from Cross H_1 X SR508	-	-	-	-
Survivor from Cross H_6 x SR508	-	-	-	-
Survivor from Cross NG 149 X SR508	-	-	-	-
508 control	Cl	Cl	Cl	Gcl
SR508 control	Cl	Cl	Cl	Scl
H_1 control	-	-	-	-
H_6 control	-	-	-	-
NG 149 control	-	-	-	-

Cl = confluent lysis :

Scl = semi confluent lysis ;

\- = no lysis.

Table 67

The patterns of growth of strains used in the cross experiments, on nutrient agar plates containing 50 µg of streptomycin per ml. of medium.

Strain	Growth produced on plate inoculated with 0.1 ml. of overnight broth culture diluted					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
H_1	-	-	-	-	-	-
H_6	-	-	-	-	-	-
NG 149	-	-	-	-	-	-
508	-	-	-	-	-	-
SR508	Conf.	++++	++++	+++	++	+

Conf. = confluent growth ;

++++, +++, ++, + = decreasing number of colonies ;

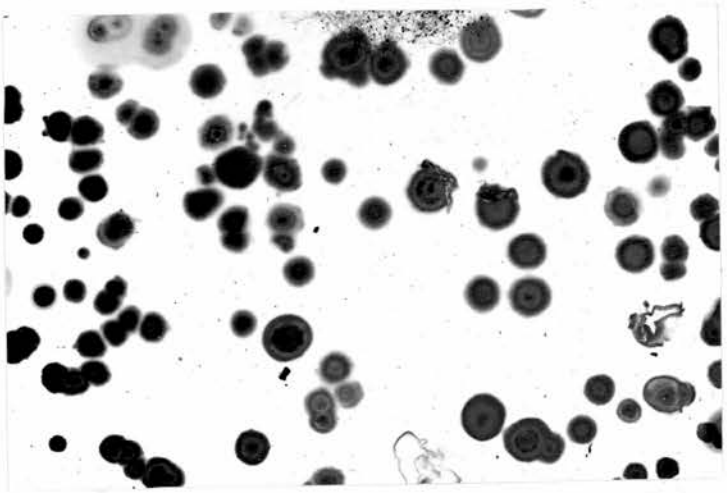
- = no growth.

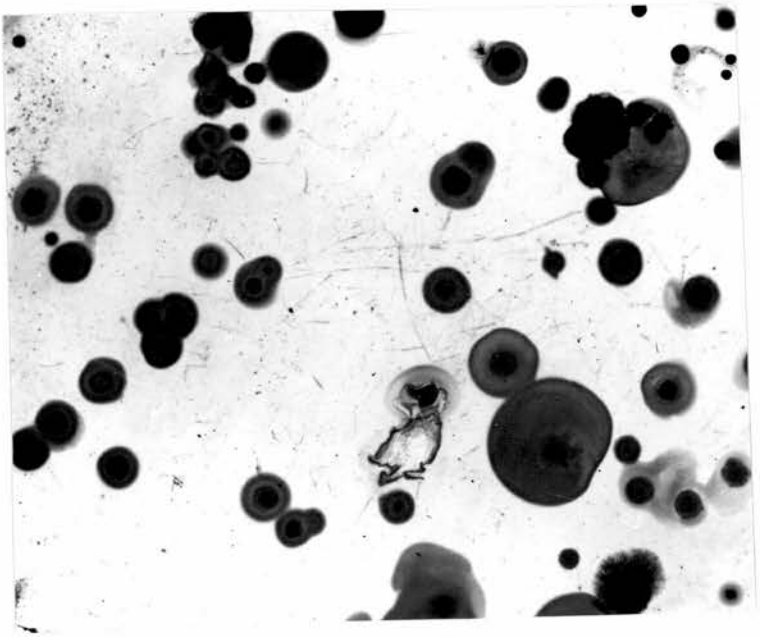
(2) In similar experiments survivors from the V. eltor - V. cholerae crosses H_4 x SR508 and H_7 x SR508 were observed to have circular areas of plaque-like clearing in their colonies. It seems that the phage particles liberated from the streptomycin-sensitive lysogenic H_4 and H_7 strains in these crosses attacked the non-lysogenic streptomycin-resistant indicator V. cholerae strain SR508 and produced zones of clearing in its colonies. Subcultures from these colonies in nutrient broth were tested against group IV phage and found to be sensitive to it. It was also noticed that in control plates containing the same concentration of streptomycin, there was no growth with either of the streptomycin-sensitive H_4 or H_7 strains. This indicates that the surviving colonies of the crosses H_4 x SR508 and H_7 x SR508 may be lysogenized, and the next step in this study attempted to prove this by showing the presence of phage in the chloroform-sterilized broth cultures obtained from these survivors. The experimental procedures are summarized as follows:

