A STUDY OF VARIATION AMONG

CHOLERA VIBRIOS

WITH REFERENCE TO EPIDEMIOLOGY OF THE DISEASE

A Thesis Presented by G.A. Garabedian in Partial Fulfilment
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by

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Abstract

Different phases of variation among 18 strains of V.cholerae including Ogawa, Inaba, Hikojima and two epidemic strains, are studied in the laboratory.

It is claimed that typical epidemic forms of V.cholerae tend to undergo thru certain changes when transferred for long periods of time on laboratory media. Such changes effect almost all phases of the organism; the morphology, which loses its characteristic vibrio shape; the colony consistency, which might undergo an S-R dissociation; the antigenic structure, which suffers a loss or alterations in its component parts; the virulence , which might be retained when tested on mice or lost completely to this animal; the biochemical activities, which are definitely weakened and the hemolytic power, which might be gained as tested by Greig's method.

Similar variations occur in the same group of organisms when subjected to the influence of a specific 'phage or immune bodies, except that changes induced thru such drastic measures, are more extensive and lead the organism to undergo thru a series of changes which effect profoundly its basic cellular activities.

It is argued that a freshly isolated epidemic strain of V.cholerae is a lactose fermenter, besides being a dextrose, sucrose and a mannite fermenter, is non-hemolytic, highly antigenic and virulent when tested on mice;and that any deviation from these characteristic features, suggests a degenerative change in the cell, brought about spontaneously or thru certain agents acting on the organisms in vitro or in vivo.

Due to the fact that both smooth and rough forms of cholera vibrios, as obtained under the conditions mentioned, might cause death in mice when injected intraperitoneally or might be equally harmless to this animal underthe same experimental conditions, it is claimed that the term "virulence", as applied to the indicate the disease producing capacity of these organisms, would not seem to represent the true property of the vibrios when such tests were to run on human beings under natural conditions.

The results obtained by the former workers that, smooth fresh epidemic strains have more protective value as vaccines when used for immunization purposes than stock strains of V.cholerae, is confirmed.

It is suggested that hemolytic properties in an organism might exist as a seperate entity, totally independent of its virulence and that the El-Tor vibrio is probably a variant of a true cholera vibrio which has undergone certain alterations in its cellular structures and virulence, the extend of such changes depending upon the effectiveness of interfering environmental conditions. A possible relationship between cholera vibrios and other allied organisms is discussed.

It is suggested that the main mode of spread of cholera would probably be from the patient or from the acute carrier to the susceptible individual, but the possibility of occurence of cholera like outbreaks at certain inter-epidemic periods as a result of vibrionic reversion is indicated. That problems in epidemiology of cholera could be enormously clarified by finding a possible method of reproducing the disease in its typical form in experimen al animals, is made apparent.

G.A.Carabedian

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INTRODUCTION

The phenomenon of variation is a common occurence among bacterial species.
Changes in certain characters of a particular strain can be brought about by
cultivating the organisms on artificial media for a prolonged period of time
or by subjecting them to the influence of certain deleterious agents, such as
treating with a specific anti-serum, mild antiseptics, bacteriophage or by
growing under adverse environmental conditions of temperature and pH that might
effect in one way or the other their normal metabolism. Under such influences
the morphology of the organisms might be seriously effected as manifested by
the appearance of numerous involution forms, the motility might be reduced or
completely lost, spore formation depressed and marked weakening of biochemical
reactions might take place. Associated with such changes or independently,
colonial dissociation may take place resulting in loss of the smooth poly-
sacharide portion of the organism and its immunological specificity. As a
result of such changes the virulence of organisms might be markedly reduced
or shifted in one direction or the other. Furthermore, by proper treatment
a particular strain might become completely refractory to the action of a
specific 'phage or a drug.

Such variations though usually trivial, at times may be so profound that
it becomes impossible even for the most experienced worker to establish any
correlation between the original strain and the variant. The typical
characteristics of the parent strain can be regained partially or fully by
animal passages, but in extreme cases when the virulence of the organism is
completely lost no such reversion is possible and the organism breeds true in
its mutant form.

Although such alterations occur among all the species of bacteria with
more or less frequency, they are probably more accentuated among the pathogenic
vibrios, where changes in almost all phases of the organism take place under
suitable conditions. Such changes at times are so extensive that the organism

loses its species specificity and attains the characters of a non-pathogenic vibrio. Thus, under such circumstances it is reasonable to assume that there is a genetic relationship between the saprophytic and the parasitic forms of vibrios, and that the latter are mutants of the free living ones that have attained virulence thru adaptation to the animal tissue. Much litterature has been accumulated for and against such an assumption with no convincing results and the relationship between the cholera vibrios and free living or non-pathogenic forms is still bound by shrewd speculation.

Historical.

V.cholerae, as originally described by Koch, are short, slightly bent organisms looking like the German mark of punctuation known as the comma (63). On twenty four hours old agar culture they usually measure $0.3-0.4\mu\times2-3\mu$. They may occur singly, in half circles, S-shaped and in long screws or spirals measuring $0.35 \times 5-8\mu$. In old cultures pleomorphism is common. The individual cell are thicker, almost straight, swollen and often showing light areas in the interior. Swollen, spindle-shaped and bladder-like forms occur. Although these distorted structures were called arthrospores by Hueppe (58), they are not spores, but probably degenerative products. Such pleomorphism appears also in strains kept for a long time on artificial media and when the organisms are cultivated on dextrose agar. In 1926, Balteanu (7) described, during the growth of the organisms, coccoid, bacillary, long-spiralled, budding and branching forms. Strong however, (111) claims that he has never observed such bizarre forms in any fresh, virulent cultures of V.cholerae.

