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CHOLERA STUDIES *†

3. Bacteriology

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SYNOPSIS

The morphological characteristics, biochemical properties, and cultural characteristics of *V. cholerae* are described in great detail in this study. The author also discusses the resistance of the organism to temperature, humidity, sunlight, and various chemicals, as well as the viability of *V. cholerae* outside the body (in faeces, contaminated material, food, beverages, water, etc.).

General Remarks

As referred to in the first of these studies,[a] several observers had considered cholera to be due to a specific gastro-intestinal infection before proof of this assumption was obtained through the discovery of the *Vibrio cholerae* by Koch in 1883.

Discussing the history of the 1817-19 cholera outbreak in India, Macnamara (1876), himself one of the pioneers in this field, recorded that, according to a conclusion arrived at in 1819 by the Bengal Medical Board,

" the proximate cause of the disease consisted in a pestilential virus, which acted primarily upon the stomach and small intestines ; and that the depressed state of the circulatory powers and diminished action of the heart were consequent on the severe shock which the system had received in one of its principal organs ".

Basically sound though this concept was, the Board refuted the idea that cholera was a contagious disease and it seems to have been only in 1831 that a contagium vivum was incriminated as the cause of the infection by Neale and a few other writers enumerated by Sticker (1912). It would appear, however, that Boehm (1838) was the first who actually claimed

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a See *Bull. Wld Hlth Org.* 1954, **10**, 421

to have seen the causative organisms in the dejecta of cholera patients. Since, according to Sticker, Boehm spoke of

" spherical organic particles, which adhered to one another like the parts of a cactus plant and resembled the yeast fungi detected by Theodor Schwann as the causative organisms of wine fermentation " [Trans.],

the claim of this author cannot be accepted. The same is true of similar findings recorded by some workers during the 1849 cholera outbreaks.

Two years after Boehm's findings had been published, Henle (1840), in an essay on miasma and contagion (" Von den Miasmen und Kontagien "), supported the idea that, with the exception of malaria, the infectious diseases were due to a living contagion, being perhaps caused by minute vegetable organisms (see Greenwood, 1949). An identical view was taken in 1849 by Snow in a pamphlet entitled " On the mode of communication of cholera ". As Snow stated in the second (1855) edition of this publication:

" Diseases which are communicated from person to person are caused by some material which passes from the sick to the healthy, and which has the property of increasing and multiplying in the systems of the persons it attacks."

Applying this concept to the pathogenesis of cholera, Snow came to the conclusion that

" ... the morbid matter of cholera having the property of reproducing its own kind, must necessarily have some sort of structure, most likely that of a cell. It is no objection to this view that the structure of the cholera poison cannot be recognised by the microscope, for the matter of smallpox and of chancre can only be recognised by their effects, and not by their physical properties ".

A statement even surpassing in importance that of Snow was made in 1849 by Budd who, as summarized by Macnamara, in a letter published on 5 September of that year in *The Times*, expressed the opinion that the causative organisms of cholera were

" a distinct species of fungus which, being swallowed, becomes infinitely multiplied in the intestinal canal, and the action thus excited causes the flux of cholera, which with its consequences constitute the disease ".

These organisms, Macnamara continued, Budd believed to be disseminated through society by their contact with food, and principally by the drinking water of infected places ; and consequently, he recommended as the most important means of preventing the progress of cholera that the poison which continues to be generated in the bodies of infected persons should be destroyed by mixing the discharges with some chemical fluid, such as sulfite of iron or chloride of lime, known to be fatal to beings of the fungus tribe. " As water is the principal means of the dissemination of the disease when it exists, too much care could not be exercised in procuring pure drinking water. "

As stated by Sticker (1912) Pacini, examining the intestines of cholera victims at the time of the 1854 outbreak in Florence, claimed to have found a " microbio colerigeno " which had the property of destroying the epi-

thelium and of entering into the deeper layers of the intestine, but not into the blood. Since these bodies, in the warm dejecta, showed a motility by far surpassing the velocity of Brownian movement, they represented, no doubt, a contagium animale.

Working at the same time as Pacini in St. Thomas's Hospital, London, Hassall, as quoted by Sticker, found

" myriads of vibriones ... in every drop of every sample of ricewater discharge; of these vibriones many formed threads more or less twisted while others were aggregated into masses which under the microscope presented a dotted appearance ".

These vibrios, which were depicted by Hassall and which in Sticker's opinion represented true cholera vibrios, were absent from the blood or urine of the patients, though abounding in their stools.

As claimed by Sticker, in 1866 true cholera vibrios were seen in the dejecta of patients by Leyden (see Wiewiorowski, 1866), and in the vomits as well as in the stools of these sufferers by Bruberger (1867).

Feeling convinced of the validity of the views held by Snow and by Budd, Macnamara tried to obtain proof of the presence of cholera germs in the dejecta of the patients by orally infecting experimental animals. As was to be expected, he was unsuccessful and had, moreover, the misfortune of contracting the disease himself, so that he had to go on leave to England preparatory to his retirement in 1876. However, while continuing to work as a surgeon in London, he enlarged his knowledge of bacteriology by studying for some time under Koch in Berlin. Anticipating the 1883 outbreak in Egypt, he applied to the India Office for facilities in order to continue his cholera researches there. It is tragic indeed that, as deplored by Rogers (1950) in a well documented article, officialdom failed to comply with this request, thus bereaving one of the greatest authorities on cholera of the possibility of crowning his lifework by the detection of the germ causing this disease.

However, a French commission, composed of Roux, Straus, Nocard, and Thullier[b] as well as a German commission under Koch and Gaffky were sent in 1883 to Egypt. As stated by Chambers (1938) in a fascinating account of their work

" Discovery of the guilty microbe was the goal of each commission, but they approached the problem from different angles. Koch, the pupil of Henle, who was in turn a pupil of Johannes Müller, quite naturally approached the problem as a microscopic anatomist who had turned microbist. He looked for the organisms that were invading the tissues about the intestinal lesions, culturing and isolating them. Roux, the pupil of Pasteur, whose great work in animal diseases had been done by infecting laboratory animals, set out first to reproduce the disease in animals. It just so happened that in this particular disease Roux's method could not succeed because cholera is peculiarly a disease of man and animals do not have it. On the other hand, Koch's method in this particular disease was one of promise ".

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[b] Sad to relate, Thullier, a most promising young worker, contracted cholera and succumbed.

After the termination of the Egyptian epidemic, continuing his researches in India, Koch found that the peculiar bacilli he had suspected and isolated in Alexandria were invariably present in the dejecta of the cholera patients examined by him in Calcutta, and in the intestines of victims of the disease, but were absent in any other morbid condition. The multiplication of these germs (first called " comma bacilli " on account of their curved aspect when examined under the microscope), which regularly took place as the disease progressed, and their disappearance in recovering patients, also lent strong support to the contention that the organisms in question were responsible for the causation of cholera; it still proved impossible, however, to confirm all Koch's postulates by using these organisms to induce the disease in experimental animals and isolating them again from the latter. Koch did not hesitate, therefore, to report in February 1884 to the German Government that his labours during the cholera outbreaks in Egypt and at Calcutta had been fully successful (see Kleine, 1934).

Though the validity of Koch's findings was soon widely acknowledged, misgivings were expressed because, in contrast to his initial findings made under particularly favourable conditions, it was by no means invariably possible to demonstrate the comma-like bacilli (or, as they were soon called, the cholera vibrios) in individuals who were to all appearances affected with, or had succumbed to, typical cholera. Worse still, findings such as those of Finkler & Prior (1884) during an 1884 cholera nostras outbreak at Bonn soon showed that in addition to the *Vibrio cholerae*, considered as unique by Koch, vibrios more or less resembling it do abound and might—as claimed by several observers—be of aetiological importance in the causation of gastro-intestinal affections. Indeed, one might claim that from 1884 onwards the study of cholera in the laboratory was to a large extent devoted to endeavouring to differentiate in a sufficiently accurate manner between the true cholera vibrios and cholera-like vibrios. To show to what extent this goal has been reached, is one of the main objects of the present and some of the future studies.

Classification

According to Bergey's *Manual of determinative bacteriology* (1948) the classification of the cholera vibrio is as follows:

Class	*Schizomycetes* Nägeli
Order	*Eubacteriales* Buchanan
Suborder	*Eubacteriinae* Breed, Murray and Hitchens
Family	*Pseudomonadaceae* Winslow et al.
Tribe	*Spirilleae* Kluyver and Van Niel
Genus	*Vibrio* Müller
Species	*Vibrio comma* (Schroeter) Winslow et al. (synonyms *Vibrio cholerae* Neisser; *Vibrio cholerae asiaticae* Pfeiffer)

The common characteristics of the genus *Vibrio* (a term derived from the Latin verb *vibrare*, to vibrate) are given in Bergey's manual thus:

" Cells short, curved, single or united into spirals. Motile by means of a single polar flagellum, which is usually relatively short : rarely, two or three flagella in one tuft. They grow well and rapidly on the surface of standard culture media. Aerobic to anaerobic species. Mostly water forms, a few parasites."

Morphological Characteristics

Normal forms

A description of the appearance of *V. cholerae* under the microscope must be qualified by the following statement: " As long as the usual methods of examination exclusive of flagellar staining are implemented, it is invariably impossible to distinguish between this organism and the allied members of the genus *Vibrio*, because they all appear identical in this respect ". In view of the marked pleomorphism displayed by the vibrios in general, and the cholera vibrio in particular, it is at the same time difficult to define the " typical " morphological appearance of the latter. However, as aptly described by Mackie (1929), recently isolated cholera vibrios, which had been grown at 37°C for 18-24 hours on carefully standardized agar media of an adequate alkalinity (e.g., with a pH of 8.0), are apt to appear

" as short, definitely curved cylindrical organisms with rounded or slightly tapering ends, and measuring usually 1.5 to 2μ in length by 0.3 to 0.4μ in breadth ".

It must be realized, however, that even at best the microscopic preparations made from cholera material invariably show some evidence of pleomorphism. Differences in the degree of curvature are bound to be noticeable under all cirumstances because naturally those vibrios whose plane of curvature lies parallel to the level of the field of vision, will appear more markedly bent than the organisms lying in other planes (Mackie, 1929). Moreover, individual vibrios, or even strains, may markedly vary in the degree of actual curvature, so that instead of typically curved, more or less straight forms may be present or even predominate. The length of the vibrios also varies from strain to strain so that either longer (and invariably slightly curved), or short (and markedly bent), forms are conspicuous or solely present. Even in recently isolated cultures occasionally quite short forms resembling cocco-bacilli are found (see, for example, Seal, 1935).

Shorter or longer forms resembling in appearance the letter " S " are often seen. The occurrence of the former is due to the fact that the vibrios are not merely bent in one plane, but also twisted, thus representing a part of a screw turn (Kolle & Prigge, 1928), while the longer " S " forms are the result of the adherence of two vibrios, specially those which have not parted after transversal fission. Such adherent vibrios may, however, not

only appear in the " S " form, but may also form semicircles. In old cultures, long spirals, due to the adherence of several vibrios, may be conspicuous, but such spiral filaments are absent in films made from recently isolated cultures. The occasional presence of such spirals in smears made from the dejecta of patients is apt to be due to contamination rather than to the adherence of true vibrios (Gruber, 1887).

It is noteworthy that in smears made from the flakes of typical, rice-water-like cholera stools the vibrios often show a characteristic arrangement, lying with their long axes parallel " like fish in a stream ", this being probably due to the viscosity of the mucus in which they had been embedded (see Sticker, 1912).

While the vibrios seen in stool smears, though apt to vary in their dimensions, usually show the typical curvature, those in histological sections of the intestines as a rule have the appearance of short straight rods. For this reason and also because spindle-shaped forms may be present, the vibrios in such sections may resemble glanders bacilli *(Malleomyces mallei)*, a feature noted by Koch in his initial investigations.

As far as the findings in smears from cultures are concerned, it is important to note that the above-described " typical " appearances seem indicative merely of a phase in their development. Henrici (1925), who studied this problem with particular care, distinguished between (*a*) an embryonic stage corresponding to the period of accelerated growth, in which the vibrios were large and bacillary in form; (*b*) an adult stage, characteristic of the period of a decreasing growth-rate, during which typical vibrios were found ; and (*c*) a senescent stage during which the irregular forms described later in this study became apparent or even predominant.

Earlier observations made in this connexion by Wherry (1905) showed that such morphological differences indicating successive growth-phases may be demonstrable simultaneously in one and the same culture: at the periphery of the growth, where active cell division took place, the vibrios displayed a short and almost oval form, whereas the older forms in the centre of the growth tended to be more elongated and to undergo involution.

Envelopes and capsules

Observations suggestive of the formation of an envelope or capsule by the *V. cholerae* seem to have been made under exceptional circumstances only.[c] Using the flagellar stain recommended by Yokota (1924, 1925) to study the opaque variant of this organism, Balteanu (1926) found that some of the vibrios stained in this manner were surrounded by a thick layer of pink-staining material. When, instead of using this stain, the films

[c] See, however, the statement of Kolle & Prigge (1928), quoted later in the section dealing with the staining properties of *V. cholerae* (page 784).

were coloured for two minutes with carbol fuchsin, then treated for 10 to 20 seconds with 1% hydrochloric acid and washed with water,

" the presence of a thick envelope stained pink surrounding the red vibrio was easily demonstrated. Sometimes a like covering enclosed two or several bacteria in a common matrix of mucus-like material. The opaque variant had evidently acquired the faculty of producing a slimy exudate simulating a capsule."

Further observations on this point have been recorded by Bruce White (1938) in a most valuable study on the rugose variant of *V. cholerae*. He stated to have seen truly capsulated forms only in the case of a markedly atypical strain, whereas, generally, the organisms composing the rugose colonies appeared to be enclosed in a common zooglea of gelatinous or mucoid intercellular material. However, ascribing the rugose condition of the cholera vibrios to an intensification of normal secretory processes, Bruce White maintained that no fundamental, but merely a gradual, difference existed between the truly capsulated forms and those embedded in a common matrix.

Flagella

Most cholera workers are in full agreement with Koch's original statement, confirmed by early observations in Egypt (see Kolle & Gotschlich, 1903) and ample subsequent work in India, that, in contrast with part of the cholera-like species, *V. cholerae* possesses only one polar flagellum. Statements made to the contrary by a few observers deserve no credence, because they were never based upon results obtained with strains freshly isolated from patients but upon findings made in the case of growths which had undergone the vicissitudes of prolonged storage, in variants, or in vibrios from carriers (see Seal, 1935). More important still, most, if not all, claims that the cholera vibrio may possess more than one flagellum were made before the now-available reliable methods of serological identification could be implemented.

As summarized by Mackie (1929):

" the length of the flagellum is somewhat variable, measuring up to 4 or 5 times the length of the vibrio. Long flagella are frequent but short vibrios with short flagella may be seen. Kolle & Prigge (1927) have figured these two morphological types, namely short ovoid organisms with short flagella and longer forms with long flagella."

Motility

It is generally agreed that, if examined under the conditions specified below, the cholera vibrios present in the dejecta of patients or in recently isolated cultures are invariably motile, showing a " scintillating " movement, compared by Koch to the exceedingly rapid progress of a host of gnats, and sometimes also exhibiting a " centrifugè " movement, consisting of a rotation on their short axes. Studying the rapidity of its locomotion,

Sanarelli (1919) found that *V. cholerae* was endowed with a speed three times greater than that of *Bacillus prodigiosus*, five times that of *Salmonella typhosa*, ten times surpassing that of *Escherichia coli*, and twelve times that of *B. megatherium*.

In conformity with a general rule, the cholera vibrio is most actively motile at a temperature of 37°C. Movements are slower at lower temperatures and cease altogether at 16°C. Riemsdijk (1929) found that the motility of cholera vibrios taken from the fluid beneath the surface pellicle of broth cultures was apt to be rather slight as compared to the active movements of the organisms collected from the pellicle. It appeared, therefore, that differences in oxygen supply exerted an important influence on the speed with which *V. cholerae* progressed.

As noted by some workers, the organisms composing cultures which had undergone prolonged storage, or variation leading to the formation of atypical colonies, may show a loss of motility. Nobechi (1923), who found two such immotile strains among the 88 stock-cultures of *V. cholerae* he examined, established that this loss of motility was due to the absence of flagella. Balteanu (1926), who like Baerthlein (1912) before him described a variant of the cholera vibrio characterized by the formation of opaque colonies and as a rule also by complete loss of motility, was of the opinion that the production of a slimy exudate by the vibrios in question explained " to some extent " the latter phenomenon. It is important to note, however, that the organisms were found to possess no flagella.

Modes of multiplication

Elongation of the vibrios, followed by transversal fission, is undoubtedly the usual, and according to most workers, even the only mode of multiplication of *V. cholerae*. However, Braulke (1933) felt certain that longitudinal division of the organisms may also occur. The claim that reproduction may be affected as well by the formation of gonidia, will be assessed in a later part of this study.

Staining properties

It is certain that *V. cholerae*, which stains readily with all usual laboratory dyes, is, as Mackie puts it, " definitely and uniformly Gram-negative ". The validity of this statement was corroborated by the investigations of Braulke, who was able to establish that the occurrence of Gram-positive cocci in vibrio cultures, instead of indicating the presence of the C forms postulated by Kuhn & Sternberg (1931), was actually the result of latent contaminations.

As will be discussed in a later study, several of the flagella-staining methods have been found useful in cholera laboratory-work. An interesting statement made in this connexion by Kolle & Prigge (1928) was that the

cholera vibrios stained according to these methods appear much thicker than those stained in the usual manner, because owing to the use of mordants for the former purpose, the teguments as well as the bodies of the organisms become stained.

Nuclei, granules, and filtrable stage

Discussing the morphological characteristics of the cholera and allied vibrios, Peruzzi (1926) stated that these organisms may show differentiated chromatic bodies, presenting during division not only the appearances, but the behaviour of a nuclear apparatus. Korobkova (1931, 1936), studying the morphological aspect of cholera vibrios grown on a special potato-starch medium, also noted the occurrence of well-differentiated nuclei.

In a recent publication, Paoletti (1952) claimed to have demonstrated, with the aid of the method of Robinow (1942, 1944), chromatic bodies in cholera vibrios similar to those described in other bacteria and considered by some workers to represent morphologically discrete nuclei.

The presence of polar bodies in cholera vibrios has been noted by several observers, first apparently by Finkler & Prior (quoted by Sticker, 1912) who reached the opinion that these " Polkörner ", becoming liberated and sedimented after decay of the vibrios, could give rise to typical growths even after they had been subjected to prolonged exsiccation. Similarly, Hüppe (1885) postulated that the granula observable in cholera vibrios which had become transformed into filaments after exhaustion of the media, could assume the role of resistant " arthrospores " and thus become a link in the propagation of these vibrios. However, as stressed by Kolle & Prigge (1928), subsequent observations showed that cultures rich in such granula were no more resistant to exsiccation or other untoward influences (heat, disinfectants) than growths free from " arthrospores ".

A similar view was also expressed by Braulke (1933) who found that the small spherical forms of the vibrios (" Kügelchen "), apt to become preponderant in old cultures, specially those constantly kept at 37°C, and suspected by some workers of acting as " gonidia ", were actually unable to multiply.

Braulke, like Bisceglie (1929) before him, was also unable to find evidence for the existence of a filtrable stage of the cholera vibrio, which had been postulated by a few workers. In the opinion of Braulke, defects in the filter-candles they had used were responsible for the apparently positive results reported by these observers.

Morphological variation

Ample experience has shown that the cholera vibrios in old cultures as well as those grown in the presence of substances apt to prove inimical

to their development, often display a morphological appearance markedly different from that found upon examination of fresh material.[d] As stated by Mackie (1929), under these circumstances

" A variety of shapes may be observed, e.g. straight organisms, thicker and swollen individuals, spherical forms with faintly stained centres, spindle-, club-, and pear-shaped organisms, individuals with irregular swellings, long spirals measuring up to 17μ, and cells which present a completely distorted structure ".

In addition to these forms, Mackie referred also to the observation of spherical or triangular giant forms, branched filaments, cladothrix-like forms, and " budding " forms with roundish protuberances.

In a recent article, describing the morphological changes taking place when cholera vibrios were kept in penicillin solutions (25-100 units/ml), Bruce White (1950) noted the appearance (*a*) of numerous globular forms at first 8-10μ in diameter and motile, but enlarging upon prolonged incubation, losing their motility as well as their staining properties, becoming vacuolated and apt to bulge in subsidiary masses from the periphery ; (*b*) under optimal conditions, also of star-fish-like forms with tapering branches. As noted by Bruce White,

" on staining the broader part of the branches is seen to consist of double or multiple chains of nucleus elements; the finer branches are formed by single vibrios and from these the culture may regenerate, either in its original form, or, if seeded on to fresh penicillin-agar, in the spherical forms ".

The question of what generally causes the above-described morphological changes has been the subject of considerable debate. The contention of a few authors, that some of the abnormal forms, particularly the " budding " forms, might play a role in the perpetuation of *V. cholerae*, has been generally refuted. In fact in the opinion of many observers these atypical forms were invariably the result of involution. Mackie, who was among those advocating this view, supported it by pointing out that these forms

" occur in cultures of some duration after growth has stopped and many of the individual organisms are dying and autolysing ", and adding that " the various irregular forms described are such as might reasonably be expected to result from cell degeneration and particularly autolysis following death ".

There can be no doubt that the above-described changes in morphological appearance are often the result of involution, the less so as it was sometimes possible to establish that the irregular forms which had developed under unsuitable conditions, were incapable of multiplication. At the same time, however, it would not be justifiable to claim that processes of involution play an exclusive role in this respect, since evidence is available to show that cholera vibrios which had become morphologically atypical

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d The morphological changes resulting from dissociation of the *V. cholerae* will be described in a later section of this study.

because they had been subsisting on exhausted media or in the presence of substances inimical to them, were capable of reverting to type when grown once more under suitable conditions. Particularly illuminating observations in this respect have been recorded by Braulke (1933) and, recently, by Paoletti (1952).

The former worker, replanting cholera vibrios previously grown on media containing lithium chloride on plain agar, noted that at first mainly the abnormal " lithium forms " (small or large spheres, etc.) developed on the latter medium but that upon continued subcultivation these aberrant forms became rarer and were gradually replaced by typical vibrios.

More convincingly still, Paoletti, implanting material from old cholera cultures on new media and examining these subcultures at two-hourly intervals, was actually able to observe that the originally present round forms assumed first a quadrangular and then a sausage shape, the latter forms becoming afterwards elongated, and finally converted into typical vibrios.

These and similar observations leave no room for doubt that the appearance of morphologically atypical forms of *V. cholerae*, besides being the result of involution or, as will be shown later, of dissociative processes, may be also indicative of a temporary adaptation of the organisms to unsuitable conditions (" Anpassungsformen " of the German writers).

Cultural Characteristics

Growth limits and requirements

Reaction of the media

When dealing with the reaction of the media suitable for the cultivation of *V. cholerae*, attention must be devoted to (*a*) the initial pH of the fluids or solids used for this purpose, and (*b*) the changes in the reaction of the media taking place in the course of cultivation. As will be shown later, a high initial pH is of great importance for the primary isolation of the organisms, while in addition to this, a maintenance of not too low a hydrogen-ion concentration exerts a great influence, when cultivation of the vibrios in bulk is called for, as for instance in the course of vaccine preparation.

Initial pH. Though, as summarized by Pollitzer (1934b), " the *V. cholerae* is not among the micro-organisms demanding elaborate preparations for their cultivation ", it proves indiscriminating in this respect only as long as the media used for its growth possess a suitable pH. The presence of even a slight degree of acidity, corresponding, according to Kitasato (quoted by Kolle & Prigge, 1928), to 0.07% HCl, is sufficient to impede the multiplication of the cholera vibrios. A markedly high alkalinity of the media, on the contrary, is not only well tolerated by these

and many other vibrios, but even proves most beneficial because it counteracts the growth of the contaminating bacteria often present in the specimens coming for examination.