There were 2 types of colonies in each of the cross plates, i.e. plain colonies and colonies with plaque-like clearings (see figs 51 and 52). Subcultures were made in nutrient broth from each of these colonies. A portion of each of the subcultures was sterilized with chloroform and to 1-ml. aliquots of each of the remainder, 0.3-ml. amounts of streptomycin solution containing 300 µg of streptomycin sulphate were added and left at room temperature overnight. The chloroform-sterilized portions were spot-tested on lawns of phage-sensitive indicator strain SR508. A clear halo around the spot indicated the presence of phage and this was

Fig. 51. Recombinants from cross H_4 x SR 508 showing plain and plaque-containing colonies.

Fig. 52. Recombinants from cross H_7 x SR 508 showing plain and plaque-containing colonies.





observed in all tests except those involving cultures made from the plaque-containing colony of cross H_7 x SR508.

Each of the streptomycin-treated portions were then subcultured on nutrient agar plates and incubated at 37°C overnight. These were again subcultured in nutrient broth and thereafter sterilized with chloroform and spot-tested for the presence of phage as before. Similar haloes were again produced around the spots excepting those prepared from aplaque-containing colony of cross H_7 X SR508 (see fig 53). With this exception the survivors of these crosses are thus proved to be lysogenic. In other words, it appears that genetic material from the streptomycin-sensitive lysogenic donor El Tor strains has been transferred to the streptomycin-resistant, non-lysogenic recipient V. cholerae strain SR508.

Similar cross experiments with other El Tor strains (summarized in the table 45) were unsuccessful in demonstrating this effect.

(3) Crosses were made with (i) haemolytic lysogenic and streptomycin-sensitive El Tor strains and (ii) a non-haemolytic, non-lysogenic phage-sensitive streptomycin-resistant V. cholerae strain to determine whether the haemolytic character of the El Tor strains could be transduced to the phage-sensitive V. cholerae strain.

Bacterial suspensions containing 10^8 cells per ml. in sterile normal saline were made from overnight nutrient agar slant cultures of each of the strains (D67, 34, 126, 10265, H_1, H_2, H_4, H_5, H_6, H_7, H_8, SR508). SR508 is a non-haemolytic, non-lysogenic, streptom-

Fig. 53

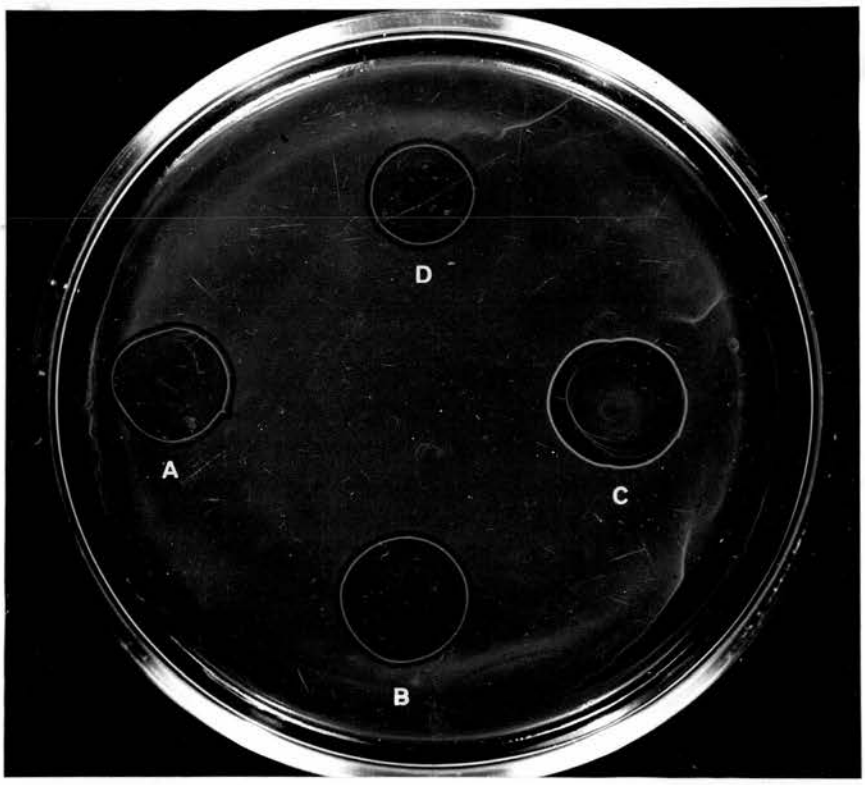
Showing clear haloes around the spots when chloroform-treated broth cultures of the surviving colonies of the following crosses were spotted on to a lawn of a sensitive indicator strain.