That similar morphological variations occur when vibrios are subjected to the effect of certain agents has been reported by Gordon (39). By growing vibrios in the presence of 0.5-1.5 per cent glycine, the author could produce swollen, spherical and oval forms; and by adding glucose or other inhibitory substances

such as phenol, p-amino-benzoic acid or glycine, he could prevent the formation of such involution forms. As in Pasteurellas, a typical morphological forms can be obtained by culturing the vibrios in the presence of certain salts, such as LiCl (10).

Morphological changes among the 'phage refractory races of cholera vibrios have been reported by a number of workers. Thus d'Herelle (55) observed cocco-bacillary and even coccus forms among such treated vibrios which were surrounded by a capsule (56); in most of the cases the normal morphology was restored when 'phage was eliminated (26) but in certain cases fixed mutation in morphology took place. In 1937, Macneal (79) observed remarkable variations in size and shape of vibrios subjected to the action of 'phage with simultaneous alterations in the internal structures. Peculiar minute granules were demonstrated in the interior of the altered bacteria and the author suggested that these could be the bacterio-phage particles. No such changes are described in vibrios treated with immune serum.

V.cholerae is motile with an active 'scintillating' or 'darting' motility and possesses a single polar flagellum. The long forms have a serpentine movement and in old cultures the motility is reduced or completely lost (31). Non-motile variants have been reported by Balteanu (7) from opaque colonies of dissociated vibrios. That loss of motility in the cholera vibrio is not associated with any deep-seated antigenic alteration in the organisms has been reported by Sugino (112) in 1935. This author claims that a non-motile and non-mannose fermenting strain of V.cholerae possessed marked antigenic powers to protect against various strains of the same organism.

Typically the surface colonies of V.cholerae on agar in twenty four hours are round, regular, 0.5-1 mm in size with central nuclei; margins are thin and the edges entire. In forty-eight hours, the colonies are 1-2 mm in size, round elevated and slightly opaque. They may be either greyish-yellow or bluish grey

in color resembling very much those of typhoid bacillus (31), (111), (60).
In old cultures the colonies are large in size, thick, opaque and with dense
centers.

Colonial dissociation among V.cholerae and other vibrios occurs readily
as in Salmonellas and is usually associated with serological changes. Thus, in
1924, Shousha (108) obtained R-forms from hemolytic and non-hemolytic strains of
V.cholerae by plating out old broth cultures. The R-variant was found to
agglutinate spontaneously in physiological salt solution and could be differentiated
from the S-form by its cultural characteristics, agglutinability and by complement
fixation tests. The smooth type was more pathogenic than its rough variant. In
1926, Balteanu (7) dissociated cultures of vibrios into three types: (1) rugose
circumvalent type of colonies with a central nodule, and thickened edge; (2) white
ringed type of colonies composed of a dense center and a thin flare bordering zone
and (3) opaque, round, hemispherical type of colonies with regular surfaces. These
colonies do not exactly correspond to the usual S-R transformations. The morphology
of vibrios in all three was not altered except that in opaque colonies the organisms
were non-motile.

Extensive changes have been reported in cultural and other physiological
characteristics of vibrios and other organisms by subjecting them to the action of
specific 'phage under suitable experimental conditions. Upon agar mixed colonies
of 'phage and bacteria of very high resistance are usually small, viscous and of
slow in growth (55). Gratia and Jaumain (49) working with 'phage treated Staphy-
lococci came across to colonies that were thick and opaque, others, discrete,like
cultures of Streptococci, some rich in pigment, others poor, some grew with a
homogeneous growth in broth, others gave a sediment. A strain thus treated in a
state of active or latent resistance shows a loss in agglutinability with specific
antiserum. However, after a series of passages the agglutinability is restored
(55). Bordet and Ciuca (9) have shown that a resistant B.coli strain does not

produce the change in color of neutral red, that it is less readily phagocytosed than a B.coli of same normal stain and that its virulence for laboratory animals is higher. Similar increase in virulence, among resistant strains of Shigella shiga, has been demonstrated by Davison (22) in 1921. D'Herelle (57), however, claims that decrease in agglutinability and increase in virulence are not absolute rules in such resistant strains. He found that some of the 'phage refractory races of P.pestis were completely avirulent to animals and suggests that increase in virulence of an organism is related to a deep-seated mutation in the bacterial cell due to action of 'phage, but does not always happen.

That such changes wrought under the action of a specific 'phage are at times so deep as to bring about complete alteration in the characters of an organism, has been demonstrated by Bordet and Ciuca (8), d'Herelle (57), Gratia (48), Fejgin (27) and others. Bordet and Ciuca noted first true mutations in bacterial cultures due to 'phage action. D'Herelle found that transmutation of B.coli into B.aerogenes might occur under similar circumstances and, that such mutants were ultrapure was shown later by Gratia. During the return of pilgrims from Mecca, while they were quarantined at the lazaretto at Tor, d'Herelle (loc.cit.) isolated from the stools of one of these pilgrims with no symptoms of cholera, a vibrio which agglutinated with V.cholerae anti-serum. However, after a time the stools revealed only non-agglutinating vibrios and eventually these also disappeared from the feces. Upon first isolation the agglutinable strain was lysed by cholera 'phage while inagglutinable vibrios isolated at a later date were perfectly refractory to its action.

Vibrionic mutability due to action of 'phage has been studied in the laboratory by a number of workers. Dealing with V.cholerae, which has an optimum temperature and a pH for growth similar to that of 'phage particle, d'Herelle found it difficult to get resistant strains by varying these factors. He observed a rapid lysis of vibrios when treated with a virulent 'phage race.

However, by using a weak 'phage and gradually increasing its virulence he could obtain strains, after such ten passages, which were resistant to most virulent races of 'phage (65).