It is not surprising to find that some variance exists in the statements made by different observers regarding the pH limits within which satisfactory growth of *V. cholerae* is apt to take place, because they worked with different, or at least with differently prepared, nutrient media. Of great importance in this respect is also that, as shown by Kabelík & Freudmann (1923), the suitability of the media for the growth of the cholera vibrio depends upon an interrelation between the hydrogen-ion concentration and the NaCl content, the optimum of the latter becoming lower as the former increases, and vice-versa. As far as the plain media routinely used for cholera diagnosis, particularly peptone water, are concerned, however, modern workers recommend an initial pH ranging from 8.0 to 9.0 or slightly higher (9.5 according to Matsuo, 1924). Vedder & Van Dam (1932), assessing the value of Dieudonné agar (a selective medium described later in these studies) for the cultivation of *V. cholerae*, found that no growth took place if the pH of the plates exceeded 9.6. This figure is fairly well in accord with the experience of Read et al. (1939) when growing cholera vibrios in 1/100 peptone water with 1% NaCl, according to which full multiplication occurred up to a pH just in excess of 9.4, but with some reduction from 9.4 upwards. In the opinion of these workers

" a pH of 9.2 may, therefore, be taken as the limit for satisfactory multiplication, a figure which is supported by the results of experience in isolating the vibrio from natural sources ".

pH changes during cultivation. That cultivation in the usual glucose-containing media leads to a marked lowering of the pH and that such a drop even takes place in the case of media into which no fermentable sugars have been incorporated, is shown by the observations of Banerjee (1939) recorded below:

Hours (or days) of growth	*Ordinary broth*	*0.2% glucose broth*
2	8.2	7.6
4	8.0	7.4
6	7.6	7.2
8	7.6	7.0
10	7.6	7.0
12	7.6	7.0
24	7.6	7.0
30 days	7.4	6.8

Remarks. (*a*) The drop of the pH was uniform regardless of whether aerobic or anaerobic cultivation was resorted to. (*b*) Banerjee found that, in contrast to the changes recorded above, no lowering of the pH took place when cultivation in Ramon's glucose medium (prepared from an HCl-digest of hog's stomach and minced veal) was resorted to, the pH remaining at 8.2, apparently the original level.

Read et al. (1939) found that a pH of 6.0 marked the lower limit of the range within which multiplication of the cholera vibrio took place in peptone water. Similarly, Jennings & Linton (1944a), working with a medium which contained a casein digest besides inorganic salts and varying amounts of glucose, found that most rapid growth of *V. cholerae* took place while the pH was falling from about 8.5 to 6.0, while lower as well as higher values were associated with inferior growth-rates. Considering their experiences, Jennings & Linton suggested with much reason

" that while a high initial pH may be optimal in the sense that it gives the best conditions for an extended period of growth, the most desirable pH for rapid multiplication may lie in the region near neutrality. Experiments showed that a pH of 10 was injurious to the vibrio and usually prevented growth altogether. Invariably growth stopped when a pH of 5.5 was attained, whether at the end of a long vigorous growth starting at high pH or at the end of a shorter period when growth was initiated at a lower pH level."

Various procedures have been used to counteract the lowering of the pH which takes places in the course of the cultivations of *V. cholerae*. Some workers resorted to the periodic addition of alkalis to the cultures, Hirsch (1928), for instance, using calcium carbonate for this purpose, Veeraraghavan (1949) sodium bicarbonate, with the aid of which it was possible to maintain the pH of the special medium he employed (see later) at a pH level of 8.0 with a marked growth increase. Various buffer substances have been incorporated in the media by other workers. Jennings & Linton (1944b) who, as will be described below, worked with a simple medium containing a casein digest besides glucose, used " aeration " with a mixture of air and CO_2 to maintain the pH of their cholera cultures at a satisfactory level. The efficacy of this procedure was confirmed by Ranta & McLeod (1950).

Gallut (1947a) admitted that vigorous bubbling of air through the media promoted to growth of *V. cholerae* by maintaining the oxidation-reduction potential of the organisms at a constant level even in the presence of glucose, which in this case was oxidized instead of being fermented. However, in a subsequent paper (1947b), he adduced doubts as to whether cultivation under enforced aeration was advantageous for the purposes of cholera-vaccine manufacture because the vibrios grown with the aid of this procedure showed an atypical morphology (prevalence of filamentous forms and early appearance of global forms) as well as diminution of their nitrogen content.

Further reference to the pH adjustment of the media used for the cultivation of *V. cholerae* will be made in a subsequent study, where the practical aspects of cholera laboratory work will be discussed.

Salt requirements

Beauverie (1916), cultivating cholera vibrios in broth with an NaCl concentration of 7, 9, 15, 20, 30, 50, 90, and 100 per thousand respectively,

found growth to become visible within 24 hours in all concentrations up to, but not above, 50 per thousand. To judge from the formation of a particularly thick pellicle, an NaCl concentration of 30 per thousand was particularly favourable for the multiplication of the organisms. However, Beauverie found that the cholera strains cultivated in broth with an NaCl-content of 30-50 per thousand, while being at first favoured in their growth, aged quickly, showing within a few days evidence of involution and loss of motility.

As noted by Kabelík & Freudmann (1923), Sierakowski, in an article published in the *Przeglad epidemiologi* in 1922, had recorded that an NaCl concentration of 0.5% was optimal for the growth of *V. cholerae*. In Sierakowski's opinion, the discrepancy between his findings and those of Beauverie was due to the fact that, in contrast to Beauverie, he had worked with solid media. However, Kabelík & Freudmann, making comparative tests with 2% peptone water, ordinary broth, agar, and gelatin with a varying NaCl content (0%-6%), found that no fundamental differences existed between the solid and fluid media, as assumed by Sierakowski. Like Beauverie they recommended the use of peptone water with an NaCl concentration of 3% for practical cholera laboratory work—a proposal also made more recently by Genevray & Bruneau (1938d).

Valuable investigations on the salt requirements of *Vibrio cholerae* were carried out by Read et al. (1939), who mainly used an artificial concentrated sea-water solution for their tests, but also experimented with the components of this preparation (sodium chloride, magnesium chloride, magnesium sulfate, and potassium chloride) and with some other substances.

Read et al. (1939) established in the course of these investigations that

> " in the absence of salt multiplication did not occur in any peptone concentration and in no case did survival reach 24 hours ".

Multiplication of *V. cholerae* was observed in the case of 1/50,000 peptone water at sea-salt concentrations of 0.5% to 3%, in the case of 1/5,000 peptone water already at a salt concentration of 0.1%, in that of 1/500 peptone water even at a salt concentration of 0.075%.

Testing individual salts (calcium chloride, sodium nitrate, and sodium sulfate, as well as the above mentioned) Read et al. found that "any one of the salts tested except magnesium sulphate can promote multiplication, but none seems to do so in any specially low concentration", and that the sea-water solution mainly used for the experiments had a somewhat higher capacity for promoting multiplication than the individual salts tested.

Magnesium sulfate, besides being shown to be incapable of promoting multiplication or survival of *V. cholerae*, was also found to be rapidly

lethal to the vibrios in the higher concentrations tested. Sodium sulfide (Na_2S), on the other hand, if added to 1/50,000 peptone water in a strength of 0.0003%, secured multiplication of the cholera vibrio at a sea-salt concentration of 0.05%, while 0.00003% Na_2S was sufficient to secure growth of the vibrios at a sea-salt concentration of 0.1%.

Oxygen requirements

The dependency of the cholera vibrio on the presence of oxygen attracted the attention of Koch (1884) who placed pieces of mica on gelatin plates inoculated with *V. cholerae*, and noted that the practical absence of growth under these platelets stood in marked contrast with the abundant development of the organisms round them. The early observers were also impressed by the fact that, when cultivated in fluid media, the vibrios grew most abundantly at the surface of these, and were led to believe that an avidity of the organisms for oxygen fully explained why, typically, a pellicle formed on the surface of these cultures. Hesse (1893), carrying out gas-analytic studies, reached the conclusion that the *V. cholerae* was unable to grow in the total absence of oxygen.

However, carrying out studies on the oxygen requirements of the cholera vibrio, Hirsch (1926a) found that this organism was able to grow anaerobically as well as aerobically in a simple chemically-defined medium, provided that a fermentable sugar (glucose) had been added to the latter. These observations, he maintained, went a long way to explain how the vibrios could multiply in the intestine under what amounted practically to anaerobic conditions.

Carrying out further investigations on the metabolism of the cholera vibrio under aerobic and anaerobic conditions, Hirsch (1928) reached the following conclusions:

> " 1. The aerophilic behaviour of the *V. cholerae* in carbohydrate-free amino-acid solutions or in peptone solutions is conditioned in an obligatory manner by the chemical composition of the substrate and can not be considered as a specific property of the organism.
>
> 2. The *V. cholerae* is capable of multiplication under strictly anaerobic conditions provided that carbohydrates are available as an anoxybiotic source of energy." [Trans.]

Working with broth media, Banerjee (1939) fully confirmed the contention of Hirsch that the *V. cholerae* was capable of growing luxuriantly under anaerobic as well as under aerobic conditions in the presence of glucose. Moreover, comparing the growth of this organism in ordinary broth tubes and in tubes in which a layer of vaseline oil had been superimposed, Banerjee found that a restricted growth of *V. cholerae* took place in glucose-free broth even when the access of air to the culture medium

had been prevented. The evidence Banerjee obtained in this respect may thus be summarized:

Hours of growth	*Growth of V. cholerae in millions*	
	Aerobic	*Anaerobic*
3	50	12
6	500	40
9	2,200	100
12	4,500	240

In view of the evidence adduced above, the cholera vibrio must be considered as a facultatively anaerobic, rather than as an obligatorily aerobic, organism.

Temperature requirements

In contrast to certain other vibrio species (e.g., the one found to be the causative organism of a fish epizootic by David (1927), which grew best at 8°-20°C), mutiplication of *V. cholerae* is most abundant within a temperature range of 30°-40°C with an optimum at about 37°C. Growth at 22°-25°C, i.e., at temperatures used mainly for the incubation of gelatin plates, is still fairly satisfactory.

As claimed by Koch (1884), the cholera vibrios are unable to multiply at temperatures below 16°C. However, as summarized by Kasansky (1895), it was soon shown by some other workers that a slow growth of *V. cholerae* may still take place at temperatures ranging from 8°C to 15°C. In Kasansky's own experience a multiplication of the organisms still occurred at 10°C to 12°C. Moreover, as will be seen in the concluding part of this study, the cholera vibrios show a most remarkable resistance to low temperatures, which are apt to prolong the life-span of the organisms even though no longer permitting their multiplication.

Nutritional requirements

The efforts made by a number of workers to determine basic nutritional requirements of the *V. cholerae* by cultivating it in simple, chemically defined media, may be said to fall into two categories: (*a*) attempts to grow this organism in media containing only ammonium salts but no amino-acids; (*b*) use of media in which amino-acids were the essential constituents. These two classes of investigations will now be dealt with seriatim.

(*a*) *Ammonium salts.* Anderson, in *An introduction to bacteriological chemistry* (1946), stated that, like certain other bacteria, the vibrios comprise two types of strains: (1) " exacting " strains, which require amino-acids for their growth, and (2) " non-exacting " strains, capable of growing on ammonium salts as well as on amino-acids. Anderson added that " the 'exacting' strains are usually pathogenic ".

The evidence available in this respect regarding the *V. cholerae* may thus be summarized: Kisch (1919), in contrast with some other early observers, was able to cultivate this organism on a basic agar-medium to which 0.19% ammonium sulfate or 0.262% ammonium tartrate had been added, while he obtained no growth on the basic agar alone. He postulated, therefore, that the cholera vibrio was facultatively capable to grow in the presence of ammonium salts instead of organic nitrogen compounds.

Linton & Jennings (1944) and Jennings & Linton (1944a), who cultivated cholera vibrios in media containing, besides ammonium sulfate, other organic salts and glucose, as well as either peptone or a casein digest, came, on the contrary, to the conclusion that ammonium sulfate acted as a buffer rather than as an essential nutrient. As stated by Linton & Jennings, growth took place if this chemical had been omitted from the media, while, on the other hand, *V. cholerae* failed to grow in the presence of ammonium sulfate but the absence of either peptone or casein digest solution. However, in a later paper, Jennings & Linton (1944b) admitted to have found

" that very good growth could be obtained occasionally when no nitrogeneous matter other than supplied by the inoculum was incorporated in the medium. The irregularity of results, however, prompted us to include the additional casein digest as a routine procedure, since the material could be completely removed by dialysis when desired."

It is of great interest to note that recently Saxena et al. (1953) recorded constant success when cultivating 14 cholera strains as well as one El Tor strain, one strain of water vibrios, and one " rough " (? cholera) strain in a medium of pH 8.0 made up according to the following formula:

Ammonium phosphate ($NH_4H_2PO_4$)	0.1 g
Glucose	0.1 g
Sodium chloride (NaCl)	0.5 g
Magnesium sulfate ($MgSO_4,7H_2O$)	0.02 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.1 g
Distilled water to make	100 ml

Note. Ammonium phosphate was found to give a better yield after 24-hour incubation at 37°C than ammonium sulfate.

While the observations of Saxena et al. prove that cholera vibrios can utilize ammonium salts as their sole nitrogen source, it is important to note that growth in the above-mentioned medium took place only when glucose was present and when large inocula (" at or above 100 million of organisms per cc. of medium ") were used.

(*b*) *Amino-acids.* As summarized by Hirsch (1926b), Uschinsky (1893) was the first to cultivate cholera vibrios in a chemically defined fluid medium which contained NaCl, calcium chloride, magnesium sulfate, and dipotassium hydrogen phosphate, as well as glycerol, ammonium lactate, and

sodium aspartate. Fraenkel (1894) established, however, that out of these substances only three, namely, NaCl, dipotassium hydrogen phosphate, and a salt of aspartic acid were indispensable for the growth of *V. cholerae*.

Hirsch (1926b), making further careful studies of this subject, found that this organism was able to use *l*-aspartic acid as the sole nitrogen and carbon source for its metabolic and energy requirements, and that the decomposition of this acid was the result of an oxidative process, the end products of which were ammonia, acetic acid, and carbonic acid.

Further exhaustive investigations in this field were recently undertaken by Ranta & McLeod (1950) who tested 20 different amino-acids by adding them singly, or in combination, to a basic medium containing 5 g of sodium chloride, 0.75 g of dipotassium hydrogen phosphate, and 0.1 g of magnesium sulfate in a litre of distilled water. While confirming that asparagine gave relatively the best results, if used singly, combinations of two or several amino-acids proved more satisfactory. Ranta & McLeod recommended ultimately a medium containing 0.067% tyrosine, 0.051% glycine, 0.042% asparagine, and 1% glucose.

Agarwala et al. (1953a) established in the course of a recent study on the oxidative metabolism of cholera and allied vibrios that cysteine, threonine, and asparagine were oxidized as at fast a rate as glucose, lactate, and pyruvate. Basic amino-acids showed very little oxygen consumption.

Accessory growth factors

As will be gathered from the statements made above, accessory growth factors (" bacterial vitamins ") are not indispensable for the cultivation of the *V. cholerae*. However, Veeraraghavan (1949), using a chemically defined medium which contained, besides different salts, ammonium sulfate and *l*-cystine, noted that addition of marmite (an autolyzed yeast product containing anti-neuritic vitamin) had a growth promoting effect. Substitution of this substance by thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium panthothenate, riboflavin, biotin, and yeast nucleic acid, singly or in combination, did not give equally good results. It has to be noted however, that, as stated by Anderson (1946), *V. cholerae* is capable of synthesizing some of these substances, such as nicotinic acid and biotin, in the course of cultivation in chemically defined media which originally contained none of these compounds.

Role of glucose

As will be gathered from some of the observations recorded above, the incorporation of glucose into the media used for the cultivation of *V. cholerae* was apt not only considerably to promote the multiplication of the organisms but even to render their growth possible under conditions

otherwise unsuitable for their propagation. The disadvantage that addition of this sugar to the media is apt to lead in the course of cultivation to a particularly rapid and marked drop of their initial pH, is more than compensated by the impetus the presence of glucose gives to the growth of the cholera vibrio.

Hirsch (1928), devoting particular attention to the role of glucose in a valuable study on the metabolism of the *V. cholerae* during aerobic and anaerobic cultivation, established, in addition to the findings already recorded (see section on oxygen requirements, page 791), that, under aerobic as well as under anaerobic conditions, the cholera vibrios by far preferred carbohydrates to amino-acids as sources of energy. Accordingly, the amino-acids (or the peptone) in media which also contained glucose were mainly important as nitrogen sources. As already referred to, Hirsch considered the aerobic growth of *V. cholerae* in glucose-free media as a conditioned process forced upon the organisms by the limitation of the nutritive substances at their disposal. He felt convinced that not this process, but growth under anaerobic conditions in the presence of glucose represented the natural mode of existence of the cholera vibrios in the infected intestine. The present writer, for one, is in full agreement with this contention.

Describing their experiences with the casein-digest they originally used without aeration, Jennings & Linton (1944a) concluded

" that glucose serves as an important source of the energy needed for reproduction (of the *V. cholerae*), and that it is utilized in a manner which results in the accumulation of acid in the medium. The organisms are capable of using about 3 grams of glucose per liter before growth is stopped."

However, when growing cholera vibrios in their modified aerated medium, Jennings & Linton (1944b) found that the new cultures could utilize as much as 10 g of glucose per litre, lesser concentrations of the sugar resulting in smaller final crops. Addition of more than 10 g glucose per litre did not lead to an appreciable increase of the yield.

Growth in plain media

While it is proposed to deal with the special media recommended for the rapid isolation or bulk cultivation of *V. cholerae* later in these studies, it seems indicated at the present juncture to describe the cultural appearances of this organism on plain media.

Peptone water

The great value of the method of cultivation or, one should rather say, of enrichment in alkaline peptone-water for the laboratory diagnosis of

cholera is due to two fundamental properties of the *V. cholerae*, namely, (*a*) that this organism rapidly grows in the medium, thus out-distancing the contaminating bacteria usually present in the specimens coming for examination, and (*b*) that the cholera vibrios have a most marked tendency to grow on and near the surface of fluid media.[e] Hence, if one puts a particle of cholera-suspect stools, preferably a mucus-flake, or a minute quantity of other materials to be examined into a tube containing 1%-2% alkaline peptone water with an NaCl concentration of 0.5% (or more up to 3%), incubates the tube for a few hours at 37°C, then takes one loopful of material from the surface of the culture, transfers this into a fresh peptone water tube, and, after further incubation for some hours, takes a loopful from the surface of the subculture and uses this material to inoculate plates containing a suitable solid medium, one may expect, in positive cases, to find on the plates the cholera vibrios in pure culture or at least in a sufficient degree of purity to permit the immediate application of confirmatory tests.

Considerable divergence of opinion exists as to the length of time during which the initial peptone-water cultures and the subcultures made from these should be kept in the incubator before material for cultivation on solid media is taken from them. As far as the original publications of the pioneers in this field could be consulted, early workers such as Bujwid (1888) and Laser (1892), while taking full advantage of serial transfer, incubated their peptone-water cultures and subcultures for 24 hours before making use of them. Koch, in an 1893 article on the state of cholera diagnosis (" Der augenblickliche Stand der Choleradiagnose ") postulated in this connexion that

" the best time for examining the peptone solution is 6-12 hours after inoculation, [but] sometimes one must wait longer. As a matter of fact it is necessary to examine a specimen from time to time, in order to establish the maximal development of the cholera bacteria. Later these are overgrown and replaced by other bacteria even in the upper layers of the fluid, and it may happen that they cannot be demonstrated by too late an examination." [Trans.]

Kolle & Prigge (1928) recommended incubation of the peptone-water cultures and subcultures for six hours, Mackie (1929) advocated a period of six to eight hours. However, in the experience of the present writer it is permissible, during epidemics particularly, to use an incubation period of three hours only, if one takes the precaution of continuing incubation of the peptone-water cultures and subcultures, so that material may be taken from them once more, should exceptionally the need arise. Enriched surface-water samples examined with the aid of such shortened incubation

[e] Schottelius (1885), who seems to have been the first to have noted this tendency of *V. cholerae* for surface growth, took diagnostic advantage of it by mixing 100-200 ml of suspect dejecta with 200-500 ml of alkaline broth or 10 times diluted meat-peptone-gelatin. The mixture was put into a beaker or even into a beer glass and left standing on or near a stove at a temperature of less than 40°C for 10-12 hours. Small quantities where then taken with the aid of a glass rod from the surface of the growth and used for examination in hanging-drop or fixed and stained preparations.

yielded analogously satisfactory growth of the cholera-like vibrios abounding in the rivers, ponds, etc., of China.

That the growth of *V. cholerae* in peptone water is rapid indeed, has been demonstrated with the aid of biochemical methods by Dunham (1887) and Wakamiya (1940). The former found that weakly alkaline 1% peptone water with an NaCl concentration of 0.5% gave a definite, though slight, cholera-red reaction, if incubated for 4½ hours after inoculation with cholera vibrios. Wakamiya, making comparative test cultivations of *V. cholerae* and *E. coli* in peptone water into which an indicator system had been incorporated, found colour changes in the case of the former organism after 1 hour's incubation, and in the case of *E. coli* after about 3 hours of growth.

The sensitivity of tests with peptone water is well shown by observations quoted by Mackie from a report issued by the British Medical Research Council in 1920, according to which *V. cholerae* could be demonstrated by enrichment in this medium when only four to eight vibrios were present in 25 ml of a dense faecal emulsion.

Since the growth appearances of the cholera vibrio in peptone water are identical with those in nutrient broth, they will be described when dealing with the latter medium. Further reference to the practical use of peptone water for the laboratory diagnosis of cholera will be made in a later study.

Nutrient broth

As has been noted above, multiplication of the *V. cholerae* in suitable fluid media takes place with such rapidity that it is possible to start subcultivation from them as early as three hours after inoculation. This is all the more remarkable because, as confirmed by the experiences of the present writer, as a rule during the first few hours of incubation no gross evidence of growth becomes manifest in the broth or peptone water tubes or flasks inoculated with cholera materials. It is but gradually, usually after a growth of 12-24 hours at 37°C, that a uniform turbidity develops in such cultures. If one takes care not to shake the tubes, one may sometimes see that at first this turbidity is restricted to the uppermost stratum of the fluids, where the most active growth of the vibrios takes place.

Besides producing a general turbidity in broth or peptone water, growth of *V. cholerae* may, under suitable conditions, also lead even more rapidly to the formation of a pellicle on the surface of these fluid media which, as described by Mackie (1929), is at first semi-transparent and fragile, but gradually becomes thicker and more coherent, and may after an incubation of several days finally sink down in the media.

It was often held that this formation of a surface pellicle was " a phenomenon of surface growth by an organism greedy of oxygen " (Iyengar, 1920), but, as shown by the systematic studies of this worker with broth

media prepared with different ingredients and possessing varying degrees of alkalinity or acidity, the presence or absence of a pellicle depended on the one hand of the reaction of the media used, and on the other on the degree of nutrition they afforded to the organisms. Thus in mutton broth prepared by tryptic digestion with an alkalinity above the neutral point of Eyre's scale (corresponding to a pH of about 8.2), pellicle formation as well as growth of the cholera vibrio were marked, while the slight growth of the organisms in acid mutton broth was not accompanied by formation of a pellicle. The latter remained absent in all broth cultures prepared with beef extract and Witte peptone, in which weak growth of the cholera vibrios took place when the reaction was alkaline, but no growth occurred when the media were acid.

Mackie, besides shortly summarizing the above findings of Iyengar and noting that in the experience of Beauverie (1916) an NaCl concentration of 3% promoted the surface growth of the cholera vibrio, also drew attention to the observation of Wherry (1905) that " the property of pellicle formation could be established by serial transfers from the surface growth, i.e., by a process of artificial selection ". Presumably, this phenomenon was the result of cultural variation which, as will be discussed later, may lead to marked changes in the growth appearances of *V. cholerae* in fluid as well as on solid media.

Agar

The colonies of cholera and allied vibrios cultivated on agar plates from fresh materials, such as the dejecta of patients or contaminated water, typically show a rather characteristic appearance, which permits their macroscopic distinction from the often simultaneously developing colonies of contaminating bacteria such as *E. coli.* The vibrio colonies, after 18-24 hours' incubation at 37°C, appear as regularly circular, pale discs, 1-2 mm in diameter, which show a peculiar bluish lustre (" opalescierendes Irisieren " according to Kolle & Prigge, 1928), when viewed in transparent light. If incubation is continued, the colonies attain a larger diameter (5-7 mm) and may eventually assume a yellowish-brown coloration.

As described by Mackie (1929):

" *stroke growth on an agar slope* after 24 hours' incubation consists of an abundant, moist, semi-transparent confluent layer which is greyish-white in colour; on continued incubation it becomes more raised and assumes a greyish-yellow tint which deepens after about 10 days to a brown colour ".