A = Chloroform-treated broth culture of plain colony from cross H_4 x SR 508.

B = Chloroform-treated broth culture of plain colony from cross H_7 x SR 508.

C = Chloroform-treated broth culture of plaque-containing colony from cross H_7 x SR 508.

D = Chloroform-treated broth culture of plaque-containing colony from cross H_4 x SR 508.


D
A
C
B

ycin-resistant derivative of V. cholerae strain 508 whereas the other strains involved were lysogenic, haemolytic, streptomycin-sensitive El Tor strains. A 1-ml. volume of SR508 suspension was added to a 1-ml. volume of each of the aforementioned suspensions. Each of these combinations was then mixed separately and added to 10 ml. of nutrient broth in a 50 ml. conical flask and incubated at 37°C overnight. The cultures were centrifuged and the deposits were resuspended in small volumes of peptone saline. Serial tenfold dilutions were made in peptone saline from each of the suspensions of the deposits and 0.1 ml. from each of the dilutions was plated on to nutrient agar plates containing 100 µg. of streptomycin per ml. of the medium. The plates were incubated at 37°C overnight. Random subcultures from surviving colonies were made from each plate in nutrient broth and these were incubated at 37°C for 48 hr. A portion of each of these subcultures was tested for susceptibility to group IV cholera phage and the other portion was investigated for haemolytic activity.

Results. All of the subcultures were susceptible to group IV cholera phage and none of them was found to be haemolytic. Thus the haemolytic character of the El Tor strains could not be transduced in these experiments.

Attempts to change the character of NAG vibrios. Mutation of

V. cholerae into a non-agglutinable vibrio and back-mutation into the agglutinating type has been reported by Tomb and Maitra (1927), Brahmachari (1929), and Bhattacharji and Bose (1964). A study of the literature quoted by Pollitzer (1959) also confirms that there were many instances of such loss of agglutinability of true vibrios or acquisition of agglutinability by NAG vibrios. This type of change was also reported by Zlatogoroff (1909) and Morowitz (1911). Such variations may be caused by environmental conditions, mutations or dissociations.

In the present work we studied the character of NAG vibrios treated with bacteria-free phage lysates derived from lysogenic strains of V. cholerae and V. eltor in nutrient broth containing varying concentrations of anticholera serum. There was no indication of change of characters in these NAG vibrios.

Procedure.

To 0.5-ml. volumes of undiluted samples and to 1 in 2 and 1 in 4 dilutions of polyvalent anticholera serum, 0.5-ml. volumes of sterile phage lysates from lysogenic strainswere added. To each of these 1-ml. volumes of serum-phage mixture, 0.5 ml. of a NAG suspension matching Brown's opacity tube No. 5 was added and mixed well. The tubes were then incubated in the water bath at 37°C for 2 hr and thereafter they were held in the cold room overnight. Subcultures were made from each of the tubes on to nutrient agar plates. After overnight incubation they were tested for agglutinability with anticholera serum.

By these methods each of the NAG strains was treated separately with each of the sera and phage lysates. Details of the sera, NAG strains and phage lysates used in these experiments are as follows:

Sera. The agglutinating sera used for this experiment were (i) Inaba, (ii) Ogawa, and (iii) Polyvalent, these were commercial preparations (Wellcome & Co. Ltd.).

Donor strains : Phage lysates were produced for this experiment from the following strains:

(A) Lysogenic V. cholerae M7 (Ogawa), M8 (Ogawa), M9 (Inaba), M10 (Inaba), M11 (Inaba), M12 (Inaba), B37 (Ogawa).

(B) Lysogenic V. eltor H1 (Ogawa), H2 (Ogawa), H3 (Ogawa), H4 (Inaba), H5 (Ogawa), H6 (Ogawa), H7 (Inaba), H8 (Ogawa).