In 1930, Asheshov et al. (5), described three races of 'phage acting on different groups of cholera vibrios. Type A. quick acting 'phage producing lysis in less than two hours. Lysis produced was not permanent and was followed by abundant secondary growth resistant to Type A 'phage. This race attacked the smooth elements and not the rough, was very unstable, losing its virulence within a very short time, sometimes within few days. Type B.- causing lysis of organisms in three hours followed by secondary growth resistant to type B'phage, was more stable than type A'phage, kept its activity for several months and acted both on smooth and rough cultures. Type C.- Slow acting, seldom produced any lysis in two and a half hours. This race seemed to kill vibrios without producing any visible lysis. The resistant vibrios grew slowly when plated on agar. All races of vibrios smooth and rough were attacked by this type. However, Asheshov's observation was extended by his discovery of more and more smooth resistant strains. Thus in his later reports, he describes four groups of vibrios and in a subsequent report mentions six groups of vibrios of smooth character which were resistant to his type A 'phage. Rao (100) states that within optimum limits lysis by type A cholera bacteriophage is enhanced by increasing acidity and that of type B by increased alkalinity. He concludes that on continued existence in an acid medium the groups II, III, and IV would all become group I, in other words lysable by cholera bacteriophage original type A of Asheshov.

Further studies of vibrios subjected to the action of types A,B and C 'phage were carried on by Yang and White (136) in 1934. Comparing the ultrapure strains with their 'phage variants, the authors could not show any quantitative serological modification differentiating B and C 'phage resistant strains from ultrapure parents. However, type A resistant rough races exhibited the general characters

of classical roughness as observed in the Salmonellas. The authors, furthermore, describe a number of culturally smooth but type A resistant strains of V.cholerae that were presumably isolated from patients in the stated condition. Thus, type A 'phage resistant strains might be either smooth or rough, in the latter case roughening involves the disappearance of a non-protein and probably carbohydrate containing substance which furnishes the characteristic O-receptor of the smooth form and that a second non-protein but Molische positive substance present but masked in the smooth organism replaces in the rough vibrio the lost smooth factor and becomes the characteristic rough receptor.

In 1935, Morison (86), summarising the results of 'phage action on susceptible and refractory organisms, states that some 'phage lyse and completely destroy bacteria, others induce a resistance in it and alter its character to a greater or less degree, still others live in symbiosis with or rather in the bacteria without altering the physical and biological characters of the bacteria. Morison further claims that numerous combinations of 'phage types can be made taking two or more types at a time and that the action of such combinations are frequently different from the action of the individual components of the combination. In an experiment he describes 511 possible combinations of nine types of 'phages. Such combinations when tested on smooth V.cholerae gave rise to changes in morphology, colony characteristics on agar, growth in broth, salt stability, agglutinability and fermentation reactions. Variations were at times so profound that the resulting bacteria were quite indistinguishable as vibrios by any method available and would be rejected in any examination of cultures made from supposed cholera stools.

In 1935, White (132) dealing with two groups of observers, one that of Calcutta - Linton (71), Linton et al. (3) and the other Kasauli workers - Taylor and Ahuja (113), (114), criticised the theory of vibrionic transmutability. The Calcutta workers, by plating out cholera stools, were able to pick off two colonies (1) Rangoon smooth-giving a typical culture of V.cholerae and (2) Rangoon rough -

a rough derivative of cholera vibrio showing no serological relationship with Rangoon smooth. Later, Rangoon rough$_1$ dissociated giving rise to Rangoon rough$_2$ which was serologically distinct from Rangoon smooth and Rangoon rough$_1$. Next, Rangoon rough$_2$ upon dissociation gave Rangoon rough$_{2a}$ which was an intermediate variant between smooth and rough forms; and finally from this a fifth race was recovered which was identical with Rangoon smooth. White, after examining these cultures claimed that Rangoon rough$_1$ and Rangoon rough$_2$ were smooth vibrios possessing a smooth polysaccharide complex and had no connection with classical roughening or with rough races of vibrios. Further, he argues that since Rangoon smooth and Rangoon rough were derived from two different colonies their genetic relation is a matter of pure assumption.

The Kasauli observers stated that during a laboratory subculture a water vibrio-'Kohat original'- yielded a race,-'Kohat current' exactly simulating V.cholerae culturally and serologically, and that three other vibrios, V.metsch nikovi from Hamburg, a water vibrio and a vibrio from a healthy person, all distinct in their O-serology from V.cholerae, made the same dramatic change during serial mouse passages. White states that if such mutations indeed occurred they did so by profound catastroph. Furthermore, he argues that during mouse passages intercurrent infection of vibrios from the gut occurs. He claims of having obtained a mutant vibrio from the heart of an animal when organisms were administered orally, after an intra peritoneal injection of a lethal dose of killed culture of V.metschnikovi. White found that these mutants obtained from the heart blood were contaminated with 'phage and asks whether such mutations as reported by Kasauli observers were not due to the action of 'phage during mouse passages.

That the action of 'phage on suceptible or refractory bacteria is more or less related to the antigenic structure of the bacteria concerned is discussed at length by Burnet and Burnet et al (11), (12), (13), (14), (15) and others. According to Burnet bacteria possessing the same antigenic structure are

susceptible to the same 'phage; e.g., E.typhi, B.enteritidis, fowl typhoid and
bacillus of white diarrhea of chicks have in common an O (polysaccharide containing)
antigen but are otherwise dissimilar. All, however, are sensitive to one race of
'phage which is without action on strains lacking this antigen. The work of White
(125) and of Furth and Landsteiner (33), have made it clear that the specificity
of 'O' agglutination reactions in the Salmonellas is due to polysaccharide haptenes
and that with change to the R-form, the characteristic S-carbohydrate haptene is
converted into or replaced by an immunologically distinct R-carbohydrate. The
logical deduction therefore is that 'phage specificity, like antibody specificity,
is primarily determined by the ability of 'phage particles to be absorbed to the
carbohydrate molecule on the bacterial surface. Burnet, using bacterial crude
extracts, by allowing agar grown bacteria to autolyse in watery solution for two
days at 55° C., was able in certain cases to inhibit the action of 'phage on
organisms. He concluded that 'phages which lyse a given strain are inactivated
by extracts from that strain. Thus the development of resistance in a particular
strain of bacteria in almost all instances associated with a loss of the correspond-
ing 'phage inactivating property of the extract. Similar experiments have been
reported by Levine and Frisch (68).