Gelatin plates

As summarized by Pollitzer (1934b):

" The growth of cholera vibrios on gelatin plates is even more characteristic than that on agar. Though it is necessary to incubate gelatin dishes at the comparatively low

temperature of 22°C., one may after 24 hours macroscopically discern colonies represented by very small clear dots. Seen under low power of the microscope they show a peculiarly granulated surface, 'as if strewn with glass particles' (R. Koch). Macroscopically their transparency is in strict contrast to the opaqueness of the bacterial colonies one is apt to encounter when cultivating from faeces. On observing vibrio colonies of recent origin and typical behaviour one notes after about 48 hours a commencing liquefaction of the medium, the colonies appearing to sink into the medium and finally to lie in a small cup or funnel. The process of liquefaction continues, the whole medium becoming dissolved after about 10 days."

While at first glance these peculiar growth phenomena appear to be of great differential-diagnostic importance, it has to be realized that they are characteristic of many of the cholera-like as well as of the true cholera vibrios. Moreover, while even freshly isolated cholera strains may show some variation in the rapidity of gelatin liquefaction, the property of liquefying these media may be more or less completely lost by old, often subcultivated, strains. A still greater drawback from the practical point of view is that at the temperatures usually prevailing during cholera outbreaks it is rather difficult to work with gelatin media. It is, therefore, not surprising to find that their use, though much relied upon during the years immediately following the discovery of *V. cholerae*, has now been given up in favour of other methods of cultivation.

Gelatin stab-culture

In gelatin stab-cultures, growth occurs along the whole track of the needle but is most marked near the surface where—as a result of liquefaction and evaporation—an air bubble forms within the gelatin. In the past, great stress has been laid upon the diagnostic importance of a typical evolution of this phenomenon. It has now been realized that an important influence is exerted by the composition of the media as well as by the quantity of the inocula used, and that, moreover, cholera-like vibrios may show a behaviour in gelatin stab-cultures undistinguishable from that of true cholera vibrios (Pollitzer, 1934b).

Coagulated blood serum

Incubation at 37°C leads to a rapid growth of *V. cholerae* on coagulated blood serum which is initially similar to that on agar. An important difference, however, is that in the case of the former medium liquefaction commences after 24 hours and gradually becomes complete. As aptly stated by Mackie (1929), the property of liquefying coagulated serum, which like that of gelatin liquefaction is due to the proteolytic action of *V. cholerae*, also shows the same range of variability, recently isolated strains differing in the rapidity of liquefaction, while old, often subcultivated strains may more or less fail to produce this phenomenon.

Potato slopes

On potato slopes alkalinized by steaming in 0.7% sodium carbonate solution, fairly abundant growth of *V. cholerae* is usually obtained after incubation at 37°C. In the course of this, chromogenesis becomes frequently marked, so that finally a yellow, greyish-yellow, yellowish-brown or pink coloration results.

As has been noted above, a yellowish-brown coloration also becomes manifest when agar cultures of *V. cholerae* are kept at 37°C for several days.[f] Bearing these observations and the experiences with potato slopes in mind, Mackie was disinclined to consider pigment production as a sign characteristic enough to distinguish between the cholera vibrios and certain other vibrios exhibiting marked pigment formation, as had been proposed by Chalmers & Waterfield (1916). As Mackie pointed out with great reason:

" The property of chromogenesis seems a general one in the genus (Vibrio) though more pronounced in certain species according to the medium in which the organism is growing."

Milk

While it is generally agreed that milk is a suitable substrate for the cultivation of *V. cholerae*, markedly divergent opinions have been expressed regarding the growth appearances produced by the organisms in this medium, a few writers even maintaining that in contrast to their usual behaviour the cholera vibrios do not produce an acid reaction in milk media. It has to be noted, however, that the evidence to the contrary brought forward by early workers, such as Kitasato (1889b), Schoffer (1895), Wherry (1905), and Kendall et al. (1914), has been fully confirmed through more recent observations by Pollitzer (1935) and Genevray & Bruneau (1938c). The former worker, carrying out a systematic study at Shanghai, found that 25 cholera strains as well as a larger number of cholera-like vibrios, mostly those isolated from surface waters, invariably produced acidity in litmus milk, usually within 24 hours of incubation at 37°C. Similarly Genevray & Bruneau, studying over 500 cholera strains in Indochina, found that these " invariably turned the colour of litmus milk into pink within 24-48 hours ".

While, therefore, there is no valid reason to doubt that cultivation of *V. cholerae* in milk leads to the production of an acid reaction, to what extent this is followed by coagulation of the medium is a rather involved question.

Referring to the initial observations made in this connexion by Koch and his co-workers in India, Gaffky (1887) considered it as " most

[f] Genevray & Bruneau (1938c), studying about 500 cholera strains isolated during the 1937-8 epidemic in Tonkin, even found that when young agar growths were taken up in large quantity on a platinum loop, a salmon-pink tint became noticeable.

remarkable " that the cholera vibrios, though rapidly and abundantly multiplying in milk, did not produce coagulation or any other macroscopically observable reaction in this medium. However, as summarized by Schoffer (1895), soon afterwards some observers found that the strains at their disposal, which had been mostly isolated in Europe during 1892 and 1893, did coagulate milk. Indeed, this behaviour of the cholera strains derived from the 1892 epidemics in Paris and Hamburg led Liebreich (1893) to the assumption that these outbreaks had been caused by " comma bacilli " different from those isolated by Koch in India.

Though this postulation is now merely of historical interest, it is important to note that, while cholera strains capable of coagulating milk have practically never been met with in India, during the present century, they have been detected upon several occasions in other areas. The following observations may be quoted in this connexion:

(*a*) Wherry (1905) found that one out of the five cholera strains he had recently obtained in the Philippines, was capable of curdling milk within 48 hours.

(*b*) Examining the strains isolated during the Russian cholera epidemics of 1908-10, Buroff & Buroff (1911) noted that all these growths produced milk coagulation, which became manifest at 37°C on the second day of incubation, at room temperature after 10-12 days. It has to be added that the strains tested by these two workers were also unusual in so far as they proved to be endowed with haemolytic properties.

(*c*) Working in 1914 with 42 strains which had been isolated during the recent Balkan wars, Popoff-Tcherkasky (1914) found that only five of these growths failed to curdle milk, while the others produced coagulation within 3-11 days. Most of the strains examined by this worker were also haemolytic.

(*d*) Pollitzer (1935), while finding that the cholera strains isolated in China during cholera epidemics produced no, or only late, coagulation of milk, noted that two out of three strains isolated from sporadic cholera cases at Shanghai in 1934 as well as a strain agglutinable with cholera immune serum which had been isolated at the same time from the Whangpoo River, combined the property of rapidly curdling milk with haemolytic properties. It is important to add that the strains of *V. cholerae* isolated in China during epidemics proved non-haemolytic.

(*e*) Ali Mustapha (1936), comparing the behaviour of 37 cholera strains with that of 27 El Tor strains, recorded the following interesting results :

Kind of strains	*Number of strains*	*Behaviour in milk media*
Strains from : India	9	Grew abundantly, producing acidity, but caused with the exception of one Indian strain no coagulation in milk.
Indo-China	15	
Strains from minor outbreaks in Baghdad and Bassorah	12	More or less complete coagulation within 2-24 days.
El Tor strains *	27	Massive coagulation, mostly within 24-48 hours, in a minority within 3-15 days.

* Lomas (1916) had previously established that two El Tor strains were capable of curdling milk within 9 and 20 days respectively, while control strains of *V. cholerae* failed to do so even within 30 days.

Mustapha suggested that, since milk coagulation was produced by the El Tor strains, which were as a rule non-pathogenic, and less rapidly also by strains isolated in the Middle East during minor cholera outbreaks, but practically never in India or Indochina, tests with milk media might be a means of distinguishing between cholera strains endowed with marked and low cholerigenic powers, respectively. However, the validity of this assumption is disproved by the observations made in the case of more than 500 Indochina strains by Genevray & Bruneau (1938c), who reported on their experiences with milk media thus :

" All the strains studied change the colour of litmus milk to pink within 24-48 hours and coagulate it. This coagulation commences with the formation of a coagulum 'cap' on the surface of the medium, which often appears within 24-36 hours. The coagulum then slowly grows, reaching a thickness of 2-3 cm within 8 days. If left in the incubator, the whole of the milk becomes coagulated, and the 'cap' more or less shrinks, sometimes undergoing a slight digestion. It has then an aspect similar to that of the soft part of bread." [Trans.]

The question of whether the coagulation of milk by *V. cholerae* was the result of a rapid formation of acid alone or was partly or even wholly due to the slower action of a rennet-like ferment, which according to Fokker (1892) was produced by the cholera vibrios, has been systematically studied by Schoffer (1895). Making parallel tests with milk samples to which lactic acid alone had been added at varying concentrations and with such in which cholera vibrios were cultivated, he established that the organisms were apt to cause curdling of milk at much lower degrees of acidity than one would have expected from the tests with lactic acid. The action of ferments was, therefore, apparently of paramount, if not of sole importance in the usually slow process of coagulation caused by *V. cholerae*. At the same time, carrying out seven successive series of tests with different kinds of milk, Schoffer noted a marked inconstancy of the coagulative reactions produced by most of his 14 cholera strains. He raised the question of whether these inconstant findings, instead of being the result of a changing behaviour of the vibrios, were not actually due to changes in the composition of the various kinds of milk used successively, which it was impossible to assess. Schoffer pointed out that this ever-changing character of milk might, to quite some extent, explain the often contradictory statements made regarding the behaviour of *V. cholerae* in this medium. However, while the possible influence of such differences ought not to be disregarded, the available evidence seems to indicate, nevertheless, the existence of a parallelism between milk coagulative and haemolytic properties of the vibrios, with the result that the El Tor vibrios are far more prone to curdle milk than the classical non-haemolytic cholera vibrios. It would seem desirable further to elucidate this point by large-scale parallel tests with cholera and El Tor vibrios, particularly in India where thus far but scanty attention seems to have been paid to the behaviour of these organisms in milk media.

Eggs

Notwithstanding statements to the contrary made by a few workers such as Hüppe (1888), Kolle & Prigge (1928) maintained that only slight growth of *V. cholerae* takes place in eggs. However, the fact that Wilson (1946), infecting chick embryos via the allantoic sac with cholera and cholera-like vibrios, noted a multiplication of the organisms not only in the allantoic fluid but also in the amniotic fluid and the egg-yolk deserves attention. Moreover, as pointed out by Kolle & Prigge, the egg-broth of Besredka & Jupille (1913) proved an excellent medium for the growth of cholera vibrios as well as for tuberculosis bacilli. Similarly, as will be described in a later study, an alkaline egg-peptone medium has been recommended by Goldberger (1914) for selective enrichment in cholera diagnostic work. It also deserves attention that more recently Derkatsch (1927) utilized an alkalinized mixture of 150 ml egg-yolk with 850 ml distilled water (pH 7.8) for the differentiation of *V. cholerae* from " para-cholera " and other non-cholera vibrios. He claimed that true cholera vibrios reacted in this medium characteristically by producing within 42 to 72 hours' incubation firm clotting of the substrate, which was followed in 5-7 days by liquefaction with evidence of ammonia production.

Bile

It is of interest to add that Ottolenghi (1911) recommended an alkaline bile medium, made with fresh cattle bile, for the enrichment of *V. cholerae* instead of peptone water. Though Ottolenghi's method was considered useful by some subsequent workers, others, particularly Krombholz & Kulka (1912), found it less reliable than enrichment in peptone water. In the opinion of Kolle & Prigge there was thus no indication to take practical advantage of Ottolenghi's method.

Blood media

The complex problem of the behaviour of *V. cholerae* in fluid or on solid blood-containing media will be dealt with in a later part of the present study.

Cultural variation

Kolle & Gotschlich (1903) who were the first to stress the occurrence of variant colonial types of *V. cholerae* stated in this connexion that

" Petrie was the first to point out precisely that in some cultures there occur, besides typical, so-called atypical (colonies), which he called " lobated " colonies. Later observations by Dönitz and Pfuhl showed that, if special substances, e.g. asparagin, are added to the nutrient gelatin or if media with a low gelatin content (3-5%) are used, the cholera colonies do not appear as round, brightly refractory discs with a slightly indented margin, which appear to be bestrewn with smallest glass splinters, but show a yellowish coloration, coarser structure and an irregular margin, which sometimes looks like frayed (formation

of loops), as is common in old laboratory strains, which had been isolated during earlier epidemics.

"A careful examination, undertaken in this respect with cultures recently arrived from Egypt in Berlin has left no doubt that in all thus received fresh strains, if they had been subcultivated once or several times, one could invariably find both types of colonies, which we might call transparent and opaque colonies." [Trans.]

Kolle & Gotschlich added that, when subcultivating pure cultures on agar, one also found two types of colonies, those which were homogeneous and others which showed formation of a distinct rim or ring.

As shown by the exhaustive studies of Baerthlein (1911a, 1912), the development of variant colonies took place not only on gelatin, but also on agar plates, on which, besides the above-mentioned transparent colonies and ring colonies with an opaque centre and a transparent border, yellowish-white opaque colonies are apt to be present or even preponderant. Baerthlein found that the transparent colonies were composed mainly of slender, uniformly staining, and well curved vibrios, whereas microscopic preparations from opaque colonies revealed the presence either of short, thick, bipolar-stained vibrios, or of longer, well curved forms showing instead of a uniform staining a segmental staining. It has to be noted, however, that in the experience of subsequent observers these morphological differences between the vibrios composing the transparent and opaque colonies respectively were not obligatory.

While finding these two main colonial types remarkably stable if selectively subcultivated at frequent intervals, Baerthlein established that if growths displaying the presence of one of the types only were subcultivated after a lapse of time, once more both colonial types became manifest.

Another point made by this worker was that recently isolated cholera cultures were more prone to show colonial variations than strains kept in the laboratory for a longer time. This seemed to be explained by the observation of Haendel & Woithe (1910) that freshly isolated cholera growths were particularly sensitive to nutritional changes. However, other workers expressed the opinion that cultural variation was " more likely to occur in artificial cultures after continued growth on medium, but may be met with even in newly isolated strains " (Mackie, 1929).

In a later paper, Baerthlein (1918) distinguished between at least nine colonial variants of *V. cholerae* but, as maintained by Gildemeister (1922), most of these seemed to present merely transitory or intermediate forms between the three principal types, the transparent, opaque, and ring forms.

Examining seven cholera strains as well as two El Tor strains and one of " paracholera " vibrios, Balteanu (1926) was able to distinguish between normal, round, translucent colonies and three variants, which he described as follows:

(*a*) Circumvallate rugose colonies, which were small, opaque, whitish-yellow, and had a thickened margin as well as radially arranged ridges. They

were firmly adherent to the medium and could not be satisfactorily emulsified in saline or distilled water. Transferred to broth, these growths produced a thick wrinkled surface film, which broke up on the tube being shaken and sank to the bottom, leaving the liquid clear. Further reference to these rugose colonies will be made in the following section of this study.

(*b*) White ring colonies, which were whitish and semitranslucent and sometimes had an opaque centre and more translucent margin, thus resembling the ring-forms of Baerthlein.

(*c*) Opaque colonies, which were round, unusually prominent, firm in consistency, adherent to the medium and difficult to emulsify. Transferred to broth or peptone water, these growths produced a thick, hard pellicle, resting on a clear medium, and a large deposit. While this colonial form proved stable on agar subculture, repeated transfers in liquid media led to turbidity and gradual reversion so that, as described by Balteanu:

" Plating from the 10th or 12th daily subculture yielded colonies with translucent margins and after further subcultures colonies of the normal type occurred."

Though Balteanu was able to procure this variant from four of his cholera strains and from one El Tor strain, it was obtained regularly only from one of the former which, because found to be haemolytic for human and sheep corpuscles, perhaps ought to have been placed in the El Tor group. Studying this strain alone in detail, Balteanu found, as has been noted earlier in this study, that the vibrios composing the opaque colonies possessed no flagella and were, in the opinion of the present writer for this reason, non-motile. Reference has also been made to the presence of a thick mucous envelope round the vibrios. If the flagellar stain recommended by Yokota (1924) was used, the organisms in question were often uniformly and intensely stained, but less frequently they showed granular or even bipolar staining.

In consideration of these findings it is rather difficult to share the opinion of Balteanu that the unusual colonial form observed by him in an atypical strain was similar to the opaque variant described by Baerthlein. More probably, the appearance of the " opaque " colonies described by Balteanu was due to a process of dissociation, so that they represented the mucoid (M) colonial type. However, in order to decide this point, it would be certainly desirable to make in this respect further studies of the opaque colonies frequently met with in the course of cultivation of typical cholera strains.

The significance of the above-described cultural variations of *V. cholerae* has been the subject of considerable debate. Though it has been postulated in some quarters that they were the result of a true, inheritable mutation, there is every reason to share the opinion expressed by Mackie (1929) that these variants

" do not, however, represent stable mutants, but are to be regarded as fluctuating variations of the organism ".

The observations of several workers that the appearance of colonial variants may be promoted by artificially subjecting the cholera vibrios to unfavourable influences, e.g., to free chlorine or phenol (Genevray 1940a, 1940b, 1940c), serve as a corollary for the assumption that such variations are the result of a temporary adaptation and not of a permanent mutation.

It is important to note that colonial variation has been demonstrated not only in the case of the classical *V. cholerae*, but also in the case of the El Tor vibrio (Balteanu, 1926, Alessi, 1939) and, as has been shown for instance by Feldmann (1917) and by Pasricha et al. (1932), likewise in the case of cholera-like strains.

Dissociation

Indispensable though it is to refer to the phenomena of dissociation at the present juncture, these processes, because apt to exert a profound influence on the immunological properties of the *V. cholerae*, can not now be fully appreciated. More than that, as will be shown in due course, a change in the growth appearances of this organism is by no means obligatory to indicate the presence of dissociation, which often may be demonstrated by biochemical or serological methods in cultures presenting no atypical features as far as their macroscopic aspect is concerned. On the other hand, it is not surprising to find that, though the presence of macroscopically characteristic dissociants had been noted by some early workers, the occurrence of such atypical colonies was confuted with that of colonial variants which, as has been described, resulted from an adaptation of the cholera vibrios to unfavourable environmental conditions.

Hadley (1927), dealing comprehensively with the early observations of dissociation in the various bacterial species, mentioned a few records dating back to 1894 which, in his opinion, referred to dissociants of *V. cholerae*. It would seem, however, that Berestneff (1908) was the first who definitely noted the rough form of this organism, stating:

" that cholera vibrios, if repeatedly transferred from agar to agar, sometimes begin to grow in the form of dry, prominent and non-confluent colonies. Many such colonies show a crater-like depression and a wall-like periphery. Such colonies are markedly different from the normal ones; on account of their dryness they are difficult to emulsify and show pseudo-agglutination, being thus unfit for agglutination tests."

Though there can be little doubt that Berestneff referred to the rough form of *V. cholerae*, it was only after the pioneer studies on microbic dissociation had been published by Arkwright (1921) and De Kruif (1921) that Shousha (1924) gave a full description of the properties shown by the smooth (S) and rough (R) types of this organism respectively.

Shousha worked with two old cholera laboratory strains, one of which proved to be haemolytic when tested with sheep erythrocytes. Both were inoculated into broth tubes which, after an incubation at 37°C for 24 hours,

were stored at room temperature in the dark. When agar subcultures were made after such storage for 15 days, the haemolytic strain only showed two types of colonies similar to the S and R forms described by Arkwright (1921) in salmonellae.

While not referring to the morphological appearances of the vibrios composing these two types of colonies respectively, Shousha stated that both were equally motile. The differences in growth appearances observed by him may thus be summarized :

Medium	*S type*	*R type*
Agar	Colonies circular with well defined margins, finely granular under low power of the microscope.	Colonies larger, flat and thin, appear granular with jagged margins when seen under low power of the microscope.
Broth or peptone water	General turbidity and pellicle formation.	Deposit at bottom and pellicle formation, the body of the fluid remaining clear.*

* Uniformly turbid growth was obtained if either media prepared with less salt were used or if the usual media were diluted to one-half or one-quarter with distilled water.

Since the publication of this initial study, the phenomena of S-R dissociation of *V. cholerae* have been exhaustively examined by different observers. The following of their findings have a bearing on the problems now under review :

Referring to the morphological appearances of the vibrios from smooth and rough cholera colonies respectively, Seal (1937) maintained that

" the individual cell of the rough type has a more opaque and granular cytoplasm and a thicker outline than that of the smooth one which, on the other hand, possesses a clear cytoplasm and a thin wall. This may explain why the rough colonies usually look opaque and the smooth ones clear and translucent to the naked eye. "

Panja (1945) who was able to produce rough colonies of *V. cholerae* by subcultivating smooth strains on agar into which mepacrine had been incorporated in the proportion of 1/5,000, noted that in the case of the Inaba sub-type some of the vibrios composing the rough colonies showed straight, spherical, or ovoid forms and were sometimes immotile. Ogawa rough vibrios showed long, straight, besides typically curved forms, but were invariably motile.

Further observations on the morphological differences between vibrios composing rough and smooth colonies respectively were recorded by Wahba (1953). In his experience " unlike other organisms, the cholera vibrios in the rough state do not always appear in long or filamentous forms ; on the contrary they might even be shorter and stouter than in the smooth state ". Making impression films from well separated colonies, Wahba found the rough colonies to be composed of three zones : a central irregularly arranged core, an intermediate zone composed of radially lying

organisms, and peripherally outgrowing tufts composed of filamentous organisms. However, the outer zone was not filamentous in the case of some rough strains. Smooth cholera colonies appeared to possess two zones only, both of which were composed of radially arranged organisms.

Marked differences existing between smooth and rough cholera vibrios in regard to the mode of cell division and colony formation were described by Seal (1937) thus :

" The essential difference depends upon the degree to which the contiguous cells adhere to each other after undergoing division. The final cluster in a smooth culture is even in appearance owing to the cells sliding past each other and forming a smooth and compact mass, while in a rough culture the tendency to slip past each other is almost absent and the cells after division tend to adhere to each other more firmly leading to the formation of bending and branching chains and irregular masses with many open spaces, projections, angles and sometimes chains, sticking out from their sides, the final cluster being thus jagged and uneven in appearance."

The differences existing between smooth and rough growths of the *V. cholerae* in fluid media were in Seal's opinion due to identical causes. In the case of the smooth type, cultivation in broth or peptone water resulted in a marked and uniform turbidity with or without a thin pellicle, because the vibrios did not tend to stick together after division. The rough strains, on the contrary, formed chains and the irregular clusters thus resulting led to the formation of a thick pellicle on the surface of the fluids as well as to the formation of granules, which as a rule sank down but could remain partially suspended, then producing a slight turbidity of the media.

Observations made by Soru (1934) with 106 cholera and cholera-like vibrios showed that vibrios of the R type had a higher negative electric charge than those of the S type. These results were confirmed by Linton et al. (1938), who partly examined dissociants they had obtained from Bruce White. Linton et al. noted the interesting fact

" that electrophoretically the organisms which are quite distinct from one another in the S state are often similar or identical in the R state. This is perhaps the underlying factor to account for the observation of Bruce White who found ' R '-strains serologically more generalized than the ' S '-strains. In the case of Shillong 1077, the ρ strain showed an even higher surface potential than the rough homologue and was very much higher than the original smooth strain."

The studies of Bruce White referred to above led to a full understanding of the phenomena, underlying dissociation of the *V. cholerae* by furnishing evidence to prove the contention made in a preliminary statement by Yang & White (1934) that in case of *V. cholerae* as in that of the salmonellae and the pneumococcus " roughening involves the disappearance of a non-protein and probably carbohydrate containing substance which furnishes the characteristic O-receptor of the smooth organism ". " It seems certain,"

Yang & White continued, " that a second non-protein ... substance, present but masked in the smooth organism, replaces in the rough vibrio the lost smooth factor and becomes the characteristic rough receptor ".

In 1934 again, Bruce White established that in the case of *V. cholerae* as well as in that of the salmonella-group a ρ variant existed, which differed from the R form by loss of the dominant R receptors. Bruce White added that according to various tests

" this loss involves the bulk of those receptors which are supplied by the alkali-resistant, non-protein and richly carbohydrate soluble substance of the rough vibrio ".

Continuing his studies, Bruce White (1936) was able to establish that " in each strain of *V. cholerae* and seemingly in vibrios in general, at least four distinct groups of polysaccharide receptors or substances are concerned in the serology of the normal parent and variant forms ". All four of these substances, named C α, C β, C γ, and C δ, were present in the smooth form, but C α was dominant. In the rough form, C α was absent and C β was dominant, whereas only C γ and C δ were present, and the latter was dominant, in the ρ form. The distribution of these four polysaccharides may, therefore, be schematized thus:

Polysaccharides

Form	*Dominant*	*Also present*
Smooth (S)	C α	C β, C γ, C δ
Rough (R)	C β	C γ, C δ
Rho (ρ)	C δ	C γ

Bruce White added that, though no variant degraded below the level of the ρ form had been discovered, such forms possibly existed. Carrying out comparative studies he found that

" the true El Tor vibrio presents a polysaccharide complex serologically identical with that of *V. cholerae* (of the same absorption type); that the C γ and C δ factors seem to be common, so far as can be judged by simple precipitation tests, to all the types of vibrios so far examined; that different groups of vibrios show sharp differences in the behaviour of their C β substances; and that the C α substances determine the serological specificity of the various smooth types ".