Recipient NAG strains : 965 group I, 658 group II, 172 group V, 454 group III, H9 group I, H10 group III, H11 group 1, H12 group III, W343 group II, W255 group I, 586 group II.

Results. None of these recipient NAG strains produced variants that were agglutinable by any of the antibacterial V. cholerae sera.

Discussion.

DISCUSSION

The aim of the present bacteriophage study was to determine whether it is possible to transmit factors associated with virulence of *V. cholerae* to other non-virulent vibrios either by transduction or by phage conversion as has been observed in *C. diphtheriae* or in *Salmonella*.

It was initially necessary to isolate temperate bacteriophages of *V. cholerae* and their host lysogenic bacterial strains, to isolate and characterize biochemical mutants from El Tor and classical cholera vibrios, and finally to study the behaviour of the recombinants resulting from various cross experiments with these strains. In spite of an extensive survey of many strains, lysogenic strains were only found among the El Tor vibrios and only 1 indicator strain was found for these. This was *V. cholerae* strain 508 and it has provided a valuable basis for many of the observations concerning the planned genetic study. In addition, we have also studied some general characters of cholera bacteriophages including their morphological details as revealed by electron microscopy.

The different virulent cholera phages obtained for the present study were propagated on young susceptible strains of *V. cholerae*. To ascertain the optimal conditions for preparing phage lysates of high titre, various conditions of incubation with different media and various sensitive strains were tested. Thus, it was found that a titre of about 10^{12} phage particles per ml. could be obtained by passaging the phage for 5-6 hr in young continuously-shaken fluid

cultures of the sensitive strain of V. cholerae. Temperate cholera bacteriophages were isolated from a large number of vibrio strains using different techniques for their isolation, but attempts to raise the concentration of these phages met with failure.

Classification of cholera phages

Plaque morphology.

The plaque morphology of cholera phages is greatly influenced by a number of environmental factors. The nature of the host bacterium and the media for plating affect the character of the plaques. Prolonged adaptation of a phage under laboratory conditions is accompanied by alteration of plaque character. Working on the characteristics of plaques of cholera bacteriophage, Asheshov et al. (1933) stated "Though the morphology of the clearings is a very important characteristic of bacteriophage, we must never forget that it is not a permanent one and depends on the environment." Thus, due to the wide range of variations that may be observed in plaque morphology of cholera phages, this character is not utilized for specific identification of phage groups and it is important to investigate other characters of these phages that may help to differentiate them.

Susceptibility to heat.

The thermal death point is one of the characteristic properties

of bacteriophages which might be utilized for their identification. In the present study the heat-susceptibility of the 4 groups of cholera phages I, II, III and IV has been studied under uniform conditions. It was observed that the heat-sensitivity of these phages was not uniform. The thermal death point of group I cholera phage was within the range 64-71°C; group II was inactivated at 68-71°C; group III at 58-68°C, and group IV at 64-68°C. This confirms the observations of Mukerjee (1961b). It has been observed that V. cholerae is killed by heating at 55°C for 30 min., but cholera bacteriophages are resistant to such a temperature. Exposure to 55°C for 30 min. is therefore utilized to isolate phages from mixtures with host vibrios without resorting to filtration. The stability of group I and group II cholera phages at 37°C for a period of 20 days was observed; decreases in plaque titre of 46.6 and 29 per cent. respectively were noted after incubation for 24 hr. The subsequent decreases in titre for group I phage after 2, 4, 8, 10 and 20 days of incubation were 64.4, 88.8, 88.8, 90.7 and 94.4 per cent.

Effects of antimicrobial agents.

The effects of penicillin, streptomycin sulphate and sulphanezathine on group I and group IV cholera phages were studied because antimicrobial therapy may be combined with phage therapy. The results show that penicillin at a concentration of 2500 units/ml. had no effect on group I phage but it inactivated 29 per cent. of the group IV phage. Streptomycin sulphate at a concentration of 50 mg./ml. reduced the activities of group I and group IV phages to

10.3 and 11.5 per cent. of their initial concentrations respectively. Sulphamezathine at a concentration of 83 mg./ml. almost completely (98.5 per cent.) inhibited group IV phage but sulphamezathine had no effect on group I phage.

Effect of pH.

The effect of pH on group II and group III phages was determined by mixing equal volumes of the respective phage lysates with T_1N_1 broth at pH values varying from 4.5 to 9.5. The phages of both groups were stable over this pH range.