A more hypothetical explanation about 'phage adsorption on the bacterial
surface has been advanced by Andrewes and Elford (3). The authors suggest that
the 'phage particle surface is a mosaic of two entities, namely AC (antigenic
component) and BC. (bacterial component). The first is responsible for the
serological specificity and the second serves as a point of attachment to suscept-
ible bacteria and determines the resistant group of the 'phage. With the union
of the antibody to the former of the two entities (AC), a progressive blocking of
the second set occurs, eventually rendering it impossible for the 'phage to make
specific contact with and lyse the susceptible bacteria. Burnet further assumes
that after the adsorption of 'phage particles on the bacterial surface component

(P.I.A.-'phage inhibiting agent), there might be complete blockage of the BC
component of the 'phage surface and the 'phage particles will be totally unable
to initiate lysis.

The bacterial surface components responsible for 'phage adsorption differ
sharply in S and R variants of the same bacterial strain and this difference is
reflected in the corresponding 'phage inhibiting activity, but in both phases
there is also a common element related to those 'phages which lyse both smooth
and rough phases with equal activity (41). Thus an anti-S serum destroys all the
'phage inhibiting activities of a homologous S-extract including those against
'phages, acting equally on S and R variants but does not destroy these activities
of the corresponding R-extract. Furthermore, if an S-strain resistant to a 'phage
in the S and R form and is converted into R-form by the use of anti-S serum, the
variant is still specifically resistant to the same 'phage due to the common
component present in both S and R bacterial surfaces and extracts.

Variations in different phases of activities of cholera vibrios have been
produced by treating smooth or rough strains with their homologous antiserum.
Nadaka (89) in 1920, and Yamanouchi (135) in 1921, observed loss of agglutinability
in vibrios grown in homologous serum. Furthermore, Yamanouchi found that agglutin-
ability of vibrios diminished after their introduction into the alimentary tract of
a cholera immune animal. Goyle (43) in 1932, inoculated six different strains of
V.cholerae into peptone water containing 10 per cent immune serum and after 7-8
such passages rough races were obtained which showed only slight cross agglutination
with smooth antiserum, were Millon's positive, showed salt agglutination but were
not different biochemically from the parent strain and were non-hemolytic. In 1934,
White (127) could produce rough variants of V.cholerae by treating smooth strains
with homologous O-antiserum in presence of complement. The races obtained were
indistinguishable from R-races produced as a result of subjecting smooth strains
to the acting of type A 'phage and were without exception resistant to this 'phage.

Further treating the R-races with homologous R-O-antiserum new races were obtained
which agglutinated feebly with the antisera prepared against smooth or rough strains.
Such races known as p-races, failed to reduce the titer of R-agglutinating serum
in absorption tests, though the R-vibrios exhausted the serum of the new variant.
It appears that this new race is a variant of the already degenerate R-vibrios.
White described four such groups of R-variants which could be distinguished
immunologically (129).

More recently, Panja (93) was able to obtain rough variants of V.cholerae
by incorporating "atebrin" in a concentration of 1:5000 in the nutrient agar.
Associated with S-R changes, pleomorphic forms appeared in the culture, some of
the races were non-motile, Million's positive and agglutinated feebly with smooth
antiserum but in full titer with rough antiserum. In all cases however, the
biochemical activities and other fermentation reactions were not seriously effected.

The heterogeneity of serological reactions of cholera vibrios has been a matter
of great controversy in the past. That bacteria could be agglutinated in presence
of homologous antiserum was first demonstrated by Gruber and Durham (51) in 1896
and was applied for identification purposes by Achardi and Bensaude (2) and Kolle
and Gotschlich (64). Before the work of Meinike, Jaffe and Fleming (81), it was
assumed that V.cholerae was immunologically homogeneous. The authors, however,
in 1906 demonstrated immunological subgroups in these organisms by agglutinin
absorption tests. Following them, in 1911 Kraus, Hammerschmidt and Zia (65)
observed differences in agglutinability among different strains of V.cholerae.

Kabeshima (62), demonstrated the existence of two serological types differ-
entiated by agglutinin absorption tests. The first of his types was isolated in
Japan during an epidemic of cholera and designated as 'Japonica - 1911 and was
later termed as the 'Original' type. The second was recovered from a sporadic
case in Formosa and named 'Formosicana-1911'and later termed as the 'Variant type.
At present they are known as the Inaba and Ogawa types respectively. Similar

observations as to these types were made later by Takagi (1913), Saito and Takagi (1914), Ota (1914,1917) and Nakate (1920). ‡ A third serological type was added by Nobeshi (88) in 1923 which was designated by him as the 'Intermediate' or the 'Middle' type and is now frequently referred to as the Hikojima type. An antiserum prepared against this type agglutinated both of the former types.