Besides elucidating the phenomena of S-R dissociation of *V. cholerae*, Bruce White (1938, 1940) also dealt in a masterly manner with the rugose form of this organism. He stated in the latter connexion that Balteanu (1926), working with cholera and El Tor vibrios, had noted the occurrence of a variant colonial form which he designated as rugose on account of its corrugated appearance on agar. However, Bruce White added:

" From a detailed description of the rugose variant Balteanu was probably deflected by the fact that it proved unstable in culture : his attention was occupied with a more stable ' opaque ' variant which is perhaps a cultural form much of the same genre."

This is in accordance with what has been stated earlier in this study in regard to Balteanu's " opaque " form.

Even though, as shown in table I, the appearances and properties of rugose growths are markedly different from those of the rough forms of *V. cholerae*, some workers were inclined to regard the rugose variant as the extreme type of roughness. It is the great merit of Bruce White to have pointed out that actually a fundamental difference exists between the two modes of dissociation concerned: while, as described above, the process of roughening is due to a failure to secrete or to form specific polysaccharides, rugosity is the result of an abnormally active secretion of a mucinous material, ascribed by Bruce White to an intensification of normal secretory processes rather than to a special type of activity. In fact, a transition to the rugose state could be observed in the case of rough or even ρ growths as well as in smooth growths of cholera vibrios and particularly of El Tor vibrios. The marked tendency of the rugose derivatives to return to their original state (S, R, or ρ), as contrasted with the stability of the ordinary R form, also rendered it altogether improbable that rugosity represented a culmination of roughness.

TABLE I. CHARACTERISTICS OF S, R AND RUGOSE GROWTHS OF V. CHOLERAE *

	Normal S culture	Typical R culture	Rugose culture derived from S culture
Appearance of colonies on agar plates.	Circular, moderately raised, glistening and moist ; finely granular under lens and of variable transparence or turbidity. Occasionally, colonies may show features usually associated with roughness.	As a rule differs but slightly in general appearance from S colony, so that it cannot be identified with certainty by simple inspection. Usually duller of surface and more coarsely granular ; may show irregular outline and flattening. Outward appearances not indicative of intensity or permanence of roughness.	The 18 hours' colony is small, much raised and refractile. It increases rapidly with further incubation and develops superficial corrugation, irregular, radial, or both. Opaque yellowish or yellow in colour, opacity and tint deepening with age. In older colonies vitreous or granular " corona " may be exuded from margin of the colony.
Consistence and adherence.	Semi-fluid, never adherent to medium.	Dry, brittle, never adherent.	Tough or gelatinous, adherent to medium.
Dispersibility and agglutination in NaCl solution.	Disperses readily with perhaps some initial sliminess. Dense suspensions in 1.7% NaCl solution may show some precipitation of slime entrapping a few vibrios.	Disperses readily in 0.85% or 1.7% NaCl solution but complete agglutination follows quickly, so that clumps float in clear liquid.	Growth disperses only partially and with difficulty. Vibrios once dispersed are insensitive to saline.

* After Bruce White (1938)

Though observed in growths from cholera stools, the rugose type of colonies was particularly met with in platings from ageing peptone-water cultures, and it appeared that higher peptone concentrations (5%-10%) favoured this mode of growth. More important was the fact that rugose colonies were found to abound in platings of vibrios which had survived specific bactericidal tests, possibly because the rough variants were resistant or were protected against the activated immune serum.

Generally speaking, as stated by Bruce White (1940):

" It is difficult to escape the conclusion that the rugose substance is a protective secretion with a role in assisting the survival of the race in nature. In the laboratory it affords defense against unfavourable conditions and the action of serum : it has repeatedly been observed that rugose forms tend to grow often in pure culture, from mixtures made in specific bactericidal tests and to survive their associates in ageing cultures of vibrios."

The rugose variants did not, however, prove more resistant than normal cholera vibrios when kept in saline solutions or grown in broth to which hydrochloric acid had been added, so that there was no reason to assume that they would have a particularly good chance of resisting the acid conditions in the human stomach. Nevertheless, the evidence that they are more resistant than non-rugose vibrios is convincing. Considering this, as well as the marked tendency of rugose growths to revert to type, one might look upon rugose transition as a means of prolonging the life of infective cholera strains whereas, as will be discussed later, the stable degradation brought about by transition into the rough state is instrumental in rendering the vibrios concerned non-infective.

Though the occurrence of a third type of dissociation of *V. cholerae*, leading to the growth in the form of minute pleuropneumonia-like (L) colonies, has been established through recent observations only (Minck, 1950, 1951 ; Minck & Minck, 1951 ; Carrère & Roux, 1953), the presence of dwarf colonies has been recorded by some earlier workers, first apparently by Baerthlein & Gruenbaum (1916).

These two workers stated that some diagnostic difficulties were caused by the occurrence of minute colonies, reaching hardly pin-point size within 24 hours of incubation even on Dieudonné-agar, which often developed alone on the plates used for isolation of *V. cholerae*. Smears from such growths showed the presence of very slender vibrios which, because forming chains, resembled relapsing fever spirochaetes. Subcultivation on solid media did not lead to a change in the growth appearance of such strains. However, if the vibrios were kept for some time in broth and then sub-cultured on suitable media, typical, opaque and ring colonies developed.

Minck & Minck (1951—see also Minck, 1950, 1951) were able to produce L-dissociation of *V. cholerae* through subcultivation of primary cultures from intraperitoneally infected mice on soft serum agar containing 1,000 units of penicillin per ml, but had no success with stock cultures.

After an incubation of the penicillin-containing cultures for six to eight hours, dwarf colonies with a maximal diameter of 500μ became visible. Their centre was found to

consist mainly of minute L forms (" elementary bodies "), while on the periphery giant globular bodies (diameter 10-20μ) were seen which were more or less filled with motile granula. Intermediate forms were likewise encountered.
It was possible to maintain the strains in this dissociated condition by weekly subculture on penicillin-serum agar. Transfers from these subcultures to ordinary serum-agar led at first to the development of normal cholera colonies, but after five to six passages through penicillin-containing media, dwarf colonies developed even on ordinary serum-agar and proved stable upon subcultivation on the latter medium.
Intraperitoneal inoculation of mice with little subcultivated L growths led to no pathological changes but seemed to confer a certain degree of immunity against infection with normal cholera vibrios. Inoculation with " fixed " L growths (i.e., those showing no tendency to revert to type on ordinary serum-agar) often led to the death of the animals. Autopsy showed the presence of acute peritonitis and necrotic enteritis. On two occasions only, a few L colonies developed in cultures from the peritoneal exudate, while, as a rule, enteric bacteria alone seemed to be present.

The findings of Carrère & Roux (1953) were on the whole similar to those described above. It is noteworthy, however, that according to their observations (*a*) a stock Inaba strain of *V. cholerae* was found to produce numerous L forms and globular bodies in ordinary broth ; (*b*) L forms developed on semi-solid media inoculated with the filtrate of a five-day-old dissociant peptone-water culture through an L_3 Chamberland candle; and (*c*) the L forms appeared to be non-pathogenic for white mice, while percutaneous or subcutaneous as well as intraperitoneal inoculations with material containing globular bodies killed these mice in 24 hours. Carrère & Roux were inclined to assume that the globular bodies might be a resistant form of *V. cholerae*.

Biochemical Properties

Specific chemical constituents

Though Galeotti (1912) claimed to have isolated a nucleoproteid of *V. cholerae* as early as in 1896, and Landsteiner & Levine (1926) laid a firm foundation for future work by extracting from a cholera strain a carbohydrate-containing substance which reacted specifically with cholera-immune serum, it was only within the last two decades that systematic studies on the immunochemistry of the cholera vibrio have been carried out by Linton and his co-workers as well as by some other investigators, particularly Bruce White, whose observations will, however, be considered in the following of these studies.

Linton and his co-workers (see Linton et al., 1935; Linton, 1940, 1942), observing the optical activity of the proteins of numerous cholera and cholera-like strains in dilute alkali solutions, were able to distinguish between two types of protein. They also found that three types of polysaccharides occurred in these vibrios, composed respectively of (1) galactose and an aldobionic acid consisting of galactose and glycuronic acid; (2) arabinose

and an aldobionic acid identical with that of group (1); (3) glucose only. It was thus possible to class the cholera and cholera-like vibrios into six groups composed as follows :

Number	*Protein type*	*Polysaccharide type*	*Nature of vibrios composing group*
I	I	I	Majority of true cholera strains, occasionally water vibrios.
II	I	II	Cholera strains, specially those from Assam (rare in Calcutta), occasionally water vibrios.
III	II	II	Mainly water vibrios, not agglutinable with cholera immune serum.
IV	II	I	El Tor strains and some identical strains isolated from carriers in India.
V	II	III	Cholera strains from carriers.
VI	I	III	Rare in nature, mainly found in old laboratory strains of *V. cholerae*.

As will be noted, even apart from the tediousness of the procedures involved, it would not be possible to make with the aid of these tests a diagnostically valid distinction between cholera and cholera-like vibrios. It is, however, interesting to see that the El Tor vibrios, though showing in most respects features identical with those of the classical non-haemolytic cholera vibrios, fell according to the above method of classification into a separate group.

Linton et al. (1936), studying the respiration and glycolysis of cholera and cholera-like vibrios, found metabolism to be most active when the organisms belonged to Group I, less so in the case of Groups II, V and VI, least active in case of the Group III vibrios. Group IV, to which the El Tor vibrios belonged, was according to these workers "sharply marked off" by the absence of glycolysis under aerobic conditions of growth. Rough dissociants showed a lower metabolism than their smooth parent strains.

The observation that vibrios of Group I, to which most true cholera vibrios belonged, was metabolically most active, is in accord with Bernheim's observation (1943) that *V. cholerae* had 24% more reactive amino groups than *E. coli*. Since then, the presence of hitherto unknown amino-acids of the cholera vibrio has been recorded (Blass & Macheboeuf, 1945, 1947; Blass et al, 1951).

Enzymatic make-up

Proteolytic enzymes

As far as could be ascertained, Bitter (1886) was the first to establish that the liquefaction of gelatin-containing media by *V. cholerae* was due to the presence of a proteolytic enzyme or, as he called it, "ferment" which exerted an influence analogous to that of trypsin. Wherry (1905),

summarizing further early observations made in this direction, stated that, in analogy with the behaviour of trypsin, the proteolytic enzymes of the cholera vibrio as well as of other bacteria were operative only in alkaline media, the presence of even small amounts of acids hindering their action. The presence of fermentable carbohydrates in the media was found to inhibit the liquefaction of gelatin, but it deserves attention that according to Auerbach (1897) who, though devoting some attention to *V. cholerae*, experimented mainly with *Proteus vulgaris*, this absence of liquefaction was due not to the appearance of acids in the course of cultivation but to an inhibition of the formation of the proteolytic enzymes. Besides the presence of protein substances in the media, the access of free oxygen was found to be essential for the production of these enzymes, liquefaction of gelatin taking place very slowly, if at all, under anaerobic conditions (Liborius, 1886).

Summing up his own observations, Wherry stated that the type of liquefaction was influenced to a marked degree by the melting point as well as the reaction of the gelatin used, and added that

" the optimum condition for growth is furnished by an albuminous medium containing between 1/50 and 1/100 gram-molecule of NaOH or Na_2CO_3 per liter, and this corresponds fairly well with the optimum conditions for the tryptic digest of fibrin ".

In a recently published study, Agarwala & Shrivastava (1953) recorded the results of viscosimeter measurements of the "gelatinase" activity of cholera and cholera-like strains grown for 24 hours in papain-broth to which gelatin at a concentration of 5% had been added. They found that growths of cholera-like water vibrios displayed a 25% greater gelatinase activity than all other strains tested which included besides true cholera vibrios also those of the El Tor group. The pH optimum for the gelatinase activity of both true cholera and water vibrios was found to be about 8.0. Incubation of the cultures for longer periods failed to increase the activity of the enzyme which was found to be stable for a long time in growths kept at 37°C.

As recently stated by Nihoul (1952), the presence of calcium exerted an impeding effect on the proteolytic activity of *V. cholerae*.

While, as apparently first demonstrated by Beaujean (1913) and generally accepted, no correlation exists between the proteolytic and the *haemolytic* properties of the cholera vibrios, the question of the relationship between the former property and the *haemodigestive* action of these organisms referred to later in this study, has been the subject of debate. Bernard et al. (1937) reached the conclusion possibly arrived at earlier by Loewy (1915) that the proteolytic and haemodigestive properties of the *V. cholerae* were due to the action of one and the same enzyme, but Beeuwkes (1939) adduced evidence to show that probably different enzymes were responsible for the gelatin liquefaction and haemodigestion respectively produced by cholera and El Tor vibrios.

Milk-coagulating enzyme

As will be further discussed below, the production by *V. cholerae* of an enzyme identical in action to that of rennet, which was capable of coagulating milk even in the presence of a weakly acid reaction, has been demonstrated by Fokker (1892).

Collagenase

Studying the action of culture filtrates of *V. cholerae* on pure collagen prepared from buffalo tendons, Narayanan & Menon (1952) stated to have demonstrated the presence of a collagenase. This enzyme, which was found capable of acting over a wide pH range with an optimum at pH 8.0, was probably also present to some extent in the culture filtrates of cholera-like vibrios, but could not be demonstrated in those of *E. coli.*

Elastinase

In a further study published in 1953, Narayanan et al. reported on the presence of an elastinase, active against elastin prepared from buffalo ligaments, in the cultures of two out of the 7 cholera strains examined in this respect. The presence of this enzyme as well as that of collagenase was also demonstrated in cultures of cholera-like vibrios.

Lecithinase

Reporting in 1944 on the lecithinase activity of *V. cholerae*, Felsenfeld (1944) referred to earlier studies made in this respect by Ruata & Caneva (1901) and Kraaij & Wolff (1923). While the latter workers demonstrated the presence of lecithinase only in El Tor vibrios, Ruata & Caneva found this enzyme to be present in all vibrio strains examined by them. Felsenfeld's investigations also showed lecithinase activity in four true cholera strains as well as in one El Tor strain. The optimal temperature for the action of the enzyme was 36°-38°C, the optimal pH 7.4-7.6. Lecithinase activity was stimulated by calcium and magnesium, whereas formaldehyde, phenol, and fat-soluble narcotics exerted an inhibitory effect.

Deaminases

As recorded by Dudani et al. in 1952 (see also Iyer et al., 1953), *V. cholerae* possess deaminases in their enzyme make-up, the rate of deamination varying from one amino-acid to another, and differing in different strains. Among the amino-acids studied, deamination of aspartic acid and serine was maximal, but arginine, glycine, glutamic acid, lysin, and threonine were also deaminated. Deamination took place under strictly aerobic

conditions only and was optimal at a pH range of 7.0 to 8.0. It is interesting that in general cholera vibrios of the Ogawa sub-type showed a higher deaminase activity than those of the Inaba sub-type.

Dehydrogenases

Dudani et al. (1953) recently published the results of preliminary observations on the dehydrogenation of various substrates by an Ogawa strain of *V. cholerae* and the Inaba and rough variants derived from it. Almost all amino-acids and aliphatic acids employed in this study were found to be capable to act as hydrogen donors for the respiratory activity of the organism. The dehydrogenases of *V. cholerae* seemed to be linked up with the cytochrome systems present in this organism, but the possibility of other coenzyme systems taking a part in the process of dehydrogenation could not be excluded.

γ-peptidase. As stated in a preliminary report, Agarwala et al. (1953b), studying the hydrolysation of glutathione by *V. cholerae*, found evidence pointing to the probable presence of γ-peptidase in the cells of this organism.

Mucinase and tissue-disintegrating enzyme

The important studies of Burnet (1948, 1949) and Burnet and co-workers (1946, 1947) on the enzymes of *V. cholerae* go back to investigations made by Burnet et al. in 1946 on the receptors of human red blood-corpuscles for virus action. These workers found that red cells which had been treated with influenza virus, while losing their agglutinability by some or all of the viruses of the mumps-influenza group, developed an agglutinability with almost any human serum ("pan-agglutinability" of Thomsen, 1926). Since Friedenreich (1928) had shown that such a pan-agglutinability with sera of all blood groups and even with that of the donor of the red cells could be produced with the aid of cholera and cholera-like vibrios, Burnet et al. experimented with the culture filtrates of two cholera and one cholera-like strain. They found that an action almost completely analogous to that of the above-mentioned viruses, and due undoubtedly to enzyme activities, could be produced with these culture filtrates.

Following up this work, Burnet & Stone (1947) made tests with various substrates to explore the possibility that the receptor-destroying enzyme of the filtrates of cholera cultures was a collagenase. The important result of these studies was the demonstration of an actively desquamating effect exerted by these filtrates on the intestinal mucosa of guinea-pigs and rabbits. As stated by Burnet & Stone,

" it soon became evident that this action was not a function of the receptor-destroying enzyme but the possible relationship of such *in vitro* desquamation to the pathogenesis of human cholera seemed to justify an independent investigation of the phenomenon ".

Burnet & Stone summarized the results of this investigation thus:

(*a*) " Filtrates from *Vibrio cholerae* cultures are capable of producing desquamation of the intestinal epithelium *in vitro*;

(*b*) There is a well-marked gradient of diminishing susceptibility to desquamation from the jejunum to the descending colon;

(*c*) Histological and other preliminary evidence suggests that the principal agent concerned is a mucinase;

(*d*) Intestinal mucin is rapidly dissolved by active filtrates, the effect paralleling the desquamation reaction; both are similarly neutralized by rabbit immune serum (prepared with the aid of *V. cholerae* culture filtrates);

(*e*) Evidence is given for the existence of another enzyme concerned with breaking down the cement substance between cells."

Discussing the importance of these findings, Burnet & Stone pointed out that

" the mucinase described in this paper can rapidly destroy the viscosity and hence the mechanical protective and lubricating properties of intestinal mucus. Experiments in progress show that this action can take place in isolated gut segments in the living animal and if the enzyme, as seems likely, is produced in large amount in the bowel of a cholera patient, it might well play a major part in facilitating desquamation of the intestinal epithelium. The third agent (i.e. the tissue-disintegrating enzyme), on which very little work has so far been done, by breaking down some presumed components of the cement substance between cells would also favour the desquamating process."

No evidence was obtained to show that the receptor-destroying enzyme played a role in this process of desquamation and tissue-disintegration.

Reporting on further studies of the cholera mucinase Burnet (1948) stated that this enzyme was found to be active against a variety of glandular mucins but exerted no action on human synovial fluids (hyaluronic-acid type mucin). The activity of mucinase was found to be completely inhibited by sodium hexametaphosphate and (like hyaluronidases) it was inert in the absence of salts.

As recorded by Burnet in a further communication published in 1949, it had been found possible to treat the vibrio filtrates so that they contained either mucinase or the receptor-destroying enzyme alone in active form: if the filtrates were treated with an excess of $CaCl_2$, brought to a pH of 6, and heated for 30 minutes at 56°C, the mucinase alone was destroyed. However, if the filtrates were alkalinized to pH 8.5 and held for some hours at 37°C, the mucinase remained fully active while the receptor-destroying enzyme was totally inactivated.

Publishing recent observations on the intestinal-epithelium-destroying enzyme Singh & Ahuja (1953) stated that they could demonstrate its presence not only in all smooth cholera strains but also in most El Tor and cholera-like strains examined by them. These two workers concluded, therefore,

" that mucinase activity is not specifically confined to *V. cholerae* but is shared by other members of the genus vibrio. Whether or not this enzyme plays any role in the causation of [the] cholera syndrome is a moot point."

Lipase

The production of a lipase active against olive oil by *V. cholerae* as well as by cholera-like vibrios has been recently demonstrated by Narayanan et al. (1953).

Carbohydrate-converting enzymes. Bitter (1886) seems to have first drawn attention to the amylolytic activity of the *V. cholerae* due to the action of " ferments " (enzymes). His observations were soon confirmed by several other workers (see Nobechi, 1925). Wherry (1905), one of the pioneers in this field, stated that all six cholera strains examined by him produced not only amylase and maltase (as had been previously found to be the case by Buxton, 1903) as well as invertase (previously found by Sclavo, 1892), but also lactase.

Indole formation

As will be described in a later study, application of Ehrlich's rosindole test shows that cholera vibrios, if suitably cultivated, invariably produce indole. Since, however, other intestinal bacteria as well as many of the cholera-like vibrio species also react positively in this respect, such tests have no differential-diagnostic importance.

As summarized by Sticker (1912), Hoppe-Seyler (1892) found that indole accumulates in the intestine of cholera patients because it is no more destroyed by oxidation as in the healthy body. The large amounts of indican and indoxyl sulfuric acid found in the urine of cholera patients also indicated according to Hoppe-Seyler an increased indole production.

Nitroso-indole (cholera-red) reaction

It is curious to note that tests based on the phenomena underlying the nitroso-indole or, as it is commonly called, the cholera-red reaction, carried out after 1883 with cholera cultures, had been utilized well before that year with the aid of the dejecta of cholera patients. According to Sticker (1912), Kopp (1837) was the first to observe that addition of small amounts of pure nitric acid to cholera stools or their destillates produced a red colour, and similar results were recorded by some subsequent observers including Virchow (see Schuchardt, 1887), who partly used other mineral acids.

After Koch had isolated *V. cholerae*, the testing of suspicious cultures with mineral acids, so as to determine whether or not the red coloration considered as characteristic for this organism appeared, was recommended by Poehl (1886) and independently by Bujwid (1887), and Dunham (1887). Bujwid emphasized in his short note, which appeared before Dunham's article, the importance of the method for a rapid diagnosis of cholera,

since a pink to reddish-violet colour appeared quickly when a few drops of 5% to 10% hydrochloric acid had been added to broth cultures of cholera vibrios grown at 37°C for 10—12 hours. He considered the test as practically specific for *V. cholerae*.

Recording the results of an investigation into the phenomena underlying this test, Salkowski (1887) stated with admirable clearness and brevity that the "cholera reaction" is

" nothing else than a quite common indole reaction, and the explanation for the fact that the indole reaction can be produced in cholera cultures with sulfuric acid alone, is simply that the cholera vibrios constantly produce nitrous acid, which is present in the fluid in the form of nitrites. There exists no specific cholera red, as has been assumed by Brieger; this is simple indole-red and demonstrable in every decomposing peptone solution. Characteristic of the cholera bacteria is only the *simultaneous* production of indole and nitrous acid." [Trans.]

The validity of Salkowski's statement that the cholera-red reaction is due to the ability of the cholera vibrio of reducing nitrates to nitrites as well as to the production of indole by this organism has been generally accepted.

The technique of the nitroso-indole test will be duly described in a future study. It has to be noted, however, that, whereas the early workers considered this reaction as one of the principal methods, or even as the cardinal method of establishing the presence of *V. cholerae*, it has now hardly any importance in practical cholera laboratory work. For it has been established that on the one hand positive reactions are also produced by certain cholera-like vibrios and even by bacteria belonging to other genera, while on the other hand for reasons which will be specified when dealing with the problems of cholera diagnosis false negatives may be obtained even though *V. cholerae* is present. However, as will be noted later, Taylor et al. (1937) ascribed some usefulness to the cholera-red test, if used in combination with other biochemical methods.

Saccharolytic effects

While, as shown by the table inserted below, *V. cholerae* has been found capable of causing acidification of media into which certain carbohydrates had been incorporated, it has to be emphasized that this process is never accompanied by the formation of gas.

Though the whole of the available literature has been considered for compilation of the table, it is based mainly upon data furnished by Heiberg (1934), because this worker used a satisfactory modern technique: (*a*) growing the strains to be tested in peptone water (pH 8.0—8.4) into which the various carbohydrates had been incorporated at a concentration of 0.5%; (*b*) adding brom-thymol as indicator in place of litmus (which—as first shown by Müller (1899)—is apt eventually to become reduced by *V. cholerae*); and (*c*) taking initial readings not later than after an incubation

of 20 hours at 37°C so as to be able to distinguish between rapid and late acidification.