Serological relationships.

The antigenic specificity of bacteriophages forms the most important criterion in the classification of bacteriophages acting on a particular bacterium. The antigenic relationship between the 4 groups of cholera phages has been studied in the present work and it was found that groups I, II and III are serologically related, but group IV is not related. It is of interest that group IV phage was also found to differ from the others in morphological details (q.v.). These results do not agree with those of Mukerjee (1962) who reported that the 4 groups of typing phages were antigenically unrelated.

Antiphage serum was prepared by immunizing rabbits individually with each of the phage lysates of the 4 groups of cholera phages. It was found that when the immunizing dose of the phage antigen was

incorporated with an adjuvant of a mineral oil/Arlacel A mixture, a single 0.5-ml. volume of the phage suspension was quite enough to produce high-titre serum. Cholera phages of group I and group II were used with adjuvant in this way for the production of antiphage sera. A dilution of 1 in 2048 of antiphage group I serum and a dilution of 1 in 512 of the antiphage group II serum (produced in response to phage antigen plus adjuvant) neutralized about 97 and 98 per cent. of the homologous phages respectively. The incorporation of adjuvant with the phage antigen for immunizing rabbits gave much better results than when the phage was used alone.

Electron microscopic studies

The study of the ultrastructure of phages in the electron microscope is of additional value as a taxonomic aid. The cholera phages examined in the present work have received little previous attention in this respect. It is clear from the micrographs of the phages that were examined that they fall into 3 morphological groups. The first group includes 2 phages with a regular hexagonal head and a long tail. These may be subdivided into one having a contractile tail sheath and the other without such a tail sheath. The second group includes 4 phages with almost circular heads and short tails measuring about 230 Å in length. There are some differences in the head sizes of these phages but they are not considered significant because distortion of the heads is likely to occur in varying degrees from one preparation to another.

Measurements of the negative-stained phage heads may thus not be reliable, but the tail lengths show much greater consistency. The third group includes 3 phages that are similar in appearance, but differ in size.

The stages in bacterial lysis and bacteriophage proliferation in a mixture of cholera phage and a susceptible growing vibrio strain have been studied. Complete lysis of the strain has been observed to take place 180 min. after addition of phage to the culture.

Genetic studies.

In the cross experiments of each of the streptomycin-sensitive lysogenic El Tor strains H_1, H_6 and NG149 with streptomycin-resistant, non-lysogenic, phage-sensitive V. cholerae strain 508, colonies of streptomycin-resistant variants with characters of El Tor strains were obtained. It is possible that these were recombinants. The streptomycin-resistant character of strain 508 has been transferred to the streptomycin-sensitive progeny of the crosses. As strain 508 is not lysogenic, the transfer of this genetic character by phage seems to be excluded. The mechanism involved in this transfer of genetic character could possibly be conjugation. Genetic studies carried out by Bhaskaran (1958) with V. cholerae culminated in the recognition of a conjugation system in which hybrids could be isolated from 2 mating parents. It was also demonstrated that in the mating process, a parent functioned as gene donor (male) while the other behaved as gene recipient (female).

This was determined by a fertility (F) factor. As in Esch. coli and Pseudomonas systems, crosses between $F^+ \times F^-$ vibrios gave the highest recombinant yield, $F^+ \times F^+$ crosses were less fertile, and $F^- \times F^-$ crosses were completely sterile. F^+ cells appeared to act as donors and F^- as recipients, but linkage between donor markers was rarely found among recombinants so that the chromosomal fragments transferred by donors were probably very small (Bhaskaran, 1960; Bhaskaran and Iyer, 1961). Although it is therefore probable that conjugation occurred in the present experiments, it is possible that transfer of antibiotic resistance by a cytoplasmic resistance transfer factor (Watanabe, 1963; Anderson, 1965) could be involved.

The transfer of resistance to a single antibiotic has been demonstrated in the present work, but it has not been formally proved that the donor strain was resistant only to streptomycin and that the progeny acquired specific resistance to streptomycin only. Although it is likely that this was so (because the donor strain was deliberately pre-trained to be streptomycin-resistant) it could be argued that the operation of a cytoplasmic resistance transfer factor has not been excluded.