Balteanu (7) in 1926 showed that V.cholerae like other motile bacteria contains a heat labile, loose flocculating flagellar or 'H' antigen and a heat stable, granular agglutinating 'O' antigen in the body of motile and non-motile races. Shousha (109) found that the 'O' antigen of cholera vibrio was specific for the group while the 'H' antigen was shared with a number of other vibrios from water or from non-choleraic cases and suggested the use of 'O' antiserum for identification purposes. Ahuja (4) in 1939 reported that 35.5 per cent of such vibrios possess an 'H' antigen partially or completely identical with that of V.cholerae. Similar observations have been made by a number of other workers such as Abdoosh (1), Gohar (37), White (126). However the most complete work on this line has been carried on by Gardner and Venkatrañ (35) in 1935. By examining 101 races of vibrios the authors concluded that cholera group of vibrios have similar biochemical reactions and contain a common 'H' antigen. On the basis of agglutination reactions, using O-serum and unheated suspensions of organisms, they divided the vibrios into six groups. Group I, containing all the standard stock cultures of cholera vibrios, strains isolated from typical cases of cholera and many El-Tor vibrios. The para-cholera, cholera like vibrios and other El-Tor vibrios were placed in the other five groups. The authors state that a true cholera strain should be non-hemolytic and agglutinable with O groupI antiserum.

While studies of Taylor et al.(116) show that classical cholera vibrio falls

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‡ Original papers in Japanese, discussed by Nobechi in 1923 (108).

invariably in O groupI, yet the presence of non-agglutinable vibrios in some
diarrheal diseases and occasionally in true cases of cholera remains a problem.
Thus Gardner and White (36) point out that in some cases the vibrios isolated
from patients may be rough and inagglutinable or that, as Taylor (loc.cit.)
remarks, non-cholera vibrios may or may not be associated with diarrheal diseases.
Furthermore, such inagglutinable vibrios often do not fall in any one of the
groups described by Gardner and Venkatraman. Thus, Taylor found that of 558
inagglutinable strains of vibrios isolated in India, 57 only could be classified
in O group II-VI, 254 belonged to 31 newly defined serological groups and the
remainder could not be classified. Similar results have been reported by Merten
and Mochtar (82) in Nederlands East Indies.

The biochemical structure of the vibrios has been studied extensively by
Linton and his co-workers (75), (83). After subjecting various strains of vibrios
to detailed chemical analysis, the authors differentiated three types of specific
carbohydrates and two proteins incorporated in the antigenic structure of these
organisms. Using these chemical components as basis for classification they placed
the vibrios into six main groups. Groups I.- Includes most of the vibrios isolated
from cases of clinical cholera consisting of galactose, aldobionic acid and
protein$_1$ found only in the agglutinating vibrios. Group III-. Includes the
non-agglutinating water vibrios consisting of arabinose, same aldobionic acid
and protein$_2$ found in the non-agglutinating strains. Groups II, IV,V, and VI.-
Include Rangoon rough$_2$, an aberrent strain (BisrahII) both containing glucose-
as carbohydrate, and other variants.

The immunological changes associated with S-R variations of vibrios have
been studied at length by White and others. In 1934, White (128) obtained an
acid-alcohol extract from mass cultures of V.cholerae a serologically active and
strongly antigenic substance resembling Q-substances of Salmonellas. This
antigen was separated into two fractions (Q$_1$ and Q2) both apparently of protein

in nature, readily redissolved in acid-alcohol and unlike the Q-substance of Salmonellas were soluble in neutral water. The first was destroyed by trypsin and pepsin but the second was resistant to the action of trypsin. The Q-serum gave no precipitation with carbohydrate-soluble-specific substances of the vibrios but had a definite agglutinating action on the living organisms, specially on R and p cultures. Vibrios heated at $100^{\circ}C$. in saline suspension agglutinated in a generalised manner and often to a high titer with the antiserum of Q-proteins of the cholera vibrios. The antiserum of the Q_2 substance of smooth Y.cholerae seemed to possess agglutinating properties additional to those of anti-Q_1 and anti-Q_2 R-serum, rather more specific and possibly related to CHO-receptors.

The polysaccharide portion of cholera vibrios has been further studied by White (130), (131) in 1936. He demonstrated that smooth cholera vibrios possess four serologically active polysaccharides or non-protein carbohydrate containing receptors which he termed Ca,Cb,Cy and Cd. Of these C_a, the determinant of smooth type specificity, is lost in roughening exposing C_b, the characteristic rough polysaccharide. On degradation to the p-form Cb disappears byt C_y and C_d common in whole or in part to many vibrios remain. On digestion of vibrios with papain in a slightly acid medium, C_a and C_y are brought into solution while C_b and C_d are not, but may be liberated from the residue with alkali.

White, (134) on a later date, described an independent type of variation characterised in the rugose races of vibrios. The rugose strains of S,R and p variants contain an O-antigen that is common to members of group A and vibrios of other groups. He claims that the rugose growth habit of vibrios has no connection with roughness and that it may be assumed by S and R races alike. Such races when revert to more usual growth habits of vibrios, return to the S or R condition from which they were derived. Rugose condition is due to secretion by the culture of a gelatinous intra-cellular substance or a definite capsule (133).

From S, R and p-rugose cultures of cholera and El-Tor vibrios there may be
isolated in relatively large amounts of a common non-protein CHO containing
haptene absent from or inconspicuous in non-rugose cultures. These substances
react by precipitation in high dilutions with antiserum of rugose cultures.
Furthermore the rugose antibodies of O group $_I$ vibrios agglutinate rugose races
of certain vibrios belonging to other groups defined by Gardner and Venkatraman
and give intense precipitation reactions with their rugose haptenes. Such rugose
variants developed on lactose litmus agar grown at 42°C. has also been reported
by Skinn (110).