Reactions produced by Vibrio cholerae in carbohydrate-containing media

Constant and rapid acidification	*Late acidification*
Arbutin (Pergola, 1921)	Glycerol [d]
Dextrin	Lactose [d]
Erythritol (Violle, 1919)	*No change*
Galactose [a]	Adonitol
Glucose	Arabinose [c]
Glycogen	Dulcitol
Levulose	Inulin
Maltose	Inusitol
Mannitol [b]	Rhamnose
Mannose	Salicin [e]
Saccharose [c]	Sorbite
Starch	Tartrate
	Xylose

[a] Late acidification according to Heiberg (1934).
[b] Negative in eight out of nine strains according to Noury & Alalou (1923), variable according to Seal (1935).
[c] See text.
[d] Variable according to some workers.
[e] Variable according to Seal (1935).

As stated by Kauffmann (1934) when reporting upon the observations of Heiberg (1934) referred to later, the reactions produced by individual cholera strains in carbohydrate-containing media are stable, as proved by re-examination of cultures which had been kept in the laboratory for periods varying from six months to one year. Identical findings have also been recorded by some other workers but the following facts must be noted :

(*a*) According to observations by Mesnard & Genevray (1931), cholera vibrios which grew on account of variation in the form of opaque colonies with a wrinkled surface, produced more vigorous acidifications of glucose and saccharose than the parent strains.

(*b*) Seal (1935) maintained that in general the saccharolytic effects of cholera strains could undergo changes in the course of subcultivation, probably hand in hand with variation of the organisms themselves, a dissociant of a typical smooth strain in particular producing some acidification only in the presence of glucose.

An elaborate attempt to use tests with carbohydrate-containing media for the classification of cholera and cholera-like vibrios was made by Heiberg (1934) who established that it was sufficient to use three substances only, namely, saccharose, arabinose, and mannose, for this purpose. The

results obtained in this manner by Heiberg were thus summarized by Kauffmann (1934) in the *Bulletin de l'Office international d'Hygiène publique*:

Group:	*Saccharose*	*Arabinose*	*Mannose*	*Strains agglutinating with cholera immune serum*	*Strains not agglutinating with specific serum*	*Spontaneously agglutinating strains*
I	+	0	+	239	27	13
II	+	0	0	1	76	0
III	+	+	+	0	12	1
IV	+	+	0	0	3	0
V	0	0	+	0	2	0
VI	0	0	0	0	5	0

It will be noted that, with the doubtful exception of one atypical and weakly-agglutinating strain, the true cholera vibrios fell in Heiberg's Group I. The vibrios not agglutinating with cholera immune serum, on the contrary, fell in all six groups, far more in Groups II and III than in Group I which seemed thus a class rather characteristic for *V. cholerae*.

Workers in the cholera areas of India and China soon confirmed that practically always the true cholera vibrios belonged to Heiberg's Group I. In fact, the only observers recording some aberrant results were Seal (1935) in India, and Yü (1938) in China.

Seal maintained that (*a*) some cholera strains isolated from carriers failed to produce acidity in saccharose-containing media, and (*b*) arabinose was affected by a very small percentage of cholera vibrios.

Yü in 1938, examining 52 smooth cholera strains which had been isolated during the Shanghai epidemics of 1932 and 1937, found evidence of late arabinose fermentation in some instances, and noted that three of the 1937 strains failed to acidify mannose and —if the present author may venture to correct a probable misprint—apparently also saccharose even after incubation for seven days.

It has to be emphasized, however, that numerous other workers, when examining freshly isolated strains, never met such aberrant reactions. At the same time it was established, however, that a considerable number of cholera-like vibrios, including those isolated from surface waters, also gave the Group I reactions. Thus Pollitzer (1936) found that practically one third (32) of 100 Shanghai water vibrios belonged to this group. Taylor et al. (1936), comparing the reactions of 125 cholera strains with those of 369 cholera-like strains isolated from patients showing clinical signs of the disease, from carriers, and from surface waters, found that 26.8% of the cholera-like vibrios from human sources as well as 11.4% of the water vibrios showed the reactions of Heiberg's Group I and concluded "that fermentation tests with these three sugars will not provide accurate information as to the characteristics of the vibrios which can be obtained by serological tests". However, as noted below, in a subsequent paper (1937), Taylor and his co-workers ascribed some usefulness to Heiberg's method if used in combination with other biochemical tests. Possibly also, as claimed

by Heiberg, the method will prove of value for the classification of the cholera-like vibrios which are rather heterogeneous serologically.

Voges-Proskauer reaction

In the course of a study on the bacteria of the haemorrhagic septicaemia group, Voges & Proskauer (1898) found that a red colour was produced if a few drops of a strong solution of potassium hydrate were added to growths of these organisms in glucose-containing media. As was afterwards established, the reaction depends upon the formation of acethylmethylcarbinol in the course of glucose decomposition, which has been ascribed to the action of a special enzyme, " carboligase " (see O'Meara, 1931).

Lemoigne (1920), using a rather delicate reaction (nickel-dimethylglyoxine test) for the examination of culture distillates, found that small quantities of acethylmethylcarbinol were formed by both cholera and cholera-like vibrios. However, while his method thus appeared to have no differential-diagnostic value, it was in Lemoigne's opinion potentially useful for the characterization of different races of these organisms.

Attention to the possibility of using the Voges-Proskauer reaction for the examination of cholera and cholera-like vibrios was, as far as could be established, first drawn by Taylor in a report to the Scientific Advisory Board of the Indian Research Fund Association rendered in 1936 and quoted by Baars (1938). Reporting in detail on these investigations in a valuable " Study of the vibrio group and its relation to cholera ", Taylor et al. (1937) stated to have compared the modified Voges-Proskauer reaction according to Barritt (1936) with the original method, using the following technique:

(*a*) For Barritt's modified test the glucose phosphate medium recommended by the Ministry of Health (Report No. 71, 1934) with an initial pH of 7.5 was distributed in 6″ × 5/8″ tubes. These were inoculated rather heavily and incubated at 37°C for 3 days. About one ml of the culture was then transferred to a tube and to it was added, first, 0.6 ml of a 5% alcoholic solution of *a*-naphthol and then 0.2 ml of a 40% KOH solution. Results were read at the end of 4 hours. A positive result was indicated by the appearance of a pink colour on the surface of the fluid in about 5-10 minutes already, which then deepened and spread to the bottom of the tube. In negative cases the fluid usually remained colourless but sometimes a faint brownish tinge appeared.

(*b*) To carry out the original test, 40% KOH solution was added to the culture tubes in amounts of 0.25 ml after the transfers necessary for the *a*-naphthol tests had been made. Results were read after 4 and once more after 24 hours.

Carrying out these tests with 90 classical cholera strains, 6 El Tor strains and 351 cholera-like strains, Taylor et al. obtained in many instances a positive result with the aid of Barritt's modified technique only. The reverse, i.e., a positive result with the original Voges-Proskauer technique and a negative one with Barritt's modification, was never observed.

Combining the observations they made with the aid of Barritt's test, the cholera-red reaction and sugar fermentation tests according to Heiberg, Taylor et al. obtained the following important results:

(1) *Classical V. cholerae.* All non-haemolytic strains agglutinable with cholera immune serum, belonging to Heiberg's Group I, gave a cholera-red reaction, but were *negative* to the modified Voges-Proskauer test.

(2) *El Tor strains.* Out of the six haemolytic strains which were agglutinable with cholera immune serum, five differed from the classical type in so far as they gave a *positive* modified Voges-Proskauer reaction.

(3) *Cholera-like vibrios.* The vibrios inagglutinable with cholera immune serum were found to fall into two main groups:

(*a*) A larger group (240 strains), showing both a positive cholera-red and modified Voges-Proskauer reaction and consisting mainly of strains of Heiberg Groups I and II;

(*b*) A minority, cholera-red and Voges-Proskauer negative, belonging with few exceptions to Heiberg Groups III-VI, while one strain was found to fall into a hitherto unknown fermentation group (saccharose negative, arabinose, and mannose positive).

Commenting on these observations, Taylor et al. stated the following:

" Mention has already been made that agglutinable non-haemolytic vibrios tested gave the reaction C-R (cholera-red) + V-P (Voges-Proskauer)—. In the series of 351 inagglutinable strains examined, only 15 gave the same results and of these 10 were of types aberrant in their sugar reactions from the recognized Heiberg types. No inagglutinable strain of Heiberg type I has given the reactions C-R + V-P—. It is therefore possible, on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V. cholerae*; if it gives fermentation reactions of Heiberg type I, is cholera-red positive and negative to the modified V-P test, it is very probably an agglutinable vibrio."

Taylor et al. claimed in this connexion that, if rather large inocula were used, it was permissible to carry out Barritt's test after an incubation of only one day instead of the customary three days and that consequently the fermentation, cholera-red and modified Voges-Proskauer tests could be "profitably performed along with the agglutination test and read with it". It has to be pointed out, however, that, using the now available sera, great reliance can be placed upon slide-agglutination tests made as soon as suspicious colonies are found on the plates used for primary isolation of *V. cholerae.*

The important observation that, in contrast to most classical *V. cholerae* strains, the El Tor vibrios usually give a positive Voges-Proskauer reaction, was confirmed by several workers, such as De Moor (1938, 1949), Mochtar & Baars (1938), Gispen (1939), Marras (1940) and Paris & Gallut (1951). Baars (1940) maintained in this connexion that the El Tor vibrios were capable of forming acethylmethylcarbinol only under aerobic but not

under anaerobic conditions. Gallut (1946) found, like Lemoigne in 1920, that, if tests more sensitive than the Voges-Proskauer reaction were used, the cholera vibrios could be also proved to produce this substance. However, the El Tor vibrios acted far more energetically in this respect. This is in accord with the observation of Baars (1940) that these vibrios ferment sugars far more energetically than the *V. cholerae* under aerobic conditions and to some extent even under anaerobic conditions.

Haemodigestive and haemolytic properties

Observations on the reactions produced by *V. cholerae* in blood-containing media go back to a rather casual statement made in 1884 by Koch at a cholera conference in Berlin (*Berliner klinische Wochenschrift*, 1884) to the effect that in one instance, when blood-containing stools had been used to make a gelatin plate, clear zones became visible round the cholera colonies. Koch felt entitled to conclude from this observation that *V. cholerae* was capable of destroying erythrocytes and probably also other cells. Schottmüller (1904) also ascribed haemolytic properties to the cholera vibrios which facilitated the differentiation of these organisms from other intestinal bacteria.

The observations of Bitter (1886) on the action of the "ferment" of *V. cholerae* on rabbit blood-suspensions can not be considered as conclusive because he worked with culture fluids which had been heated for half an hour at 60°C. He found that under these circumstances the erythrocytes were remarkably resistant to the action of the ferment. The haemolytic action of *V. cholerae* on blood-containing gelatin plates, ascribed by other workers to the secretion of a cell-destroying toxin by the organisms, was in Bitter's opinion due to the damage caused to the erythrocytes through enclosure in the media, on account of which the blood corpuscles became amenable to the action of the ferment and other products of decomposition.

Studying 12 cholera-like as well as 9 true cholera strains, Kraus (1903) found the former alone capable of producing a soluble "haemotoxin" in broth cultures and consequently able to produce zones of clearing round their colonies on blood-agar plates. Kraus recommended, therefore, the use of the latter media for the differentiation of the non-haemolytic *V. cholerae* from haemolytic cholera-like organisms.

The problem of the haemolytic properties of the vibrios began to attract much attention after Gotschlich (1905, 1906) had isolated six peculiar strains from dead bodies of returned Mecca pilgrims at the quarantine camp of El Tor. Though these victims showed no signs of choleraic disease either during life or at post mortem, the vibrios found in their intestines were not only agglutinable with cholera-immune serum but showed, as far as the tests used by Gotschlich went, in all other respects as well, the reactions of true cholera vibrios. However, re-examining these strains,

Kraus & Přibram (1905) found to their surprise that the organisms in question produced, like the cholera-like vibrios formerly examined by Kraus, a soluble haemotoxin as well as an exotoxin rapidly lethal to experimental animals.

Since this discovery was made, diametrically opposite views have been expressed in regard to the relationship between these El Tor vibrios[g] with the true cholera vibrios responsible for epidemics, and—in connexion with this problem—regarding the question of whether or not the classical *V. cholerae* is non-haemolytic in contrast to the El Tor vibrios. Kraus and his co-workers (see the ultimate statement of Kraus, 1922) continued to assert that on account of its above-described properties the El Tor vibrios fell into a class distinct from that of the non-haemolytic *V. cholerae*. Many German workers on the contrary (see summary by Kolle & Prigge, 1928), maintained that the cholera vibrios were apt to show variability in regard to their haemolytic properties as well as in other respects and that, consequently, tests with blood-containing media were unsuitable for the characterization of this organism—an opinion which implies that the El Tor vibrios do not form a group of their own.

In order properly to assess the merits of these opposite claims, it is necessary to pay attention to the methods of examination used by the various workers and to the manner in which they interpreted their findings.

Proper choice of blood

The first point to be noted in this connexion is that the various workers have used different sorts of erythrocytes for their tests. As noted above, Koch (1884) made his initial observation on the supposed haemolytic properties of *V. cholerae* on a plate which happened to contain human blood. The use of this was recommended by Schottmüller (1904), while some other early workers (e.g., initially Kraus, 1903) used rabbit blood for their tests. Prausnitz (1905), who seems to have been the first to make comparative tests in this respect, found rabbit as well as calf blood more suitable than human blood, but worked for the sake of economy mainly with calf blood. The use of the latter was strongly recommended by Schumacher (1906), because in his experience the calf erythrocytes were least liable to become damaged by mechanical, thermic, or chemical influences and were, therefore, most resistant to the action of the vibrio "ferments". Kraus and his co-workers on the other hand (see Kraus & Prantschoff, 1906) soon adopted the use of sheep blood but considered goat blood also suitable. Goat blood has been used for the large-scale studies on the haemolytic properties of *V. cholerae* referred to below, but, as confirmed by

[g] Though a few workers have designated also haemolytic cholera-like vibrios with this name, it is imperative to use it exclusively for those haemolytic strains which are agglutinable with cholera immune serum. Otherwise utter confusion would reign.

some later observers, for instance on account of comparative tests by Finkelstein (1930), sheep blood was equally satisfactory. In fact, Krishnan & Gupta (1949), submitting in 1949 to the WHO Cholera Expert Committee a draft proposal for a standard haemolytic test to be adopted for cholera work, recommended the use of sheep blood in preference to that of goat blood.

These statements make it clear that in assessing the results of past workers, full reliance can be placed only on findings with suitable types of blood, particularly with goat, sheep, or calf blood, while those with human blood ought to be disregarded. It is of great interest to add that according to observations made by Zimmermann (1934) most cholera strains, though incapable of producing lysis of sheep erythrocytes, were found able to form a thermolabile haemolysin against human red blood-corpuscles, while the El Tor vibrios lysed both sorts of blood. These observations which have been recently confirmed by De et al. (1954), are in accordance with earlier findings made by Přibram & Russ (quoted by Kolle & Schürmann, 1912 and Kolle & Prigge, 1928) who, carrying out absorption tests, showed that the filtrates of vibrio cultures did not contain one common haemolysin but separate ones for the different sorts of erythrocytes they were able to lyse.

Methods of examination

Two fundamentally different methods are used to assess the behaviour of cholera or other vibrios in blood-containing media—cultivation of the organisms on blood-plates (nowadays invariably agar plates) and tests with blood suspensions which have been added to adequate amounts of fluid vibrio cultures, or of their filtrates or centrifugates. The technique usually adopted for the latter purpose which, as will be set forth in a later study, is still used in actual practice with some modification, is well exemplified by the following description of the classical procedure adopted by Greig (1914):

" Each strain was grown in alkaline broth, as recommended by Meinicke (1905) for 3 days at 37°C., at the end of that period falling quantities of the culture, viz., 1 c.c., 0.5 c.c., 0.1 c.c., 0.05 c.c. and 0.01 c.c. were measured with a pipette and placed in small sterile test-tubes; the quantities were brought up to exactly 1 c.c. in each tube with 0.85% NaCl. Then 1 c.c. of a 5% suspension of goat's washed red corpuscles was added to each tube. An experimental error is made if the suspension of red corpuscles is added first, since the culture, which is lighter, floats on the top; so that if a haemotoxine is present the upper layer of red corpuscles gets a very concentrated dose. The contents of the tube are very carefully mixed and the mixture is placed in the incubator at 37°C. for 2 hours. The tubes are taken out and placed in the icechest over night. Next day the presence or absence of haemolysis in each tube is noted and recorded."

No doubt, it would be more exact to use corresponding amounts of filtrates instead of materials from the fluid cultures for the above-described

tests. Unfortunately, however, as first shown by Meinecke (1905) and confirmed by later observers, the haemolytic property of the strains is greatly reduced if filtration is resorted to. Greig (1914) maintained in this respect that " the haemolysis-producing substance in the broth culture is, to a considerable extent, non-filterable ".

It will be perceived that, whereas in the case of tests performed according to Greig's or a similar technique the red blood-corpuscles are exposed almost solely to the action of the " haemotoxins " (haemolysins), in the case of cultivation on solid blood-containing media, they are also exposed to the action of the enzymes of the vibrios. It is not surprising, therefore, that, as will be shown below, the results obtained with these two categories of tests respectively are as markedly different as the technique adopted in each case. It is obvious that the results of tests aiming to show the presence or absence of haemolysis will be far more clear-cut if, by using Greig's or a similar technique, or by working with filtrates, the additional influence of the vibrio enzymes is practically or totally excluded.

Quality of the media used

As noted by Schumacher (1906) in the course of exhaustive studies on the behaviour of cholera and cholera-like vibrios on blood-agar plates, it is essential to pour these with a sufficient amount of the medium so as to obtain a uniformly and adequately thick layer. The reason for this was that even vibrios, which ordinarily did not produce zones on the plates, were apt to show ill-defined halos round their colonies at thin spots of improperly poured plates. Loewy (1915), besides repeating the advice given by Schumacher, also insisted upon the use of *freshly* taken and defibrinated blood, because blood kept in storage could show spontaneous haemolysis. Plates which had become dry or which showed a darkening of their initially bright-red colour, were unsuitable for haemolysis tests.

Zimmermann (1932) noted, in analogy with the experiences of Meinicke (1905) in the case of blood-plates, that the results of haemolysin tests, made by growing cholera vibrios for 48 hours in broth tubes, to which sheep blood had been added previously to obtain a concentration of 5%, were apt to be divergent, if the tests were repeated at short intervals. It was striking, however, that different strains tested at one and the same time showed a peculiarly uniform behaviour, either mostly producing haemolysis or mostly failing to do so. Since such a simultaneously occurring variation of several strains was altogether unlikely, Zimmermann postulated with much reason that the observed variations in the haemolytic properties of the strains were the result of differences in the physico-chemical state of the media or of corresponding changes taking place in the course of cultivation. Inadequacies in the defibrination of the blood were likewise apt to introduce an element of chance. In fact, consistently

negative results were obtained with the same strains if, instead of the broth, a synthetic fluid medium and, instead of defibrinated blood, citrated sterile sheep blood were used. However, significant though these findings are, in actual practice it is equally reliable and more expedient to use an up-to-date modification of Greig's method in place of that of Zimmermann. It seems unnecessary, therefore, to deal in detail with the technique of the last mentioned worker.

Status of the strains examined

The various workers postulating an inconstancy of the reactions produced by vibrios in blood-containing media or suspensions, based their claims, to a varying extent, upon an examination of recently isolated growths and of stock cultures respectively. It is of importance, therefore, to see whether, or to what extent, the inconstancies which they noted in the course of their work were due to a changing reactivity of the individual strains, caused by the process of ageing and/or by mutation or dissociation.

Studying the mutations of *V. cholerae*, Baerthlein (1911b, 1912, 1918) noted that the opaque variants of ordinarily non-haemolytic cholera vibrios were able to produce haemolysis in blood suspensions as well as clear zones round their colonies on blood-agar plates. Since, however, this worker continued to keep both the suspensions and the plates at 37°C in the incubator and extended the period of observation to 72 hours, no reliance can be placed on his findings. Further observations made by Goyle & Gupta (1932) with spontaneously-agglutinating cholera strains which had obviously undergone dissociation, and by Genevray (1940) with variants of *V. cholerae* obtained through the action of chlorine or phenol showed that, like their smooth parent-strains, these dissociants and variants respectively failed to produce haemolysis in blood suspensions.

Van Loghem (1913b) and Snapper (1921) asserted in general that, while the haemodigestive properties of *V. cholerae* were apt to show variation, the incapability of these organisms of producing haemolysis in fluid substrates, as well as the haemolytic properties of the El Tor vibrios, were stable characteristics. This is in accord with previous observations made by Meinecke (1905) who stated that

" the haemolysins of the vibrios are but little apt to undergo spontaneous decomposition. Out of 20 filtrates of different vibrios cultures, to which phenol had been added and which had then been kept in the ice-box for 6 months, 17 retained their original titre and three only had become less haemolytic."

Analogous observations were made by Zimmermann (1933) who found that, with the exception of one variable El Tor strain, none of the vibrio strains studied by him showed evidence of a short-term variation of their

haemolytic properties. Re-examining these strains once more after a period of observation totalling one year and nine months, Zimmermann (1934) likewise observed no instance of a fundamental change in their haemolytic properties, and only in a limited number of instances a variation in the intensity of the reactions produced by haemolytic strains. The haemolytic properties of Zimmermann's strains were not influenced by animal passage, nor by bacteriophage action as had been claimed by Doorenbos (1932).

Though, as will be gathered from the evidence set forth above, the presence or absence of haemolysis may be considered as stable characteristics of practically all strains of the cholera and allied vibrios,[h] it is nevertheless desirable to use freshly isolated growths rather than stock cultures to assess the reactions produced in this respect by a given strain, or series of strains. Van Loghem (quoted by Zimmermann, 1932) no doubt went rather far when ascribing the occurrence of aberrant haemolytic reactions shown by stock cultures of *V. cholerae* to contaminations with haemolytic vibrios. But even apart from this possibility, the uncertainties arising from the use of stock cultures, the source of origin and character of which are quite often not or not exactly known, may be great and this absence of exact information was no doubt responsible to quite a considerable extent for the statements made to the effect that the classical *V. cholerae* may produce true haemolysis. The difficulties apt to arise in this respect are well exemplified by the experiences of Zimmermann (1932). This worker found among the 70 strains labelled in his material as *V. cholerae* two which were not agglutinable with cholera-immune serum but were haemolytic, and which, therefore, as he cautiously put it, could not be considered as " typical " cholera vibrios. Out of Zimmermann's 21 strains labelled as El Tor, on the other hand, one proved to be non-haemolytic, thus reacting like a cholera vibrio and not like an El Tor vibrio. A minority of his other El Tor strains were but slightly or even almost not agglutinable with cholera-immune serum, but—as Zimmermann argued—" they must have been found agglutinable with cholera serum by Gotschlich and Doorenbos, because they had been diagnosed on account of this fact ".

Interpretation of results

The fundamental difference between the phenomenon of true haemolysis observable in fluid substrates and the appearances apt to become manifest when the vibrios were grown on blood-plates was clearly recognized by Schumacher (1906) who maintained in this respect that

" there can be no doubt that the halo formation of cholera strains on blood agar prepared with human, pigeon-, rabbit-, guinea-pig-, horse- and dog-blood, is not due to a haemo-

[h] The only recent observations recorded to the contrary were those of Del Favero (1938) who stated that laboratory strains of *V. cholerae*, subjected 15 times to subcultivation at 20°C, became, in contrast to their initial behaviour, strongly haemolytic for sheep-erythrocytes. Since Del Favero's original paper could not be consulted, details of his methods could not be ascertained.

lysin production by the cholera colonies, but is solely due to the action of the proteolytic ferment excreted by the cholera bacteria ".

However, even though Schumacher and also some other early workers emphasized that, in order to decide whether or not a given strain was haemolytic, tests should be made with blood suspensions and not with blood plates, many investigators not only mainly paid attention to the latter category of tests but often took the appearance of clear zones round the vibrio colonies on the blood plates as proof that the strains in question were endowed with haemolytic properties.