It was also observed that the streptomycin-resistant, non-lysogenic, phage-sensitive V. cholerae strain 508 was lysogenized when it was crossed with either the streptomycin-sensitive lysogenic El Tor strains H_4 or H_7. It thus appears that the genetic material from the streptomycin-sensitive lysogenic El Tor strains has been transferred to the streptomycin-resistant, non-lysogenic recipient V. cholerae strain 508. The mechanism involved in this case may be phage conversion. It is tempting to suggest that phage-mediated

transduction was involved, but the evidence so far does not allow this conclusion because no reliable markers were available.

Cross experiments with more genetic markers call for the isolation of biochemical mutants. Various procedures were followed extensively in attempts to isolate and characterize such mutants. There seemed to be very low frequencies of mutation in the strains tested and successful results could not be obtained. A cystine-requirement marker was developed with 2 strains but this model failed after 2-3 subcultures. The results of the cross experiments that have been performed suggest that the temperate phages of the lysogenic El Tor strains may take part in the transfer of genetic material from donor to recipient strains. However, unless cross experiments with more genetic markers are performed this cannot be considered proven.

The unsolved problem.

The problem of endemicity of cholera in the countries where the disease is prevalent still awaits solution. A true carrier stage for this disease has not yet been demonstrated in man and no extra-human reservoir of infection is known in nature. The problems of how and where the cholera organism persists during the inter-epidemic or quiescent period in an endemic area are unsolved. We do not yet know the complete natural cycle of infection in this disease.

A probable solution of the problem has been suggested following observations of loss of specific somatic group of agglutinability of true vibrios

and acquisition of specific somatic group OI agglutinability by NAG vibrios. Mutation of V. cholerae into a "non-agglutinable vibrio", and back-mutation into the "agglutinable" type has been reported by Tomb and Maitra (1927), Brahmachari (1929) and Bhattacharji and Bose (1964). Instances of such loss of agglutinability of true vibrios or acquisition of agglutinability by NAG vibrios are also quoted by Pollitzer (1959). The term "NAG (non-agglutinable) vibrio" is misleading because, although vibrios in this category are not agglutinable by somatic group OI antisera, they are certainly agglutinable by various other sera such as flagellar H antisera.

Various non-pathogenic vibrios have been frequently isolated from surface water sources, from house flies and also occasionally from human cases of cholera, both during epidemic and non-epidemic periods, and it seems that these vibrios are in circulation in the community. Bhattacharji and Bose (1964) passaged non-pathogenic vibrios in rabbits and these workers claimed that it was possible for NAG vibrios to change to typical V. cholerae after successive passages. If the cultural habits and environment of a population brings people into frequent contact with such non-pathogenic vibrios that are present in nature, it may be that classical V. cholerae could apparently emerge spontaneously. On the other hand, where the people have been able to put up a barrier between themselves and these vibrios as a result of better socio-economic conditions, the non-pathogenic vibrios do not get a chance to be passaged in the human intestine. More data are needed before this theory can be postulated with any confidence.

Accordingly, in the present work, the writer has attempted to change the characters of NAG vibrios by treatment with phage lysates from lysogenic El Tor strains in broth containing varying concentrations

of anticholera serum. There was no indication of change of characters in these NAG vibrios, despite a prolonged and intensive series of experiments with many strains.

Future prospects

This section of the work has been arduous and relatively disappointing in not producing more positive results. However, various aspects of cholera bacteriophage including genetic mechanisms in vibrio strains have been profitably studied. Useful experience has thus been gained in many procedures and some new facts have emerged. The difficulties encountered endorse the reports of other workers that this field of research is demanding. A further period of study with an adequate and continuing supply of recent isolates of vibrio strains may bring success.

In view of the current prevalence of V. cholerae and V. eltor in epidemic outbreaks of cholera, their differentiation and possible inter-relationships are matters of considerable importance in the study of the epidemiology of the disease. A number of tests are already reported to distinguish V. cholerae from V. eltor. Of these the group IV phage sensitivity test is generally preferred because V. cholerae strains are uniformly sensitive to the phage in optimal test dilutions while V. eltor strains are resistant. As the group IV phage sensitivity test is therefore of importance, studies should be carried out to see if group IV phage resistance could mutate to sensitivity in V. eltor strains. Haemolysin production

should also be investigated on the same lines. There is a possibility that V. cholerae and V. eltor may be mutant forms of one another. Proof of this would have important epidemiological implications.