The antigenic formula of cholera vibrios has been a matter of considerable
discussion. Nobechi (88) gave the antigenic formula AX to the Inaba type, A(B)X
to the Hikojima type and BX to the Ogawa type. Heiberg (53), in 1935, modifying
Nobechi's formula gave AX,ABX and BCX to the three types respectively. Further
studies by Scholtens (105) suggests that cholera vibrios may be divided into two
main serological groups by cross absorption tests. One containing an antigenic
factor A and the other a factor B. Factor A is common to both groups and both
groups have been isolated in the same place without any indication of predominance.
According to Scholtens the antiserum of <u>V.cholerae</u> contains two agglutinins A' and
B'. A' agglutinates all cholera strains while B' only one third of the strains.

Recently, Burrows et al. (16), (17), analysing the antigenic components of
<u>V.cholerae</u>, El-Tor vibrios of O group I and non-cholera vibrios of O group II-VI,
came to the conclusion that the O group I vibrios of Gardner and Venkatraman are
characterised by the presence of a group specific A-antigen and type specific B
and C antigens associated with the Japanese types. In accordance with such findings
the antigenic formula AB is suggested to the Ogawa type, AC to the Inaba type and
assumed that ABC corresponds to the Hikojima type. Thirteen antigenic components
are described in the vibrios studied, of which, they consider five as being major.
Besides the three mentioned, antigens designated as D and E could be used to

create new types. All these major antigens with the exception of A are found in other types of O group other than I, and so the authors suggest using a monospecific A-antiserum for diagnostic purposes. They seem to have found no immunological distinction between cholera and El-Tor vibrios of O group I.

Fermentation reactions of vibrios have been studied in detail by Heiberg (52) in 1934. After examining 375 strains of vibrios collected from different institutions, Heiberg found that, all strains agglutinable with cholera antiserum, with few exceptions, ferment glucose, levulose, mannose, saccharose, maltose, dextrine, glycogen, amidon and mannite within 6-20 hours at 37°C. but not arbinose, xylose, rhamnose, adonite, dulcite, sorbitol, salicin and tartrate. Late acidity (in less that 8 days) was observed in lactose, galactose and glycerine. Using saccharose, arabinose and mannose as basis for classification, he divided the vibrios into six fermentation groups. ~~Group I.- fermentation groups~~. Group I. fermenting saccharose and mannose but not arabinose, cholera red test positive; includes 239 agglutinating strains and 27 non-agglutinating ones, of the latter two were para-cholera strains isolated in Egypt by Mackie, three isolated by Dorenbcos in El-Tor and the rest from patients manifesting typical symptoms of cholera. Group II.- fermenting saccharose but not mannose or arabinose, cholera test positive; includes one agglutinating strain and 76 non-agglutinating ones; among the non-agglutinating strains, one was El-Tor, three were water vibrios and the rest from patients with typical cholera symptoms. Group III.- fermenting saccharose, arabinose and mannose. Group IV.- fermenting saccharose arabinose but not mannose. Group V.-fermenting mannose but not saccharose or arabinose and Group VI.- not fermenting any of these sugars. Cholera red test was negative in the last four groups and included a number of vibrios from water and from non-cholera sources. Thus Heiberg concludes that a true cholera strain should ferment saccharose and mannose but not arabinose.

In 1937, Combiesco et al. (18), studying the fermentation reactions of 107 vibrios, concluded that the vibrios isolated from true clinical cases of cholera and agglutinated with O-antiserum fell in Heiberg's group I, non-agglutinating forms from such cases belonged to group I or group II; the para-cholera strains failed to ferment any of the three sugars (group VI-Heiberg). Similar observations have been made by Taylor et al. (115) and Pandit and Mitra (92). That, due to their extreme variability, fermentation reactions could not be used as a basis for the classification of vibrios has been brought into view by Mertens and Mochtar (82) and Gardner and Venkatraman (35).

The hemolytic power of V.cholerae has also been a matter of some controversy. An important discovery was made by Gotschlich (40) in 1906 at the quarantine station at El-Tor by isolating a vibrio from sick and healthy pilgrims which was identical culturally and biochemically with that of V.cholerae but differed from it in being hemolytic. The organism was found in the absence of cholera in the region and was considered for sometime as a non-pathogenic species. De Moor (84), (85), however, in 1938 reported an important outbreak of cholera in the Celebes in which El-Tor vibrios were isolated from 47 patients. A little later, van Loghem (77) confirmed the findings of de Moor. Otten (90), in 1939 examined the Celebes strain and found that it was less hemolytic than the El-Tor strain but otherwise similar to it. He further believed that V.cholerae could give rise to a hemolysin which was often less stable than that of Celebes vibrio and absent in three days old broth cultures but could be detected by adding blood to one day old broth cultures.

That cholera vibrios under certain cultural and environmental conditions might show hemolytic properties has been demonstrated by a number of workers. Parrino (94) in 1933, showed that cholera and cholera like vibrios when cultured in alkaline medium attained an increased hemolytic power, gave a more intense nitroso-indole reaction and showed a greater agglutinability, and that these

characters were depressed when the organisms were culured in an acid medium.
Gohar (37) found that by washing the growth on agar slopes in a minimum quantity
of saline or by evaporating broth cultures in vacuum, V.cholerae, like the El-Tor
vibrio, attains hemolytic properties and that this property becomes more apparent
when the organism was grown under aerobic conditions. Doorenbos (24) in 1936
examined 12 strains of cholera vibrios freshly isolated from fatal cases and found
that 24 hours growth of these organisms in broth were not hemolytic to sheep cells
but 8 hours growth, of two thirds of the strains had marked hemolytic power to such
cells. His results were confirmed by Fournier (32) in 1940. Flu (30) in 1934
claimed that a true cholera vibrio might attain hemolytic properties like that of
El-Tor vibrio after long sojourn in a given host. More recently, it has been urged
by Doorenbos (25) that El-Tor vibrio is a modified endemic type of the true cholera
vibrio and may revert to the epidemic type under suitable conditions.