It was the great merit of Van Loghem (1911, 1913a, 1913b) to have reaffirmed through studies commenced in about 1909 that the classical cholera and the El Tor vibrios respectively produced qualitatively distinct reactions in blood-agar plates besides being distinguishable by their behaviour in blood-containing fluid substrates, in which, in contrast to *V. cholerae*, the El Tors produced haemolysis. It is true that *V. cholerae* was capable of producing clear zones round its colonies on goat-blood agar plates as the El Tor vibrios invariably did. However, Van Loghem emphasized, in the case of the latter organisms, that these zones appeared quickly, were not quite transparent, and showed a reddish tint. In the case of *V. cholerae*, on the contrary, the zones appeared more slowly, were quite clear and had a greenish hue. Spectroscopically, it could be shown that oxyhaemoglobin, while absent in the zones around the cholera colonies, was present in the zones surrounding the El Tor colonies, because in their case true *haemolysis* took place which led to the penetration of haemoglobin into the zones. The process of *haemodigestion*, of which alone the cholera vibrios were capable, led to a decomposition (Abbau) of the components of the erythrocytes and, as a consequence, no haemoglobin was present in the zones.

Haemolysis, produced by the El Tor vibrios, was an eminently stable property of the organisms, haemodigestion was an unstable property possessed by the *V. cholerae* as well as by the El Tor vibrios. Van Loghem found a close parallelism between the haemodigestive and gelatin-liquefying properties of the various vibrio strains, which indeed, in the opinion of some observers, are due to the action of one and the same enzyme.

The old El Tor strains of Van Loghem's series liquified gelatin very slowly and it seemed likely, therefore, that in their case the process of haemolysis quite overshadowed that of haemodigestion.[i]

The validity of Van Loghem's findings was questioned by Baerthlein (1914, 1918), but since the conclusions of the latter were disproved by Snapper (1921), it seems unnecessary to discuss Baerthlein's rather involved postulations in detail. Van Loghem's observations were confirmed by Kämmerer in the course of a profound study on bacteria and red blood-

[i] Further reference to Van Loghem's observations on cholera and El Tor vibrios will be made in the following study where the vibrio haemolysins will be dealt with from the immunological point of view.

corpuscles (1920) and supported as well as amplified by Snapper (1918, 1921), who (1921) thus summarized his interesting findings:

[*a*] " In the halo round a cholera culture on a blood-agar plate and in the culture itself haematin is formed; consequently the halo is produced through a decomposition of haemoglobin.
In the halo round an El Tor culture at most traces of haematin are formed; the halo is produced through haemolysis and diffusion of the liberated haemoglobin throughout the plate. As proof for this serves that on haemoglobin plates, which contain only dissolved blood-colouring substances, the El Tor vibrios form *no* halo, the cholera vibrios a *distinct* halo.

[*b*] In El Tor growths on blood-agar plates inorganic iron compounds are formed, whereas in the cholera growths no inorganic iron but haematin is produced.

[*c*] The halo-formation by the cholera bacilli is much more marked on *blood-bile-agar* than on ordinary blood plates. On the former even cholera strains which seem to have lost the ability of forming halos, can still produce wide halos, in which a further decomposition of the haematin spontaneously formed in such plates takes place.
El Tor vibrios form on blood-bile-agar plates usually no, exceptionally indistinct halos." [Trans.]

In Snapper's opinion the explanation of the last-mentioned phenomenon was that in the bile-containing blood-agar all haemoglobin had been transformed into haematin substances, no unchanged erythrocytes having been left.

Final confirmation of Van Loghem's findings was furnished through a series of fine studies by Bernard et al. (1937). These workers were able to extract from agar and broth media used for the cultivation of *V. cholerae* a protease which (*a*) while possessing no haemolytic power was found to be capable to bring about in successive stages " the phenomenon of haemodigestion" as described by Van Loghem, and (*b*) was also found to exert a tryptic action both on denatured proteins, such as coagulated horse-serum, gelatin, and milk, and natural proteins such as egg-white and fibrin. The results of these investigations as well as those of the extraction of an exohaemolysin from culture media used for growing El Tor vibrios, which were recorded by Bernard et al. in 1939 and will receive full attention in the next study, definitely confirm the difference of the reactions produced by cholera and El Tor vibrios in blood-containing media or substrates, thus ending a controversy lasting for more than thirty years.

The experiences of Greig (1914) and other modern workers in testing representative series of vibrio strains, almost invariably[j] supported the validity of the above-mentioned observations.

Greig (1914) found that the 333 cholera strains which he examined according to the above-mentioned technique, all proved non-haemolytic,

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[j] A divergent opinion was expressed by Kabeshima who, in a short note published in 1918, maintained that 91.6% of the 206 cholera strains investigated by him showed haemolysis in fluid as well as on solid media. It was not possible to consult the more exhaustive publication in a Japanese medical journal which Kabeshima referred to in his note. There can be hardly any doubt, however, that deviations from the standard technique used by the other workers were responsible for the strikingly aberrant results obtained by him.

while 100 strains of cholera-like vibrios, isolated from human stools or from surface waters, invariably produced haemolysis, some to a marked degree. A great majority of the 161 cholera strains, the behaviour of which was tested on agar plates containing 12% goat-blood, produced no definite zones of clearing in these media within 24 hours, and only a few indistinct ones. However, clear zones became manifest, if readings were taken after more prolonged incubation. Greig emphasized, therefore, that, if blood plates were used to test the haemolytic properties of cholera-suspect vibrios, positive findings becoming manifest after more than 24 hours should be disregarded.

Testing 103 cholera strains which included, besides 27 stock cultures, mainly those isolated in Rumania and Bulgaria, Loewy (1915) found no evidence of haemolysin production either in the centrifugate of 5 days' broth cultures or in the case of agar plates to which 10% sheep or goat blood had been added. However, a proteolytic "ferment" of the *V. cholerae*, which in Loewy's opinion was identical with the ferment causing gelatin liquefaction, was found by this worker to be capable of exerting a digestive action on damaged erythrocytes.

Van Loghem, summarizing in 1932 the results of the above-mentioned observers as well as those obtained by various workers in the Dutch East Indies, was able to report on the examination of over 600 strains isolated from authentic cholera cases and invariably found to be non-haemolytic.

As Zimmermann summarized in 1933, among the 69 cholera strains examined by him, two were found to possess haemolytic properties. With one exception, which has been noted above, the 28 El Tor strains of his collection produced marked haemolysis and the same was true of the 14 cholera-like strains at his disposal. Of the two haemolytic cholera strains, one was a stock strain over 20 years old which agglutinated but weakly with cholera-immune serum, the other a stock strain from Paris kept in the Berlin Institute of Hygiene.

Genevray & Bruneau (1938c), recording their observations on more than 500 Indochinese cholera strains, stated to have observed no instance of haemolysis produced by these vibrios within 24 hours. However, all the strains produced *hématolyse* (that is, apparently, haemodigestion) on sheep-blood as well as on rabbit blood-agar.

Attention has also to be drawn to the experiments made by a few workers with heated blood-agar plates ("chocolate" agar plates), on which, as was first noted by Loewy (1915), cholera vibrios were apt to produce clear zones. Kovacs (1927), exhaustively studying this phenomenon, confirmed that these vibrios, obviously because they were capable of exerting an action on the erythrocytes which were damaged through boiling, produced—usually on the second day of incubation—yellowish, quite transparent halos round their colonies on chocolate agar (Kochblutagar) plates prepared with the aid of sheep blood. El Tor vibrios on the contrary, because they

exerted at most a slight haemodigestive action, produced but occasionally indistinct halos.

Finkelstein (1930) postulated that by the combined use of blood suspensions, ordinary and heated blood-agar the vibrios could be classified into four groups thus:

Group	*Haemolysis in suspensions*	*Clearing of blood-agar*	*Clearing of heated blood-agar*
I	negative	negative	negative
II	positive	positive	positive
III	positive	positive	negative
IV	negative	positive	positive

In contrast with the findings of Loewy and Kovacs, Finkelstein claimed that the classical *V. cholerae* does not produce clearing of heated blood-agar plates, thus falling into Group I of his scheme. Since, however, he used ox blood for his tests, and took readings after 24 hours' incubation at 37°C only, and since, moreover, a study of his protocols shows that out of the total of 11 strains isolated from cases of clinical cholera which he could examine, 5 only fell into Group I, while 3 belonged to Group II, 2 to Group III and 1 to Group IV, it is impossible to place reliance in Finkelstein's findings. Generally speaking, it must be emphasized once more that tests on blood plates, however interesting their results may be, are of no decisive value in answering the practically most important question of whether or not a given vibrio strain is capable of producing true haemolysis. Tests with blood suspensions alone can furnish clear-cut evidence in this respect.

In addition to the above-recorded findings, within recent years the following important observations have been made in regard to the El Tor vibrio in particular.

As has been noted in the second of these studies,[k] in 1937-8 as well as in 1939-40 and in 1944 manifestations of a " choleriform " disease with a high fatality-rate have been observed in South Celebes, in which haemolytic vibrios agglutinable with cholera-immune serum were found to play the causative role (De Moor, 1938, 1949). Making a careful study of 370 strains isolated from sufferers and their environment, De Moor (1949) concluded that the vibrios in question, which fell in the same serological group as *V. cholerae* and belonged like the latter to Group I of Heiberg, but gave with four exceptions a positive Voges-Proskauer reaction, were true El Tor vibrios. Agreeing with Van Loghem (1938) that the El Tor vibrio fell into a group different from that of *V. cholerae*, De Moor proposed the name " Paracholera (El Tor) " for the choleraic disease in Celebes. It is thus curious to see this term once more used in the sense Kraus (1909) proposed it to designate instances of choleraic disease caused by haemolytic vibrios

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

agglutinable with cholera-immune serum. Since, however, the name of paracholera was afterwards adopted to designate clinical manifestations in which vibrios serologically different from *V. cholerae* were assumed to have played a causative role, it cannot be considered as felicitous. The term " enteritis choleriformis El Tor " proposed by Van Loghem (1938) to designate the Celebes disease seems therefore preferable.

Though the outbreaks observed in Celebes have been the most conspicuous, they were probably not the first in which El Tor vibrios were responsible for the causation of choleraic disease. A strain called Kadiköj, which showed the properties characteristic of the El Tor vibrios, was isolated in 1913 by Kraus in Bulgaria (Kovacs, 1927). Mackie (1929) maintained in general that vibrios of this type had been met in choleraic conditions as well as in carriers in countries outside India, e.g., in the Near East.

It is of great interest to note in the latter connexion that Abdoelrachman (1944/5), examining 90 water samples from different sources in the Hejaz, was able to demonstrate the presence of El Tor strains in one out of 29 specimens taken from the holy well Zam-Zam at Mecca. All other water samples examined by this worker as well as 1,109 stool samples, including those of 715 pilgrims from the Dutch East Indies and Malaya, gave negative results for the *El Tor* vibrio and, cholera being absent at the time, also for *V. cholerae.*

Though so far El Tor vibrios have not been found responsible for the causation of cholera in India, their frequent occurrence in this country has been proved through recent investigations. Thus Venkatraman et al. (1941) recorded that they isolated 15 El Tor strains from 878 specimens, collected in the Tanjore district of Madras from 237 open natural water-sources, including rivers, channels, tanks, ponds and a few wells. The examination of 1,827 stool samples gave negative results for El Tor vibrios and, since cholera was absent at the time, also for *V. cholerae.*

Read & Pandit (1941) carried out analogous investigations in (*a*) two districts of Bengal, where cholera was endemic; (*b*) a district in Bihar, where annual epidemics occurred; and (*c*) an area in Sind, which had remained largely free from cholera for the past ten years. The main conclusions reached by these two workers were:

[1] " The non-haemolytic agglutinable vibrio was found in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease.

[2] About 7 per cent of close contacts of cholera cases proved positive and about 16 per cent of water sources in direct contact with cases were positive at different periods of the epidemic. On the other hand the non-haemolytic vibrio with one or possibly two exceptions was not found (in water samples) in the absence of the disease.

[3] The haemolytic agglutinable vibrio, while detected in the presence of the disease, has been found usually in its absence. It has been found in cholera areas of two different epidemiological types in different provinces of India and in relative large numbers (i.e. in 18 out of 206 water samples) in an area which must be taken as not only free from

cholera during the period of investigation but free from cholera during the decade previous."

Continuing these investigations, Read et al. (1942) tested the haemolytic properties of cholera and El Tor strains from various sources. Growing these organisms in Douglas's broth (see Douglas, 1914) instead of in untrypzined broth as had been done by Greig, but otherwise using the technique recommended by the latter worker, Read et al. obtained the following results:

Character of strains *	*Haemolysis*	
	Produced	*Not produced*
Case strains, India	0	15
Case strains, Celebes	7	0
Contacts, India	0	4
Contacts, Celebes	3	0
Water, India	15	6
Water, Celebes	6	0
El Tor stock strains	5	1
Total	36	26

* All agglutinable with cholera-immune serum.

It will be noted that while the strains isolated from cases and contacts in India, in contrast to those from Celebes, were invariably non-haemolytic, the majority of the strains isolated from water sources in India showed the haemolytic properties characteristic of the El Tor vibrios. This was particularly true of the growths obtained from "non-contact" water sources, 14 out of 15 of which proved haemolytic.

In their classical study on the antigens of the cholera group of vibrios Gardner & Venkatraman (1935), after an exhaustive examination of about 100 strains, postulated

" that the absence of haemolytic power and the possession of a characteristic O antigen are the chief distinctive characters of the vibrios most undoubtedly causative in epidemic cholera ".

There is no doubt that the evidence brought forward in the foregoing pages, particularly the recent observations in India, lend full support to this judicious contention.

Vital Resistance

Heat

It is generally agreed that *V. cholerae* is not at all resistant to high temperatures. As Kolle & Schürmann (1912) summarized in this connexion,

" Boiling temperature destroys the vibrios immediately. At 80°C. they are killed with certainty within five minutes, and heating for half an hour at 56°C. suffices to terminate the life of the cholera vibrios."

Babes (1885) established that rapid heating of gelatin cultures to even only 75°C rendered the growths sterile. Good growths could be obtained from gelatin cultures slowly heated (? in the water-bath) up to 45°C. Exposure at 46-48°C for two days rendered the cultures sterile, but temperatures of 40-41°C were well tolerated by the cholera cultures for three days.

Kitasato (1889a), heating gelatin tubes, which after liquefaction had been inoculated with *V. cholerae*, at various temperatures and for different lengths of time in the water-bath and then making roll cultures, found that (*a*) exposure of the inoculated tubes for 15 minutes to 55°C usually prevented growth, and (*b*) heating for 10 minutes at 60°C or for 5 minutes at 65°C invariably did so.

In the experience of Borntraeger (1892) dry heat of 80°C killed the cholera vibrios within a few minutes while exposure to higher degrees of dry heat (80°-100°C) led to the death of the organisms within a few seconds. Borntraeger considered it feasible under these circumstances to use, in emergencies, dry heat generated in baking stoves for the disinfection of objects such as clothing and bedding contaminated with *V. cholerae*.

It is of interest to add that Shousha (1924) found the rough dissociants of *V. cholerae* somewhat more resistant to heat than the smooth parent strains.

Cold

Though not very resistant to heat, the cholera vibrios show a remarkable tolerance for low temperatures, even for those well below the freezing point. Uffelmann (1893a) established in this connexion that suspensions of *V. cholerae* in water as well as cholera cultures, if exposed in the open during winter, remained viable for 3-4 days even when the minimal temperature became as low as −24.8°C. Still far more remarkable experiments carried out by Kasansky (1895) showed that broth, gelatin, and agar cultures of *V. cholerae* (*a*) tolerated temperatures down to −31.8°C; (*b*) remained viable if kept completely frozen for 20 days or if subjected to repeated freezing and thawing; (*c*) survived exposure to the cold of the winter at Kasan, Russia, for four months. However, cultures exposed to the cold in November proved to be no longer viable when re-examined in April or May.

Drying

As Koch recorded at a cholera conference held in Berlin in 1884 (*Berliner klinische Wochenschrift*, 1884), cholera vibrios, grown in peptone broth and spread in thin layers on cover-glasses, withstood drying for periods

of up to one hour, but were sometimes found to have succumbed after two hours and were invariably incapable to survive drying for periods exceeding three hours. If compact masses of vibrios, scrapings from potato-cultures for instance, were dried, the organisms could survive for periods of up to 24 hours, evidently because under these circumstances no rapid drying took place.

Similar experiments were made by Kitasato (1889a) who found that on silk threads, which had been dipped into fluid cultures, the cholera vibrios withstood drying better than on cover-glasses, obviously because in the latter case desiccation took place more slowly. Working at room temperature (20-22°C), Kitasato found that even on the silk threads the vibrios survived for a few days only, longest (up to seven days) on those kept in the exsiccator, apparently because in the latter case rapid drying of the outer layers led to a more prolonged retention of some moisture inside the threads.

However, in an additional note Kitasato (1890) stated that, if kept on moist filter paper in closed Petri-dishes, cholera vibrios on cover-glasses were capable of surviving for 85-100 days, and for 200 days or even longer on silk threads.

Suzuki (1922), again studying the resistance of *V. cholerae* to drying, found that if the organisms were smeared on a silk thread which was then placed in a jar with calcium chloride, the organisms survived no longer than 18-28 hours. However, longer survival took place if suspensions of the vibrios in saline solutions containing some horse-serum, egg-white, or undiluted horse-serum were used for such tests.

Observations made by some workers have shown that, when undergoing exsiccation, cholera vibrios from layers which apparently had become quite dry may remain subcultivable. Thus Gildemeister & Baerthlein (1915), studying the survival of *V. cholerae* in the faeces of patients and carriers (see below) obtained sometimes positive results when making subcultures from apparently exsiccated specimens.

Laigret & Auburtin (1938) recorded to have obtained broth subcultures from cholera vibrios which had been dried in vacuo over calcium chloride, ground in a mortar, and then kept in rubber-stoppered test tubes at temperatures ranging from 25°C to 39°C for five weeks. Still far more remarkable results were recorded by Campbell-Renton (1942) who, drying single drops of peptone-water cultures of cholera and El Tor vibrios in vacuo over phosphoric oxide (P_2O_5), still obtained positive results when making subcultures from six out of seven *V. cholerae* and three out of five El Tor specimens tested after four years' storage in vacuo at room temperature. However, interesting though this observation is, the highly artificial conditions under which the experiment was made, must be kept in mind. It is, however, of great importance that according to recent experiences (Hornibrook, 1949, 1950; Sokhey, 1949; Sokhey & Habbu, 1950) freeze-

drying (lyophilization) is an excellent means of preserving not only the viability but also the immunogenic properties of the *V. cholerae*.

Sunlight

Orsi (1907), carrying out systematic studies with cultures of *V. cholerae* and *Salmonella typhosa*, found that sunlight exerted a damaging action on these organisms without, however, invariably leading to their total destruction. The cholera vibrios in particular remained viable in fairly considerable numbers after exposures to sunlight averaging 8-10 hours (temperature in the shade 23°-31°C). In the experience of Conor (1912), however, these organisms, if suspended in canal water and exposed to the action of sunlight in transparent glass flasks or tubes, were no more demonstrable after an exposure of 5-7 hours at temperatures ranging from 27°C to 34.6°C. Yasukawa (1933), working with suspensions of *V. cholerae* in sterilized sea-water, even found that a two hours' exposure of these specimens to sunlight in boxes covered with transparent or with cobalt glass was sufficient to kill the vibrios.

Other rays

Schiavone & Trerotoli (1913; see also Galeotti, 1916) found that cholera vibrios, if suspended in saline and exposed in glass dishes in thin layers (2-3 mm) to the rays of a mercury vapour lamp 20 cm distant, were killed in one minute, even though the temperature did not exceed 20°C. Under the same conditions the vibrios in blood-serum were killed in half an hour, those in broth or urine in 2 hours, and those in milk in 2½ hours. Pieces of material soaked with suspensions of *V. cholerae* were rendered sterile by the ultraviolet rays of the lamp in 15 to 45 minutes.

As shown by Rieder (1898) exposure of cholera vibrios to X-rays for 20-30 minutes is apt to inhibit their growth or even to kill the organisms.

Acids

As alluded to earlier in this study, *V. cholerae* is extremely sensitive to the action of acids. In this connexion, Kolle & Schürmann (1912) observed that hydrochloric acid or sulfuric acid kills the vibrios in a few seconds if used in a concentration of 1/10,000, and lactic acid produces this effect even in weaker concentrations.

In the course of a study on the viability of the cholera vibrios in milk curd, which will be referred to later, Panja & Ghosh (1945) found that, besides hydrochloric acid and lactic acid, acetic acid was also immediately fatal for these organisms if present in peptone water at a concentration sufficient to produce a pH of 4.4. However, the vibrios were capable of

surviving for five minutes if, instead of these acids, citric acid was used under analogous conditions.

Interesting observations on the action of gastric juice on the cholera vibrio have been made by Napier & Gupta (1942). Whereas the vibrios added to a specimen of gastric juice taken from a patient who suffered from hyperacidity were killed immediately, the organisms were apt to survive up to 24 hours (in one sample even up to 264 hours), if gastric juice from a patient with hypochlorhydria was used for analogous tests. Generally speaking, the vibrios survived for considerable periods (24 hours to maximally 370 hours) in specimens of gastric juice from which free hydrochloric acid was absent (pH 6.0—8.0) but succumbed immediately, if the pH of the gastric juice was less than approximately 4.75 owing to the presence of free acid. However, since Napier & Gupta found that addition of distilled water to the specimens tested prolonged the life of the vibrios in spite of a high initial acidity, they believed that cholera vibrios ingested with a copious draught of water might pass the stomach in viable form even though large amounts of free hydrochloric acid were present. As pointed out by Greig (1929), when referring to earlier observations on the adverse action of gastric juice on *V. cholerae*, vibrios enclosed in a mass of food might also pass the stomach unharmed.

It may be conveniently added that, as found by Dawson & Blagg (1948, 1950), in addition to normally acid gastric juice the saliva of healthy persons appears to exert an antibacterial action on *V. cholerae* and might thus form a first line of defence against a not too massive infection.

Disinfectants

As was early noted by Koch (1885) and confirmed by ample later observations, the usual disinfectants, even if used in low concentrations, exert a rapidly lethal action on *V. cholerae* in suspensions or fluid cultures and inhibit the growth of this organism if added in small amounts to solid media destined for its cultivation. Thus Koch and his co-workers (see Gaffky, 1887) found that phenol (carbolic acid), if used in a concentration of 0.5% killed the vibrios in 10 minutes, while at a concentration of 1%, the organisms were killed in 5 minutes. Babes (1885) found that mercury perchloride, if added to gelatin at a concentration of 1/15,000, prevented the growth of the cholera vibrios, while according to Forster (1893) these organisms were killed within 5-10 minutes if incorporated in mercury perchloride dilutions of 1 in 2 or 3 millions.

Other antiseptic substances

As will be gathered from the summaries of Kolle & Schürmann (1912) and Mackie (1929) as well as from more recent publications, in addition to the usual disinfectants, a considerable number of other substances endowed

with antiseptic properties have been found to exert an inhibitory or lethal action on *V. cholerae*. The following deserve mention:

Soap. The conclusions reached by Jolles (1893) that various sorts of soap were endowed with vibriocidal power, were not confirmed by Murillo (1912), in whose experience addition of soap to nutrient media even in a concentration of 1/10 did not inhibit the growth of *V. cholerae*. Kolle & Prigge emphasized, therefore, that " even most thorough washing with soap was incapable of destroying the cholera vibrios ".

Alcohol. As maintained by Babes (1885), the maximal concentration at which alcohol added to nutrient media did not inhibit the growth of *V. cholerae* was 1/15. It is in accord with this observation that, as established by Van Ermengem (1885), broth cholera-cultures became sterile within half an hour, if absolute alcohol was added at a proportion of 1/10.

Iodine. In the experience of Babes (1885), addition of iodine to nutrient media at a concentration of 1/600 to 1/800 was just incapable of inhibiting the growth of *V. cholerae*. Bujwid (1892) found that iodine vapours retarded the growth of this organism but established, in accord with previous experiences of Neisser (1887) and Riedlin (1888), that *iodoform* exerted a far more marked action in this respect. Since various cholera-like vibrios tested by Bujwid with iodoform were far less inhibited in their growth than *V. cholerae*, he suggested that this fact might be used in differential diagnosis—a proposal which is now interesting merely from the historical point of view.

Potassium permanganate. In contrast with the statement of Babes (1885) that potassium permanganate did not inhibit the growth of *V. cholerae*, Panja & Ghosh (1943) maintained that this chemical was lethal to cholera vibrios and still more to cholera-like vibrios and that, therefore, " fruits and vegetables artificially infected with cultures of *V. cholerae*... can be effectively disinfected by soaking them in permanganate solutions of 1/5,000 to 1/10,000 dilutions for 5 minutes ". Since, however, this conclusion is not in accord with Babes' observations and also not with the results of recent experiments made with other organisms such as *S. typhosa*, one should—as justly stated by the editor of the *Tropical Diseases Bulletin* (1943)—be cautious in accepting the recommendation of Panja & Ghosh until their results are confirmed by further tests.