The author's experience and the experiences of other workers in this field indicate that genetic studies in vibrios should be pursued more extensively. Further studies on the role of temperate phage in transferring genetic material from donor to recipient strains and the mechanism of gene transfer in conjugation in V. cholerae may reveal clues to many of the questions that are at present unanswered.

THE STUDIES REPORTED
IN SECTIONS I AND II
ARE NOW SUMMARIZED
SEPARATELY IN THE
FOLLOWING PAGES.

THE COMBINED REFERENCES
ARE LISTED AT THE
END OF THIS THESIS.

SUMMARY OF SECTION I

1. The literature on cholera has been reviewed with particular reference to possible toxic mechanisms. The present knowledge regarding endotoxic and exotoxic or exoenzymic products of *V. cholerae* has been summarized.

2. Experimental models for research in cholera are described.

3. Skin-reactive factors produced in *in vitro* preparations of *V. cholerae* and *in vivo* in experimental animals have been studied in detail and the following facts have emerged:

(i) *In vitro* production of skin toxin from *V. cholerae* has been achieved equally well by culturing in 5 per cent. Difco Bacto-peptone water or in 1 per cent. peptone water.

(ii) Skin-reactive factors contained in vibrio-induced positive rabbit gut loop fluid (enterotoxin) evoked skin lesions similar to those produced with the culture filtrates of *V. cholerae*.

(iii) The production of skin toxin in cultures of the vibrio strains was first demonstrable after 4-6 hr incubation and thereafter the concentration increased gradually with maximal production at 24 - 48 hr.

(iv) Filtrates of positive loop fluid and *V. cholerae* cultures produced skin lesions characterized by marked induration and erythema beginning 2 to 3 hr after intracutaneous injection and becoming maximal at 18 - 24 hr

but presisting for 4 - 5 days.

(v) Experiments with intravenous injections of dye at varying times after the intracutaneous injection of the test toxic materials in guinea pigs showed that filtrates of positive loop fluid and V. cholerae culture filtrates that were capable of evoking induration also caused an increase in skin capillary permeability. Immediately after injection of the dye there was a marked increase in permeability of skin capillaries followed by recovery within 1 hr. During the next few hours the permeability gradually increased with maximum intensity at 18 - 24 hr. Between 24 - 48 hr the intensity faded and the skin appeared to return to normal by 72 - 96 hr.

(vi) Positive skin lesions similar to those produced by V. cholerae were obtained with culture filtrates of Esch. coli, El Tor vibrios and some NAG vibrios.

(vii) Fractional precipitation of culture filtrates of V. cholerae with ammonium sulphate produced skin lesions of varying diameters.

(viii) Both the induration - evoking factor and the capillary permeability factor of both skin toxin and entero-toxin are equally sensitive to inactivation by heat. They are stable over a wide range of pH, non-dialyzable, and they are partially inactivated by exposure to formaldehyde. They are not inactivated when they are oxidized by H_2O_2 nor

when they are reduced with thioglycollic acid.

(IX) The skin reactivity is not affected on dilution of the toxin in saline, phosphate-buffered saline, citrate-phosphate and Tris buffers, or in the presence of calcium ions.

(X) The toxin retained its full activity after exposure to trypsin and chymotrypsin. It was completely inactivated by treatment with pancreatin and lipase. Phosphorylase did not inactivate or enhance the cutaneous effects of skin toxin.

(XI) The crude toxin preparations possessed gelatinase activity.

(XII) The skin toxin can be adsorbed with activated charcoal but the adsorbed toxin does not appear to be eluted. This finding does not exclude other possible reasons for the partial inactivation of skin toxin following exposure to charcoal.

(XIII) The in vitro prepared skin toxin is actively haemolytic whereas loop fluid preparations with comparable cutaneous activity are only weakly haemolytic. The haemolysin does not therefore appear to be the skin-reactive factor.

(XIV) The action of the toxins on guinea pig skin is not inhibited by prior and sustained treatment of the animal with mepyramine maleate.

(XV) Both of the toxin preparations are non-lethal to mice and chick embryos and they are also non-toxic to epithelial

cells in culture.

(XVI) Antitoxic sera prepared against skin toxin completely neutralize both skin toxin and loop fluid. The normal sera of rabbits obtained before immunization have no effect on these toxins. Potent homologous antibacterial serum also had no effect on the skin-reactive components of skin toxin or enterotoxin.