Whether cholera cases arise from previous cases thru direct contact with
patients in the acute stage, in incubation period or in carrier stage, or that
true epidemic vibrios, after undergoing mutations in nature and becoming undetect-
able by known cultural or serological methods, revert into typical virulent forms
under suitable conditions, has been an important epidemiological problem with much
confusing results. In 1913, Greig (45) stated that chronic carriers harbor the
vibrios in their gall bladder and that in three out of eleven cases examined daily
cholera vibrios were discovered intermittently in the stools for a long period of
time. Two years later, Goulter (42) reported that 17 cases out of 226 autopsies
showed V.cholerae in their bile. Similar investigations by Cromwell and Johston
(21) showed that 65.2 per cent of such cases harbor cholera vibrios in their bile.
Contacsene and Marie (20) found that by whatever route the cholera vibrio enters
the body, it finally reaches the walls of the small gut.

That the convalescent carrier stage is short and unlike enteric fevers no

permanent carriers exist in cholera, has been shown by a number of workers.
Wesskopf and Herschmann (124) in 1915, examined 247 cases of cholera in Slavonia
and found that 80 per cent were free of vibrios at the end of illness. In 1916,
Levi (67) found that the stools of 93 per cent of cases and 99 per cent of
contacts were free of vibrios within 14 days. Conseil (19) reported that vibrios
actually disappear from the stools of patients on the ninth or tenth day after
recovery and that the longest carrier period was 20 days. Galambos (34) in 1916,
found that in a series of 89 cases, except in 2-3 cases, vibrios disappeared from
the stools in 7-8 days; in the exceptions they were found for 2-3 weeks. Sergent
et al. (107) reported a case of a native woman who was carrying vibrios 53 days
after the recovery. Jatta (59) found that healthy carriers usually stopped
passing vibrios in their feces 3-5 days after recovery but 15 per cent of such
cases continued to carry the organisms for 6-12 days. Greig (45) stated that
the largest period of a carrier stage was 44 days. Pottevin (97) in 1913, reported
that on 300 cases in Italy in the year 1910-1911, 97 per cent were free of vibrios
at the end of a month. Defressine and Cazeneuve (23) found that the number of
vibrios in the stools of healthy carriers was relatively small and that the duration
of the carrier state was in no case more than 8 days. Livierato (76) in the same
year found that out of 97 cases about 13 per cent carried the vibrios for the
period of 10-12 days, about 79 per cent for 13-41 days and only 2-3 per cent carried
for a longer period, the longest period being 48 days. Similar results have been
reported by Schoble (104) in 1915. Greig (44) cites the case of a man who gave
rise to an epidemic of cholera in a jail 18 days after the onset of the disease.

Further investigations have been carried on to detect agglutinating or non-
agglutinating vibrios in stools of cases and carriers, in water wells and elsewhere
in nature with the idea of establishing a possible epidemiological relationship
between these two groups of vibrios. The results are confusing. Flu (29) in 1913,
Mackie and Storer (78) in 1916 and Jorge Ricardo (61) in 1920, have reported cases

of cholera like disease, the stools of which upon culture yielded only non-agglutinating vibrios. In 1921, Tomb and Mitra (119) examined large number of stool specimens from healthy people in the endemic area of Asansol, Bengal, but could not detect any carriers. Later (120) they reported that in no instance were they able to isolate agglutinating vibrios in the stools of either survivors or of contact carriers 3-4 weeks after the cessation of an epidemic. Tomb and Mitra further stated that they have been able to isolate agglutinating vibrios from waters contaminated with cholera dejecta only 24 hours after pollution, after this period the vibrios were changed into non-agglutinating forms. According to these authors, 30 per cent of the inhabitants of the endemic area of Asansol are carriers of non-agglutinating vibrios, but there has been no true cholera cases in the locality for a long period of time, and that the non-agglutinating vibrios had no effect in immunizing the population, because when the true disease came it was as fatal as ever.

In 1930, Khan (103) after studying the carrier problem of cholera, concluded that 95 per cent of all cases and contacts were free of vibrios within a fortnight. He claims that, judging from the number of vibrios voided in the stools, it would not be an overestimation to say that a carrier is 15 times less infective than the patient. He believes that the limited geographical distribution of cholera is evidence against the assumption that cholera is spread by such carriers. Furthermore Khan argues that the inagglutinable vibrios do not cause epidemic cholera, they can only do so if they get changed into agglutinable forms and "it is doubtful if such a change takes place at all". He asserts that the maximum period of carrying the inagglutinable vibrios is also very short, such carriers are free of vibrios within 3-4 months. Thus, Khan concludes, that the epidemics of cholera arise from actual cases and from those in the incubation period or in convalesence, but not from chronic carriers.

That vibrionic transmutability is a possible occurence in nature during

interepidemic periods and that such mutants, under certain conditions as yet
unknown, might give rise to serious epidemics de novo, has been suggested by
a number of workers. Ouchi (91) in 1933, stated that the reappearance of cholera
at Shangai during the summer might not be due to healthy carriers and that the
vibrios were possibly harbored in some as yet unkown cultural reservoirs between
the epidemics and reappeared during the hot season to give rise to new outbreaks
of the disease. Nicholls (87) reported that in about 11.000 estate laborars and
third class passengers travelling from India to Ceylan, he found 84 carriers of
agglutinating vibrios, 2839 carriers of inagglutinable ones otherwise indisting-
uishable from the first group and 992 carriers of vibrios which were inagglutinable
and differed biochemically and morphologically from the first two groups. The
author suggests that the V.cholerae is the virulent variant of an organism
commonly found in the intestines of people living under unsanitary conditions.
Taylor and Ahuja (118) examined 91 water sources consisting of shallow wells, tanks,
rivers and concluded that vibrios were almost universally present in such unprotected
places in areas where cholera was not endemic and their presence was not related to
contamination from cholera sources. The vibrios isolated were of heterogeneous
types as determined by their biochemical and serological reactions but none showed
O-serological relationship with the cholera vibrio. Linton et al. (74) in 1938
reported that the strains obtained from the early part of the epidemic contain a
lipoid-polysaccharide complex which was absent in organisms isolated from the
latter part of the epidemic, from carriers, wells and from those maintained for a
long time in the laboratory.