Copper sulfate. Copper sulfate, found effective against *V. cholerae* in a concentration of 1/600 by Van Ermengem (1885) and in higher dilutions by Babes (tolerance limit 1/3,000-1/5,000), was recently again recommended by Halawani & Omar (1947) who found that this compound

" is lethal in dilutions ranging from 20-45 parts per million to *Vibrio cholerae* in concentrations ranging from 10 to 1,000 million per cc. of Nile water ".

It is of interest to add that Bose & Chakraborty (1948) found metallic copper to be vibriocidal. In the presence of strips of copper foil, *V. cholerae*

could not be recovered from suspensions in distilled water, in which the vibrios normally survived for two days. In filtered tank water, the presence of copper foil shortened the life of the organisms to about 1½ hours as against a survival for 15 days in the controls.

Water which had been in contact with copper foil for periods of two or four hours, also proved bactericidal within 30 minutes and 15 minutes respectively, if cholera vibrios were added subsequent to the removal of the metal. However, no bactericidal effect was noted if water which had been in contact with copper foil for 48 hours was used for the preparation of peptone water or Douglas broth, cholera vibrios cultivated in such fluid media remaining viable for 15 days.

Since satisfactory results were also obtained in tests carried out without the use of copper foil in polished copper vessels, Bose & Chakraborty recommended the use of these containers during cholera epidemics for the temporary storage (4-6 hours) of water. It is important to note in this connexion that chemical tests with the water samples used in these experiments failed to show the presence of copper.

Essential oils

Since—as will be seen in a later part of these studies—essential oils have been used with some success for the treatment of cholera, it is of importance to note that in the experience of some workers, such as Babes (1885) and Riedlin (1888) such oils were found capable of inhibiting the growth of *V. cholerae*. The former of these observers found mustard-oil far more effective in this respect than peppermint-oil, oil of cloves, bergamot-oil or turpentine-oil, while Riedlin (who did not test mustard-oil) found turpentine-oil to be most antiseptic, followed in order of efficacy first by lavender-, eucalyptus- and rosmarin-oil, then by oil of cloves. Other essential oils, including those of anise, fennel, juniper, peppermint, and thyme, were in Riedlin's experience of " subordinate importance ".

Aniline dyes

Shiga (1913) found that some aniline dyes, particularly methylene blue and thionin, even if used in concentrations of 1/33,000 and 1/25,000 respectively, inhibited the growth of cholera vibrios, but that by growing them in the presence of still lesser amounts of these dyes the organisms could be made dye-fast, the strains then becoming capable of tolerating the action of the dyes at much higher concentrations.

Exploring the possibility of incorporating aniline dyes into agar media destined for the cultivation of *V. cholerae*, Signorelli (1912) found that addition of dahlia, erythrosin, orcein, or safranin led to a loss of virulence of the cholera vibrios developing on such media, which became decolorized while the colonies became intensely coloured. Growth of *V. cholerae* on agar containing methyl green or azolitmin also led to a decolorization

of the media but, the colonies not taking up these dyes, no loss of virulence resulted.

Further interesting observations on the vibriostatic and vibriocidal properties of aniline dyes were made by Panja & Ghosh (1943), whose most important findings may be summarized thus:

(*a*) Brilliant green, crystal violet, methylene violet, added to agar in a concentration of 1/100,000 exerted a bacteriostatic effect on *V. cholerae* and El Tor vibrios. The same result was obtained with 1/50,000 concentrations of malachite green, acriflavin, gentian violet, methyl violet, methylene blue, fluorescein and pyronin yellowish, with thionin at a concentration of 1/25,000, with mercurochrome, safranin, and basic fuchsin in concentrations of 1/5,000 respectively.

(*b*) Brilliant green and malachite green, if incorporated into peptone water at concentrations of 1/100,000 exerted a selective bactericidal effect on most cholera strains as well as on a large number of " paracholera " strains isolated from patients with clinical signs of choleraic disease, but these dyes did not affect the cholera-like vibrios from river water.

(*c*) Added in a final dilution of 1/5,000, brilliant green killed the vibrios in cholera stools. These vibrios also disappeared earlier than in untreated cases from the stools of cholera patients who had been given the dye orally, but clinical improvement was not marked. Since it had been found that an excess of alkali prevented the bactericidal action of the dyes in vitro, this comparative failure of treatment stood probably in connexion with the alkaline reaction prevailing in the intestines of the patients.

Charcoal

Mackie (1929) stated that " one hundred c.cm. water to which has been added a loopful of *V. cholerae* and 1 gm. of charcoal is sterile in 15 minutes ".

Milk of lime

Milk of lime, which had been found vibriocidal in a concentration of 20% by Giaxa (1890), was, according to Pfuhl (1892), prescribed for the disinfection of cholera stools in an instruction (" Anweisung zur Desinfektion bei Cholera") issued in 1892 by the Prussian authorities. According to this, one was advised to prepare milk of lime with 1 l of pure quicklime reduced to small pieces and 4 l of water, to add the finished product to an equal amount of the stools to be disinfected, and to let the mixture stand for at least one hour before disposal. Since, however, observers in Java claimed to have had bad results with this method in actual cholera work, Pfuhl made further investigations with fresh stools from patients in which *V. cholerae* abounded. He found the method effective, provided that the milk of lime was not merely poured over the stools but actually mixed with them. However, prolonged stirring was unnecessary.

Chlorine and chloride of lime

Harding (1910), experimenting with water to which 1-2 drops of a 24 hours' cholera culture had been added per litre, concluded that most

samples of contaminated water, if treated with one part of chlorine per million for 15 minutes, are apt to be free from cholera vibrios. However, if organic matter is plentiful, it is advisable to use higher concentrations, .so as to leave, after oxidation of the organic matter, 0.5-1.0 parts of chlorine per million available for the purpose of sterilization. Ditthorn (1915), judging from experiments in which a whole slant of a 24 hour-old cholera culture had been added per litre of the water samples tested, found that even in the presence of such a marked contamination chlorine, used at the rate of 0.28 mg per litre of water killed the vibrios in 10 minutes.

Satisfactory experiments with chlorine compounds were made by Conor (1912), who found that cholera-contaminated samples of canal-water from Tunis were freed from the vibrios if chloride of lime or chloride of soda were added to the water at the rate of 2 mg per litre and allowed to act for eight hours. Conor considered it essential, however, to utilize freshly prepared solutions of these compounds with a content of free chlorine amounting to 30-40 per thousand.

In contrast to the experiences mentioned above, Genevray (1940a) laid stress upon the fact that in peptone water the cholera vibrio was found to resist considerable doses of free chlorine, since an excess of 2 mg of free chlorine per 10 ml of the medium (i.e., 200 mg of free chlorine per litre) did not destroy it. Genevray found it pertinent to ask, therefore, what action chloride of lime, used in Indochina for the disinfection of ponds during cholera epidemics, could exert under these circumstances. To answer this question it would be certainly desirable to make further investigations on the action of chloride of lime on *V. cholerae* in areas where cholera prevails.

Ozone

Investigating the value of ozone for water sterilization, Schubert (1914) found this method preferable to that of sand filtration for rendering the water supplies free from cholera vibrios and from typhoid bacilli.

Symbiosis

Voicing an often-expressed opinion, Kolle & Schürmann (1912) maintained that:

" In the case of a simultaneous presence of bacteria causing decay or rapidly growing saprophytes no appreciable development of the cholera vibrios takes place under most natural conditions—indeed in most instances decay and decomposition are factors which rapidly destroy the cholera bacteria."

In support of this contention Kolle & Schürmann pointed out that in the experience of Koch (1885) and subsequent workers, as a rule the

cholera vibrios did not survive long in highly contaminated and decaying substrates such as the contents of cess-pools or sewers.

That also under other circumstances the presence of other bacterial species was apt to handicap or even to shorten the existence of *V. cholerae*, has been shown by (*a*) the observation of Rosenthal (1910) to the effect that cholera vibrios were unable to grow in milk or other suitable media in the presence of *Lactobacillus bulgaricus*, which produced a markedly acid reaction; and (*b*) investigations by Panayotatou (1913), demonstrating in the water of the Nile the presence of four (not further identified) bacterial species which were markedly antagonistic to *V. cholerae*, and thus apparently responsible for the failure of the latter to persist under laboratory conditions in water samples from that river.

It is important to note, however, that symbiosis with other bacterial species was by no means always found to be unfavourable to the persistence of *V. cholerae*. Kabelík & Freudmann (1923) noted in this connexion that, when cultivated in peptone water together with *E. coli*, cholera vibrios grew far more luxuriantly than did *E. coli*. Sarkar & Tribedi (1953), again studying the relation between these two organisms, found that when a loopful of a cholera culture was added to a 24-hour old broth culture of *E. coli*, and daily platings were made, during an initial period lasting from three to fourteen days, *E. coli* colonies only grew on the plates. Subsequently, however, cholera colonies appeared in increasing proportions, to become finally solely present. Sarkar & Tribedi found that this evolution was paralleled by changes in the pH of the broth culture: the preliminary cultivation of *E. coli* led, after 24 hours, to a lowering of the initial pH of 7.6 to 7.2. After addition of the cholera vibrios, the pH rose, vibrio colonies appearing as soon as it had reached 8.8 and being solely present, when the pH had reached 9.2. However, in the opinion of the two workers, this rise of the pH alone was not responsible for the disappearance of *E. coli* because it was found that (*a*) prolonged cultivation of this organism alone in broth led to a pH of 9.0, at which it was able to survive; and (*b*) it was viable for several days in broth with an initial pH of 9.8, which it lowered within 24 hours to 9.3. It was also noted that, while *E. coli* was unable to multiply in a broth culture in which cholera vibrios alone had survived, it could multiply in these cultures if the vibrios had been killed through heating for one hour at 60°C. Sarkar & Tribedi postulated, therefore, that the antagonistic action exerted by *V. cholerae* and (as they also established) by cholera-like vibrios on *E. coli* was due to the presence of a thermolabile colicidal substance. The antagonism exerted by the cholera vibrios was also manifest if they were present in stools together with *E. coli*.

Carrying out exhaustive and exact studies on the effect on *V. cholerae* of the concurrent presence not only of *E. coli*, but also of *Aerobacter aerogenes*, of *Proteus vulgaris*, of *Streptococcus faecalis*, a Gram-positive coccus isolated from water, and of water-vibrios not agglutinable with

cholera-immune serum, Read et al. (1939) established that under these circumstances

" the agglutinable vibrio can survive in weak peptone water and salt solutions even when present in smaller inoculum, except in the presence of certain inagglutinable vibrios. In several experiments it survived for two weeks or more in the presence of the latter vibrios."

The observations recorded above as well as those to be dealt with now show that the cholera vibrio is by no means as invariably frail an organism as it is assumed to be by some authorities.

Viability of V. cholerae Outside the Body [1]

Faeces

Describing the experiments made by Koch and his co-workers during their initial work, Gaffky (1887) stated that

" if intestinal contents or faeces rich in cholera bacilli but containing other bacteria as well were put on moist earth or linen and were kept in a manner preventing exsiccation, at first the cholera bacilli grew most luxuriantly so that after 24-48 hours specimens taken from the surface contained—as proved by microscopic examination—the cholera bacilli practically in pure culture. However, after a few days already they began to die and the other bacteria started to multiply."

Though unable to state definitely what the maximal period of survival of the vibrios under these and similar conditions was, the observations of Koch and his co-workers indicated that—particularly if decomposition took place and/or other bacteria were present—the *V. cholerae* did not persist long.

While Koch and his co-workers made their observations with what Greig (1914) afterwards called " uncultivated " strains of *V. cholerae*, i.e., directly with the faeces of cholera sufferers, many of the numerous other workers investigating the survival of this organism used for their tests the dejecta of individuals free from cholera, to which they added cultivated vibrios. While one must fully agree with Abel & Claussen (1895), Greig (1914), and Gildemeister & Baerthlein (1915), who made the most valuable investigations in this field, that only observations with cholera-faeces can be considered as valid, it is interesting to see that they adduced different reasons why the results obtained with artificially infected stool specimens should be rejected. Abel & Claussen (1895) maintained in this connexion that quite possibly the " comma bacilli " in the cholera faeces, where they are often present in almost pure culture, possessed a higher vitality than those in artificial stool mixtures, where they were subjected to competition with other bacteria. Greig (1914), on the contrary, recommended " uncultivated " material because experiments previously made with typhoid bacilli seemed to indicate that stock cultures of *V. cholerae* were more resistant

[1] The occurrence and persistence of cholera vibrios in animals (e.g., in flies and aquatic animals) will be dealt with later in these studies.

than the vibrios in the faeces. Gildemeister & Baerthlein (1915) stressed with much reason the importance of results with rice-water stools because, owing to the abundance of mucous material, these were less prone to undergo exsiccation than other kinds of faeces.

Several of the early workers who could examine genuine cholera stools in Europe, found that the average period of persistence of the vibrios was longer than the preliminary findings of Koch and his co-workers in India had indicated, and some reported instances of an excessively long survival of the organisms. While in view of the limitation of the differential-diagnostic methods available to the early observers the latter records must be interpreted with great caution, the fully reliable findings of Abel & Claussen (1895) and of Gildemeister & Baerthlein (1915) deserve great attention.

Abel & Claussen (1895) worked with 31 cholera stools which had been sent for diagnostic purposes to the Institute of Hygiene in Königsberg in Prussia. Once the diagnosis of cholera had been established, these samples, kept in the well-closed bottles in which they had arrived, and protected against direct sunlight, were stored at room temperature (13°-16°C). Re-examinations with the aid of peptone-water enrichment were made daily or at least every few days. If loopfuls of the stools gave no results, larger amounts up to 50 ml were used for peptone-water enrichment and platings and only if these also proved negative, the cholera vibrios were considered to have disappeared.

Abel & Claussen found under these circumstances a persistence of the cholera vibrios for 1-5 days in eleven instances, for 6-10 days in six, up to 15 days in nine, for 15-17 days in three, for 24 and for 29 days in one instance respectively. It will be noted, therefore, that (*a*) the cholera vibrios disappeared from about one third of the samples within five days and (*b*) that a survival of the organisms for more than 15 days was not frequent (16.1%).

Working during the first World War at Posen (now Poznan), Gildemeister & Baerthlein (1915) examined 70 stools derived partly from cholera patients, partly from supposedly healthy carriers, only few of the samples showing a typical rice-water-like appearance. Their technique differed in some details from that of Abel & Claussen, particularly because (*a*) they kept their specimens (protected from direct sunlight and at room temperatures ranging from 12°-21°C with an average of 18°) in covered Petri-dishes, so that they were apt to undergo exsiccation; and (*b*) they used for their platings blood-alkali agar as recommended by Dieudonné (1909), whereas Abel & Claussen had worked with gelatin-plates.

The results obtained by Gildemeister & Baerthlein may be summarized thus:

Period of survival (days)	*Number of specimens*	%
1	18	25.7
2-5	8	11.4
6-10	9	12.8
11-15	8	11.4
16-20	11	15.7
21-30	11	15.7
31	1	7.1
34	1	
36	1	
37	1	
51	1	
Total . . .	70	99.8

Gildemeister & Baerthlein concluded, therefore, that

" (1) Cholera vibrios succumb in a major part of the cholera stools within a short time;

(2) However, in a not inconsiderable part of the stools they remain viable for several weeks, sometimes more than 30 days."

Greig (1914), using for his observations on the persistence of *V. cholerae* 94 typical cholera stools freshly collected in Calcutta and applying a technique similar to that of Abel & Claussen, but taking advantage of Dieudonné as well as of agar plates, recorded the following results:

Month of examination	*Number of stools examined*	*Duration of life of cholera vibrios (days)*			*Average temperature*
		Minimum	*Maximum*	*Average*	
December 1912	9	1	10	3.6	72°F (22°C)
January 1913	6	1	12	6.6	
February	13	3	17	7.7	
March	20	1	13	6.5	85°F (29.5°C)
April	22	1	5	2.8	
May	10	1	3	1.4	
June	15	1	2	1.2	
July	—	—	—	—	
August	4	1	12	6.0	83°F (28°C)
September	3	4	5	4.3	
October	3	3	4	3.7	

Greig concluded from these investigations that, though there was considerable variation between individual strains, " the life of the cholera vibrio outside the human host under natural conditions in India is short ", and added the equally important statement that

" Temperature has a powerful influence on the vitality of the cholera vibrio outside the human host. Thus as the hot season, in Calcutta from March to June advances, the life of the organism becomes shorter : in the present research the minimum duration of life was reached in June. On the other hand, from December to February, the cold season, the vitality is greater, and the maximum duration of life occurred in February. Again in August when the monsoon has fully developed and the temperature has fallen somewhat the life is longer than in the hot season but as the number of cases of cholera during August and September is small my observations during this period were fewer."

That the prevailing temperature exerts an important influence on the period of survival of *V. cholerae* in the faeces of the patients, was also shown by observations in Japan. In addition to findings recorded in this respect by Takano et al. (1926), Soda et al. (1936) noted that if specimens of one and the same cholera stool were kept at 37°C, at room temperature and in the ice-box respectively, survival of the vibrios was longest (up to eight days) in the last case, shortest (sometimes only 3 hours) at body temperature (37°C). Identical experimental observations had been previously recorded by Shoda et al. (1934).

These observations as well as the fine studies of Greig leave little room for doubt that differences in the prevailing temperature were largely, if not solely, responsible for the marked differences observed in regard to the length of survival of the causative organisms in cholera stools in India and Europe respectively. It is significant that analogous differences were noted when the length of survival of the cholera vibrios in sewage, cess-pools or septic tanks and the like was studied. Flu (1921) established in this connexion that, in contrast with *S. typhosa*, cholera vibrios persisted in the septic tanks of Batavia not, or not much, longer than 24 hours. In Europe on the contrary, to judge from the summary of Fürbringer & Stietzel (1908), survival periods of *V. cholerae* in cess-pools, manure, and the like, for one to two weeks have been recorded by several observers. That in these cases temperature differences also played an important role, is suggested by observations of Ohwada (1924) which showed that *V. cholerae* survived in sewage at 37°C for one day, at room temperature for four days, and in the ice-chest for twelve days.

Contaminated material

Besides the work of Koch and his co-workers, whose experiments with moist earth and linen were referred to above, the fate of *V. cholerae* in these and other substrates contaminated with faeces or with material from cultures has been studied by numerous other workers. While the experience they gained with foodstuffs will be dealt with separately below, the following findings obtained with other materials deserve attention :

Earth and dust. Nicati & Rietsch (1885) claimed that, if cholera stools were sprinkled on moist earth, the vibrios remained viable for 14-16 days. However, Uffelmann (1893b) concluded from numerous experiments that *V. cholerae*, if added at high concentration to samples of garden earth, survived at room temperature for two to three days only. The viability of the organisms could be prolonged to 12 days, if the samples were kept at 6°C, and to 16 days at 0° to + 1°C, i.e., at temperatures hardly ever met with in the regions where cholera is prevalent.

Studying the possibilities of aerial transmission of infectious diseases, Germano (1897) exhaustively experimented with different dusts to which cholera vibrios in suspensions or incorporated in normal faeces had been mixed. He found that the vibrios survived well if the dust was kept moist but died rapidly (maximally within three days in the case of brick-dust), if exsiccation took place. Germano concluded, therefore, that the chances for a transmission of cholera through the air were " extremely slight ".

It may be conveniently added that, according to the summary of Takano et al. (1926), investigations on the survival of *V. cholerae* on coal have been made by Hata & Matsuda (1906). These two workers noted that the organisms persisted in a mass of moistened coal for seven days, and in a mass of

dry coal for 21 days, moisture apparently hastening the death of the organisms by facilitating the growth of other bacteria.

Cloth and cotton. A few workers, e.g., Gamaleia (1893) and Karlinski (1895) found that under highly artificial conditions counteracting exsiccation the cholera vibrios on pieces of cotton or cloth which had been soaked in suspensions of these organisms could survive for several weeks or even months. However, most observers, who performed their tests under conditions comparable to those actually obtaining, agree that the cholera vibrios persist on these contamined materials for a few days at the most (one to five days according to the summary of Jettmar, 1927). The earlier observations made to this effect have been confirmed by those made during the 1947 cholera outbreak in Egypt, when, as recorded by Shousha (1948), it was shown by tests with faeces of the cholera patients that *V. cholerae* survived on depurated cotton for two days only, and on raw cotton and cloth for three days.

Paper. To judge from the scanty information available, the survival period of *V. cholerae* on paper or objects made of paper is short. Uffelmann (1892) stated in this connexion that if cholera stools were left to dry on the printed page of a book (which took about 10 minutes), and the book was then closed and kept in a cupboard, the vibrios survived for at least 17 hours. Under the same conditions the organisms remained viable for at least 23½ hours on letter-paper enclosed in an envelope, and for at least 20 hours on a post-card.

Germano (1897) found that the *V. cholerae* was able to survive for one day only on blotting-paper which was let to dry after it had been soaked in a suspension of the organisms. If exsiccation was prevented, the vibrios were still viable on the 20th day.

Tests with Chinese bank-notes which had been handled by fingers contaminated with cholera stools showed that, after the bank-notes appeared to be dry, the vibrios remained alive for maximally four hours (Jettmar, 1927). However, recent observations in Egypt (Shousha, 1948) showed a survival period of *V. cholerae* on bank-notes contaminated with cholera stools for two days, and on postage stamps treated in the same manner for one day.

Metals. Uffelmann (1892) found that if cholera stools were put on copper and silver coins and permitted to dry, the vibrios remained viable for 10-30 minutes only. Identical results were obtained with brass plates.

Tests made during the 1947 cholera epidemic in Egypt with faeces-contaminated coins showed a survival of *V. cholerae* for seven hours (Shousha, 1948).

Tobacco. Wernicke (1892) found that even on moist and snuff-tobacco cholera vibrios succumbed within 24 hours.

Food

For obvious reasons, the fate of *V. cholerae* in food materials contaminated with cholera stools or cultures has attracted the attention of numerous workers. Babes (1885) who seems to have been the first to make systematic studies in this respect, noted that the cholera vibrios remained alive up to 48 hours on fresh non-acid vegetables, potatos, and cheese, but not longer than 24 hours on sour fruit and vegetables.

Essential findings made by subsequent workers may thus be summarized:

Meat. There can be no doubt that under suitable environmental conditions meat and meat products form a favourable substrate for the survival of *V. cholerae.* Thus Uffelmann (1892) found that on roast pork, kept under a glass-bell, the vibrios survived for at least eight days. Lal & Yacob (1926), testing various Indian foodstuffs, placed meat high among those found potentially suitable for the cultivation of the cholera vibrios. Japanese observers (see Takano et al., 1926) found meat a suitable substrate for the survival and, during the first 20 hours after contamination, for the multiplication of the *V. cholerae.* Cholera vibrios on the surface of meat which was kept in the open during mid-winter were found to be able to survive for one to two weeks.

Fish and shell-fish. As shown by numerous observations, fish and shell-fish, stored pending consumption, form a suitable substrate for quite prolonged survival of *V. cholerae.*

Systematic studies made by Friedrich (1893) showed that cholera vibrios were apt to survive on fresh fish for two days, on smoked herrings for one day (according to Uffelmann (1892) even for four days), on caviar for three to six days or, if the latter was kept in the ice-box, even longer.

Takano (1913) found that cholera vibrios smeared on fish meat, which was then kept at room temperature during the month of October, survived for three to four days, but that storage of the material in the ice-box prolonged the viability of the organisms to 10-12 days.

As quoted by Takano et al. (1926), Toyama, working mainly with fresh-water fish, established that cholera vibrios smeared on the meat of these animals remained viable for two to three days during mid-summer, for seven to ten days in early summer, for one to two weeks in mid-winter. Storage of the fish in an ice-box at 3°-8°C prolonged the survival period of the vibrios to 14-19 days, occasionally even to 25 days.

Cholera vibrios which, as will be discussed later in these studies, may be ingested by oysters or other shell-fish and are then apt to persist in the intestinal tract of these animals, have been also found capable of surviving on shelled oysters kept at room temperature (20°C). As stated by Takano et al. (1926), under these conditions the number of organisms first decreases but soon an increase sets in, which becomes maximal 68 hours

after contamination. The vibrios which, as emphasized by Takano et al., grow " very much better on an oyster than an ordinary fish ", disappeared in 171 hours, i.e., seven days and three hours after contamination. As shown by tests with contaminated oysters which had been soaked in dilute acetic acid, it was comparatively easy to kill the cholera vibrios on their surface, but the organisms survived in the intestinal tract of the oysters for seven hours, when 1%-2% acetic acid was used, for two hours in the case of 3% acetic acid, and for 45 minutes if the concentration was increased to 4%-5%.