(XVII) Skin toxin which produces a skin induration of 14-15 mm diameter is also capable of producing a positive loop lesion in a normal rabbit when a 0.5-ml. volume of a dilution up to 1 in 4 in saline is injected into a rabbit loop.

(XVIII) Skin toxin or vibrio cell suspension in peptone saline treated in vitro with either antitoxic or antibacterial serum did not produce a loop lesion in a non-immunized rabbit. The untreated skin toxin or the vibrio cell suspension was also unable to produce any loop lesions in rabbits immunized with skin toxin.

(XIX) "Anti-toxic" serum was not only capable of neutralizing skin toxin derived from both homologous and heterologous strains but it was also capable of agglutinating both homologous and heterologous vibrio cells.

(XX) Skin toxin prepared in vitro produced precipitin bands in agar gel diffusion plates with either homologous or heterologous antitoxic serum, but no such precipitin bands were produced by loop fluid. Gel diffusion studies also

confirmed that the culture medium that is used for production of skin toxin did not give any precipitin bands with serum of rabbit immunized with skin toxin. Normal sera of rabbits obtained before immunization with skin toxin did not produce precipitin bands on an agar gel diffusion plate against skin toxin. It is therefore evident that refined fractionation of the crude toxin preparations is now indicated.

4. The above findings are discussed with a view to their further development in cholera research.

SUMMARY of SECTION II

1. The literature on bacteriophage in general and cholera phage in particular has been reviewed with special reference to the uses of phage in the treatment of cholera and in the differentiation of V. cholerae from V. eltor and other vibrios.
2. A phage-sensitive non-lysogenic indicator strain of V. cholerae and lysogenic strains of El Tor vibrios were isolated after an extensive survey of many vibrio strains.
3. High-titre phage lysates have been prepared with 9 different virulent cholera phages and the ultrastructure of these has been studied with the electron microscope. These investigations revealed short tails varying from 198Å-297Å attached to the head of each of the phages representative of groups I, II and III; a long tail of 2240Å was attached to phage of group IV. The

cholera phage 326 possessed a long tail with a contracted sheath. Some cholera phages were shown to have no tails. The heads of the phages are variable in size and shape.

4. A comparative study of procedures for the production of potent anti-phage antisera in rabbits showed that best results were obtained by incorporating an adjuvant of mineral oil and Arlacel A in the cholera phage antigen. Antiphage sera produced in this way have been used in cholera phage growth experiments and in studies of the kinetics of phage neutralization in various phage-antiserum mixtures. Serological relationships appear to exist between phages of Mukerjee's groups I, II and III, but phage of group IV is unrelated to the others.

5. Work on the characterization of cholera phages has been confirmed and expanded.

The thermal death point of group I phage was within the range of 64-71°C; group II was inactivated at 68-71°C; group III at 58-68°C and group IV at 64-68°C.

Both group II and group III phages were stable over a pH range of 4.5 to 9.5.

Stability tests for group I and group II cholera phages at 37°C revealed a decrease in plaque titre of 46.6 and 29 per cent. respectively after incubation for 24 hr. The subsequent decreases in titre for group I phage after 2, 4, 8, 10 and 20 days of incubation were 64.4, 88.8, 88.8, 90.7 and 94.4 per cent.

The effects of treatment of group I and group IV cholera phages with penicillin, streptomycin sulphate and sulphamezathine showed that penicillin at a concentration of 2500 units/ml. had no effect on group I phage but it inactivated 29 per cent. of group IV phage. Streptomycin sulphate at a concentration of 50mcg/ml. reduced the activities of group I and group IV phages to 10.3 and 11.5 per cent. of their initial concentrations respectively. Sulphamezathine at a concentration of 83 mg/ml. almost completely inhibited group IV phage but sulphamezathine had no effect on group I phage.

6. Genetic cross experiments with streptomycin-sensitive lysogenic El Tor vibrios and a streptomycin-resistant non-lysogenic phage-sensitive strain of V. cholerae yielded streptomycin-resistant recombinants with the characters of El Tor strains. It was initially thought that conjugation may have been the mechanism of transfer of genetic material in these experiments, but it seems possibly that a resistance transfer factor has operated.

7. Recombinants obtained from crosses between other lysogenic El Tor vibrios and the non-lysogenic V. cholerae strain may have been produced by transduction , cytoplasmic resistance transfer mechanism has not been excluded in the present work.

8. Intensive attempts to change the character of NAG vibrios by transduction with temperate bacteriophages produced no positive evidence that this is possible.

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