Milk. In a classical study on the behaviour of *V. cholerae* in milk, Kitasato (1889b) established the following periods of survival of the organisms:

Temperature	*Raw milk*	*Raw milk with 10% sodium carbonate*	*Steam-sterilized milk*
36°C	14 hours	55 hours	2 weeks
22°-25°C	1-1½ days	Still fairly numerous after 78 hours	Still viable after 3 weeks
8°-18°C	2-3 days	.	.

Noting an incessantly progressing acidification of the milk media in the course of these tests, Kitasato emphasized that

" the length of survival of the cholera bacteria is dependent upon the reaction of the milk; the more rapidly the milk sours, the more rapidly the cholera bacteria therein perish; however, the cholera bacteria survive until the milk becomes strongly acid ".

Since heating of samples of raw or raw alkaline milk inoculated with cholera vibrios for five minutes at temperatures ranging from 96°C to 100°C rendered the samples sterile, Kitasato also concluded that " boiling is the simplest and most effective method to free milk from cholera germs ".

Most subsequent observers confirmed that under the ordinarily prevailing temperatures cholera vibrios could survive in raw milk for at least one to two days, regardless of whether or not the milk became in the meanwhile acid or, as some workers such as Heim (1889) and Basenau (1895) expressly stated, even regardless of whether or not the milk had curdled. It is, however, interesting to note that in the experience of Panja & Ghosh (1945) cholera vibrios added to Indian milk-curd (dahi) were killed within five minutes and that according to recent observations in Egypt (Shousha, 1948) the life-span of *V. cholerae* added to already sour milk was only one hour. These observations seem in accordance with experimental findings of Heinemann (1915) who established that cholera vibrios were immediately killed if added to samples of sterilized milk which contained 0.45% lactic acid or, as seems indicated by the protocols of this worker, even at lower concentrations of the acid.

It is, on the other hand, important to realize that boiled milk, if contaminated by *V. cholerae* pending storage, is at suitable temperatures a substrate favourable for the initial multiplication and survival of this organism. Observations made in this respect in the Berlin Gesundheits-Amt (1892) showed that, whereas cholera vibrios added to raw milk survived for less than 24 hours, they remained viable for nine days in milk which had been boiled for one hour and again cooled before contamination.

Butter. Heim (1889) found that cholera vibrios, while surviving for a day only on low-grade, slightly acid butter, remained viable on butter of better quality for over one month. However, other observers, such as Laser (1891) and Uffelmann (1892) recorded periods of survival of *V. cholerae* on butter not exceeding one week.

Cheese. As stated by Babes (1885) and some subsequent European observers, cholera vibrios were apt to remain viable on cheese for 48 hours. Shousha (1948) in Egypt noted a survival period of the organisms on " white " cheese (probably a local product) for only two hours.

Salt. As maintained by Takano et al. (1926), experiments carried out in Japan had shown that the cholera vibrios do not multiply in salt solutions and are even gradually killed. The data they furnished to support this contention are summarized in table II. Takano et al. added that

" In salting fish, if impure salt be used and left at a room temperature, the cholera vibrios survive for 2 weeks. The effect is better if the abdominal viscera of the fish be removed and the fish be packed in salt."

TABLE II. PERIODS OF SURVIVAL OF V. CHOLERAE IN SALT SOLUTIONS

Chemically pure NaCl				Common cooking salt
Concentration (%)	37° C	Room temperature*	2° - 4° C	2° - 4° C
1	1 month	1 month	10 days	15 days
5	20 days	1 week	15 hours	15 days
10	1 day	1 day	15 hours	3 days
15 - 25	.	.	7½ hours	2 days
Saturated solution	.	.	.	14 hours

*Autumn season.

Analogous investigations by Genevray & Bruneau (1938a) showed the survival of *V. cholerae* in solutions of either NaCl or sea-salt to be as follows:

Concentration (%)	*Period of survival*
2.5	One month or more
5.0-7.0	More than three weeks
8.0	More than two weeks
9.0-11.0	24-48 hours

Since no multiplication of the vibrios was observable at salt concentrations exceeding 8% (80 per 1,000), Genevray & Bruneau felt certain that sea-salt in bulk did not play a role in the spread of cholera.

However, though high salt concentrations exert an unfavourable influence on the survival of *V. cholerae*, Venkatraman & Ramakrishnan (1941) found 2% solutions of sea-salt (or of the impure salt obtainable in the bazaars of India) in carefully buffered saline, prepared as will be described in a later part of these studies, excellent vehicles for the transmission of cholera-suspect stools to distant laboratories. In the experience of these two workers, cholera vibrios in artificially contaminated stool samples remained viable in such solutions for 62 days, while the vibrios in actual cholera stools were preserved even up to 92 days, the pH of the solutions remaining at its original level of 9.2.

Sugar and honey. Shousha (1948) noted that contamination of sugar with cholera stools resulted in a survival of the vibrios for three days. To judge from tests made with cultures, *V. cholerae* survived for one day only on honey.

Bread and cakes. As noted by Uffelmann (1892), *V. cholerae* was apt to survive for at least one day on slices of unwrapped rye-bread, for up to three days on rye-bread wrapped in paper, for at least one week on bread kept under a glass-bell.

According to Friedrich (1893), cholera vibrios survived on pastry not longer than 24 hours but were able to persist on biscuits for periods up to four days.

Cereals. Observations made during the 1947 outbreak in Egypt (Shousha, 1948) showed a *V. cholerae* survival of two days on rice and lentils contaminated with the stools of patients. A much shorter survival (7 hours) was noted in tests made with cholera cultures, thus lending support to the assumption that the organisms in the actual stools are better protected against adverse conditions than those grown on artificial media.

There can be no doubt that, as summarized by Sticker (1912), rice-gruel and similar dishes prepared from cereals, if kept under suitable temperatures, form a favourable substrate for the growth of *V. cholerae.*

Potatoes. As summarized by Sticker, cholera vibrios were apt to survive on the surface of raw potatos for at least 48 hours. The acid reaction initially present on the cut surfaces of potatoes was unfavourable for the organisms, but in the case of some kinds of potatoes a change to an alkaline reaction could take place spontaneously which favoured the persistence or even the multiplication of *V. cholerae.* Sticker added that cold potato dishes were a favourable substrate for this organism which could multiply there without causing visible changes.

Onions and garlic. Contrary to the popular belief that their consumption is apt to confer protection against cholera infection, onions and garlic actually form fairly good substrates for the survival of *V. cholerae.* Tests carried out in this respect with faeces of patients during the 1947 outbreak in Egypt showed, according to Shousha (1948), the following survival periods:

Onions — outside	2 days	Garlic — outside	1 day
— inside	3 days	— inside	2 days

Green vegetables. While according to earlier experiments, such as those of Babes (1885) and Uffelmann (1892), cholera vibrios were able to persist on green vegetables for two to three days only, longer periods of survival (up to 22 days on spinach, even up to 29 days in the case of one lettuce specimen) have been recorded by Pollak (1912). Though it has to be noted that the conditions under which this worker experimented did not correspond well with those actually prevailing, he was certainly right in stressing that persistence of an adequate degree of moisture was apt to promote a prolonged survival of *V. cholerae* on green vegetables (and also on fruit).

It is important to note that cholera vibrios can survive on cucumbers, which have a mildly acid reaction, for some days—as found by Mackie & Trasler (1922) in Mesopotamia, for three days, according to observations made in the Berlin Gesundheits-Amt (1892), even for 5-7 days. For, as quoted by Sticker, Hankin (1896b) not only incriminated cucumbers to be instrumental in the causation of some cholera cases in India but supported this contention by demonstrating the presence of *V. cholerae* on the cucumbers in question. There can be no doubt that the use of human manure for fertilizing cucumbers, which are often eaten uncooked, renders them potentially rather dangerous for the transmission of cholera.

Fruit. Systematic investigations made with a series of different fruits and berries in the Berlin Gesundheits-Amt (1892) yielded rather variable results with survival periods of *V. cholerae* ranging from one hour to between three and seven days at room temperature, for somewhat shorter periods (up to four days) at 37°C. It was noted that the organisms could survive on the surface of dried European fruit for one to two days.

The results obtained under somewhat unrealistic conditions by Pollak (1912) may thus be compared with recent experiences made with cholera stools during the 1947 outbreak in Egypt:

Kind of fruit	*Pollak (1912)a*	*Shousha (1948)*	
Apples	16 days	—	
Dates	—	Outside:	2 days
		Inside:	3 days
Grapes	— [b]	Outside:	2 days
Lemons	14 days	Skin:	3 hours
		Inside:	1 hour
Oranges	10 days	Skin:	3 hours
		Inside:	1 hour

[a] Maximal periods observed.

[b] Pollak quoted Dobroklonski (1910) to the effect that *V. cholerae*, while surviving inside the berries for not longer than 24 hours, could persist on the outside of grapes for four days, and on their stalks for even 12 days.

Since, as will be discussed later in these studies, in China at least cut melons have been found to play quite an ominous role in the transmission of cholera infection, it is important to note that according to the laboratory observations of Friedrich (1893) the inside of these fruits was an excellent substrate not merely for the survival but for the multiplication of *V. cholerae* as long as exsiccation could be prevented. It has to be added, however, that according to the experience of Mackie & Trasler (1922) in Mesopotamia, cholera vibrios were able to survive for only three days on melons, which had a strongly acid reaction at all stages of ripening.

Local foods. Lal & Yacob (1926), testing the relative suitability of certain foodstuffs used in India as substrates for the growth of *V. cholerae*, recorded the following:

(1) Articles containing salt and animal or vegetable proteins, such as meats, fresh fish, boiled rice, fresh milk, bazaar biscuits, oven-baked Indian bread, halwa, as well as raddish, cooked greens, and water melons were specially suitable for the growth of *V. cholerae*.

(2) Contrary to popular beliefs, chillies and onions did not inhibit the growth of the vibrios.

(3) Sugared foodstuffs gave variable results.

(4) Fats proved poor culture media, while sour articles, such as pickles and beer, were not likely to convey the infection.

Takano et al. (1926) recorded the following results with Japanese condiments:

" The [cholera] vibrio smeared on the body of fish is not killed in sugared vinegar for 2 hours. It survives for over 20 hours at room temperature in vinegar-bean-paste, and soy (soy-bean sauce) has even less disinfecting power than has vinegar-bean-paste. In short, it may be said that fresh fish prepared with vinegar, bean paste or soy is dangerous food during a cholera epidemic."

As found by Genevray & Bruneau (1938b) in Indochina, soy-bean milk and soya cheese, in which cholera vibrios survived for 12 hours and which were consumed on the day of preparation, were dangerous food articles at the time of epidemics. The length of survival of *V. cholerae* was less than one hour in fermented soya sauce, four to five hours in prawn paste and three to six hours in an Annamite dish (*nuoc-mam*) made from macerated fish, and since these articles were invariably stocked for some time before they were sold, it seems unlikely that they played a role in the conveyance of cholera infection.

Beverages

Babes (1885) maintained that cholera vibrios could stay alive in coffee, chocolate, and fruit juices for 48 hours, and in beer and wine for less than 24 hours. Systematic investigations made in this direction in the Berlin Gesundheits-Amt (1892; see also Friedrich, 1893) gave the following results :

Drink	*Viability of V. cholerae*	*Drink*	*Viability of V. cholerae*
Beer	Up to 3 hours	Coffee with milk*	8 hours
Wine	5-15 minutes	Tea (1%)*	8 days
Coffee (6%)*	2 hours	Tea (4%)*	1 hour
Coffee with chicory*	5 hours	Cocoa (1-2%)*	7 days

* Contaminated after cooling.

Panja & Ghosh (1945) found that undiluted lime juice (pH 2.8) killed cholera vibrios within five minutes, and lime juice freshly diluted with 1% peptone water (final pH 4.4) within half an hour.

Interesting results were recorded by Yacob & Chaudhri (1945), when studying the survival of *V. cholerae* in aerated drinks. These two workers found that the organisms, when added to soda water (pH 6.8), were no more demonstrable after two hours, and also noted an absence of the vibrios 10 minutes after blocks of cholera-infected ice had been added to samples of non-contaminated soda water. These ice cubes had been prepared by freezing for 24 hours in a refrigerator one litre of water, into which 7 ml of a 24-hour culture of *V. cholerae* in peptone-water had been incorporated previously.

Survival in water

Fresh water. While, as described in detail by Gaffky (1887), in the course of their work at Calcutta Koch and his colleagues were able to demonstrate the presence of *V. cholerae* in some tanks which served as a source of water-supply in a cholera focus, they could not reach definite conclusions regarding the possibilities of survival and multiplication of cholera vibrios in water in general, because the few investigations they could make in this direction gave discrepant results.

However, ample observations made by other workers soon filled this gap. Reviewing the results these investigators had obtained, Gotschlich summarized in 1903:

(*a*) Sterile distilled water was not a suitable substrate for the survival of *V. cholerae*, but addition of minimal quantities of nutritive substances or of NaCl created more favourable conditions.

(*b*) In the experience of some observers, the cholera vibrios could survive for prolonged periods in sterilized spring or well water, according to Wolfhügel & Riedel (1886) for periods of up to a year.

(*c*) On the contrary, the survival of the organisms in the filtered and/or sterilized water supplied by water-works was short, e.g., 7 days in Berlin tap-water according to Babes (1885).

(*d*) The periods of survival observed in the case of the raw water of wells and springs varied from about one day to several weeks (up to 10 weeks according to Kruse, 1894).

(*e*) The length of survival of *V. cholerae* in raw river-, pond- or tank-water also varied within wide limits, being for instance, according to Cunningham (1889), not more than four days in the water of Calcutta tanks, varying according to Uffelmann (1892, 1893b) in the port and river water of Rostock from 1 to 20 days, amounting under peculiar conditions in the deposits at the bottom of an aquarium to three months (Wernicke, 1895).

Some of the early workers clearly recognized that the marked differences noted in regard to the persistence of *V. cholerae* in different water-samples were due either to variations in their character, particularly their varying content of organic matter (Koch, 1884), or to extrinsic conditions, especially differences in the temperature of the water (Nicati & Rietsch, 1885), or to both these factors. The great importance of the latter factor was demonstrated by Uffelmann (1892, 1893b) who found that cholera vibrios survived in the water of the Rostock (river) port for one day at 30°C, for two to three days at 20°C and for five days at 10°C. At a temperature of 6°C the vibrios were found to survive for at least 20 days in the river-water and for at least 23 days in tap water.

In marked contrast with the results obtained in the case of rivers in Europe, Hankin (1896a) found that *V. cholerae* could not persist even for two hours in the raw water of the Ganges and Jumna Rivers in India. Since boiled water-samples proved no more inimical to the growth of the organisms, Hankin postulated that the raw water of these rivers exerted a vibriocidal action due to the presence of volatile acid substances.

That river water which is unsuitable for the persistence of *V. cholerae* may also be met with in other areas where cholera is apt to prevail, was shown by observations made later by Pollitzer (1934a) who found that cholera vibrios added to specimens of the Shanghai River and creek-water were no more demonstrable in 66% of the 50 samples tested, after they had been kept in the dark at room temperature for 24 hours. A survival of 24 hours was noted in 26% of the samples, longer periods of survival up to maximally four days being exceptional. There was no doubt that the short persistence of the vibrios was due to the heavy contamination

of the Shanghai surface-waters with various bacteria. Cholera vibrios added not only to sterilized but also to Seitz-filtered specimens of the waters used for the above-mentioned tests persisted for months, and in some instances almost a year.

Panayotatou (1913) considered the water of the Nile River in Egypt, in which in contrast to many rivers in other parts of the world no cholera-like vibrios could be found, as rather unsuitable for the persistence of *V. cholerae*. As noted before, she ascribed this unsuitability (which was only relative in degree, as the vibrios were found capable of surviving in raw Nile water for periods ranging from 1 to 13 days as against at least 30 days in boiled or sterilized samples) to the presence of bacteria antagonistic to the cholera vibrios. Gohar & Makkawi (1948), recently finding that when cholera stools were added to crude Nile water, the vibrios survived for four days only as against nine days if pure cultures of *V. cholerae* were used for such tests, ascribed the difference to an inimical action of the bacterial species normally present in the faeces.

Further observations on the vitality of cholera vibrios in the water of India may be summarized as follows:

Jolly (1926) raised an interesting point when drawing attention to observations which showed " the occurrence of a change in the reaction of Ganges river-water from alcaline to acid beginning about April before the onset of the rains, and swinging back from acid to alcaline in October or later ". Since the two waves of cholera in Eastern Bengal and Assam coincided with the " neutrality points " twice reached by the pH values of the Ganges water, Jolly suggested that a causal connexion might exist between the two phenomena, the infection dying out " rapidly at times when the reaction of the water is either too acid or too alcaline for the organisms and when the temperature is also unfavourable ".

Though it is difficult to share Jolly's belief that an alkaline reaction of the Ganges water might have proved inimical to *V. cholerae*, one must admit that the presence of an acid reaction of the water might have played a role, in addition to the prevalence of temperatures unsuitable for the survival of the organisms.

Khan & Agarwal (1929), comparing the survival of cholera vibrios in unboiled and boiled samples of the Ganges and Jumna water, recorded the following results:

	Survival (days)	
Kind of water	*Unboiled*	*Boiled*
Ganges	1 ±0.62	2 ±0.46
Jumna	1 ±0.62	7 ±1.97
Well-water	1 ±0.47	3 ±0.86

Comparing the results which they obtained in the four individual sets of tests they made during a period lasting from February to April,

Khan & Agarwal noted that the duration of life of *V. cholerae* in their samples became shorter hand in hand with an increase in temperature and absolute humidity. They recorded in this connexion that at the time of their first experiment the mean temperature was 73°F (approximately 23°C) and the mean average humidity 0.388, whereas the corresponding figures for the third set of tests were 86°F (30°C) and 0.607.

In a further study Khan (1930) tried to determine to what cause the supposed vibriocidal action of the Ganges water was due. He could find no evidence of the existence of volatile vibriocidal substances, noting in particular that water-samples heated under conditions which would have prevented the escape of volatile substances exerted no more an untoward influence on the survival of *V. cholerae*. Khan ascribed, therefore, the apparent loss of vibriocidal power, occurring already when the samples had been heated for half an hour at 55°C before they were used for tests with *V. cholerae*, to the fact that during the process of heating the bacteria naturally present in the water were killed. Thus, instead of competing with the cholera vibrios for the available food material, as they did in raw water, they furnished in their dead bodies additional nutritive substances for the vibrios. It served as corollary for this assumption that (*a*) in Ganges water-samples, which had been passed through Chamberland filters, the cholera vibrios, though persisting longer than in raw water, died markedly more rapidly than in heated water-samples, apparently because the filtered water was less rich in food materials than the heated water ; and (*b*) in the experience of Khan as well as of most other observers sterile distilled water was an unsuitable substrate for the survival of *V. cholerae*.

D'Hérelle et al. (1930), testing various Indian water-samples (mainly well-water, no river-water), found that the period of survival of *V. cholerae* in these specimens was short, as a rule not exceeding 24 hours, and lasting maximally two to four days. They stressed the fact that the lifespan of these organisms appeared to be much shorter in India, where cholera was prevalent, than in Europe, which was usually free from the infection.

Studying once more the viability of *V. cholerae* in certain waters of India, Lahiri et al. (1939) recorded the following findings:

Kind of water	*Raw*		*Candle-filtered*	
	Untreated	*Autoclaved*	*Untreated*	*Autoclaved*
hill spring	1 hour	18 hours	—	—
Calcutta tap-water	18 hours	24 hours	2 days	12 days
Hooghly River	18 hours	3 days	2 days	2 days
tanks *	up to 72 hours	up to 12 days	7 days	18 days

* With a high salt content and rich in organic matter.

Lahiri et al. were inclined to ascribe the considerably longer survival of the cholera vibrios in autoclaved samples of this water to a breakdown of suspended organic matters facilitating the nutrition of the cholera vibrios.

It should be noted that further tests with Hooghly river-water carried out in 1942 under the auspices of the Indian Research Fund Association, showed somewhat longer periods of survival of *V. cholerae*, amounting to three to four days in the case of raw water, to periods up to three weeks in the case of specimens which had been passed through L_3 Chamberland candles.

In the course of their investigations on the growth and survival of *V. cholerae* in water, Read et al. (1939), to whose important work attention has been paid already above, did not test raw water-samples. However, they carried out tests with autoclaved samples of water from Calcutta tanks, in which they noted survival periods of the cholera vibrios ranging from about five to over 30 days. A general conclusion reached by these workers was that

" Available figures of analyses of natural waters in the Calcutta area suggest that the requisite conditions for multiplication and survival [of *V. cholerae*] as far as salt content and organic matter are concerned are present in most of the natural sources."

Read et al. also adduced evidence to show that there was a relationship between the prevalence of cholera and a high monthly average of total solids in the Calcutta waters.

Since the important observations of Read & Pandit (1941) on the persistence of *V. cholerae* in the natural waters of rural areas in India are of epidemiological significance rather than being germane to the subject of bacteriology, they will be discussed in a later part of these studies.

Sea-water. Observations on the survival of *V. cholerae* in sea-water seem to have been made first by Nicati & Rietsch (1885) who, as quoted by Gotschlich (1903), succeeded in isolating cholera vibrios from the much-contaminated sea-water of Marseilles (France) harbour and establishing that these organisms could survive in sterilized sea-water for periods up to 81 days.

Jacobsen (1910), working with water from the Copenhagen, Denmark, harbour (salt content varying from 8.9 to 16 per thousand), found that in this substrate cholera vibrios persisted for 7-17 days during August and September, up to 47 days in November and December, and ascribed this difference to a higher microbial content of the water during warm weather.

Long periods of survival of *V. cholerae* in artificially prepared sea-water were recorded in 1933 by Yasukawa (22 days at the surface and 30 days at the bottom of a tank) and in 1939 by Venkatraman (at least 74 days survival in 2% salt-water). On the other hand, Flu (1921) noted a survival of the vibrios in the water of the port of Batavia (Java) for four days only, while Gohar et al. (1948), working with samples from some Egyptian ports found that the vibrios disappeared from raw sea-water in about 24 hours.

A moderately long persistence of the organisms in sea-water was noted by some early Japanese observers quoted by Takano et al. (1926) thus:

Author	*Length of survival of* V. cholerae *in sea-water*
Nogami (1902)	Raw sea-water: 3-4 days at 37°C, 9-10 days in the ice-box. Sterile sea-water: 30-42 days at 37°C, 53-65 days in the ice-box.
Yano et al. (1904)	At 37°C, 12 days in raw and 83 days in sterile sea-water; at room temperature, 26 and 152 days respectively, in a dark room at room temperature, 41 and 209 days respectively, in the ice-chest (3-8°C) 27 and 230 days respectively.
Matsuda (1920)	7-10 days in raw sea-water not exposed to direct sunlight, depending upon the degree of contamination of the water. Exposure to direct sunlight rapidly killed the vibrios.
Remarks.	(*a*) According to Yasuhara (1926), whom Takano et al. could not quote, cholera vibrios survived during winter for 11 days in the sea-water of Katsuura port, for 8 days in the water of the river-mouth, for 11 days in the river-water.
	(*b*) Some of these workers, carrying out parallel tests with fresh water, found that here also survival of *V. cholerae* was considerably longer at lower than at higher temperatures.

Concluding remarks. The evidence adduced above makes it clear that the chances of a survival of cholera vibrios in water depend upon an interplay of a number of variable factors, such as the temperature and pH of the water, its salt content, the amount of organic matters present, and the degree of bacterial contamination. There is little room for doubt that, provided conditions for the subsistence of *V. cholerae* do exist, the temperature of the water, which in turn depends upon the prevailing season, is one of the main factors, perhaps the principal factor, determining the length of survival of the organisms.

RÉSUMÉ

Cet article est consacré à l'étude de la morphologie et des propriétés biochimiques de *Vibrio cholerae*, ainsi qu'à celle de ses cultures qui comprennent, outre les formes S et R, un variant peu stable mais apparemment plus résistant aux conditions adverses, et des formes L.

L'auteur étudie l'action des facteurs physiques et chimiques sur le vibrion, la durée de sa survie hors de l'organisme humain, dans les fèces, la terre, les tissus, les aliments et l'eau. Au cours de l'exposé sont discutés les caractères qui peuvent contribuer à différencier le vibrion cholérique des vibrions cholériformes, en particulier les propriétés hémolytiques et hémodigestives de ces divers micro-organismes.

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