In 1938, Pasricha et al. (96) examined 300 samples of Calcutta waters, 640
flies and 94 cockroaches from the same region. They could isolate vibrios from
all samples of river water, 86 per cent of tank waters, 19 per cent of flies and
17 per cent of cockroaches examined. The vibrios isolated from these sources
resembled morphologically and in their main biochemical reactions the cholera vibrio

except that they were inagglutinable with O-serum and only 7 per cent showed H-antigenic relationship with V.cholerae. Lal, Ghosal and Mukherjee (66) in 1939, bred houseflies in the laboratory under aseptic conditions and fed the insects on 'phage free vibrios of known characters. After such series of passages thru same strains of flies, they observed changes in vibrios from Linton's chemical group I to group V in one case, and from groups III and V to group I in two cases. Changes in serological reactions never occurred but in one instance a transient shift in the fermentation reactions from Heiberg's group II to group I was observed.

In 1939, Tomb (121) reported that 30 per cent of the inhabitants of the mining settlement in Bengal were permanent carriers of non-agglutinating vibrios and he believes that these constituted the reservoir of endemic cholera cases in that region. Marras (80) was able to isolate El-Tor vibrios from healthy Mohamedan pilgrims during the years 1936 to 1939, Read and Pandit (101) studied the distribution of the agglutinable vibrios in the general population and in water sources in a rural endemic area of Bengal. V.cholerae was isolated from the stools of 96 per cent of the early clinical cases, 7 per cent of close contacts and 16 per cent of water sources exposed to the dejecta of patients. In the absence of the disease the organisms were never isolated from the stools and only once or twice from water sources. The El-Tor vibrio was similarly absent from the stools but was readily obtained from 3 per cent of surrounding waters. Venkatraman et al. (122) in 1941, examining large specimens of water from 237 open natural water sources in Cauvery Delta, could isolate agglutinating vibrios on 21 occasions in the course of 878 examinations; 19 of the vibrios isolated were hemolytic to the goat erythrocytes and belonged to the El-Tor group, while the remaining two were indistinguishable from V.cholerae. They claim that the region was free from cholera throughout the period of investigation and for several months previously.

That the problem of epidemiology of cholera is not yet solved is emphasized by Seal (106) in 1945. Seal argues that if cholera vibrio could bot be isolated

from stools of the general population or from water sources in an endemic area, that cases and "acute carriers" are responsible for the dessimination of the disease and these only for short periods and ranges, then, where and how does the cholera vibrio exist before a case occurs in an endemic area?

In view of such a conflicting literature, a review of the vibrionic variability seemed indicated. The main purpose of the present research has been to study correlative changes in the cholera group of organisms and interpret the results of such alterations in the light of the epidemiological significance of the disease. Having no access to a choleraic region, all the work was carried on in the laboratory and thus, the conclusions reached in some passages are nothing more than logical deductions based on vitro or vivo experiments.

METHODS AND MATERIALS

The phenomenon of variation in cultures of cholera vibrios was studied as it occurred:

I.- Normally on ordinary culture media,

II.- As a result of subjecting vibrios to the action of specific 'phage or

III.- As a result of treating with homologous immune bodies. The last problem was attacked:

A.- In vitro. By subjecting smooth vibrios to the action of immune serum in presence of complement and,

B.- In vivo. By bringing them in contact with tissues of immune animals. Finally, the immunological relationship of the variants was studied by:

IV.- Cross protection tests on animals.

At the start of the experiments described herein, two very recent epidemic strains of V.cholerae were received; one from Egypt, isolated from an epidemic that started at El-Korein in 1947 and the other from a monor epidemic in the region of Damascus (Syria) in the same year. Both strains were obtained thru the kindness of Public Health Service of Syria in Damascus. The Egyptian strain will be referred to as El-Korein strain and the Syrian as M-4 strain. Ogawa strains PA-550, D-643, E-521, C-487, D-492, C-601 and A-510 were isolated from stools in Calcutta and received in 1945, while Ogawa stock strain, Ogawa E-593, E-569, Inaba stock strain, Inaba E-644, Hooghly River strain and Hikojima stock strains arrived in the same year, yet no information as to their source or date of isolation was available. Inaho-Haffkins 168 and Jenkins strains were obtained by the A.U.B. Laboratories in 1938 and 1933 respectively.

I.- Normal Variations in Cholera Vibrios on Laboratory Media.

It is a known fact that V.cholerae, like the Salmonellas, by passing for a prolonged period of time on artificial media, under goes variation which effects to a greater or a less extend its species characteristics. Such variants when

detected during research have been reported as "rough", "inagglutinable" or
"atypical", but no systematic study, as to the nature of such variations has been
carried on as yet; and it is unfortunate that most of the work on the fermentation
reactions and serological grouping of vibrios has been carried on stock cultures
(i.e. Heiberg 1934, Gardner and Venkatraman 1934, Burrows et al 1946). Taking
advantage of the acquisition of two recent epidemic strains, El-Korein and M-4,
a comparative study was carried on, their biochemical behaviour, serological
reactions and virulence, of together with the stock strains already mentioned.
The two epidemic strains were studied as soon as they were received and again a
year later; passages were made on meat extract agar at weekly intervals. The
results of such studies are shown on Tables I_a, I_b, II, and III.

For fermentation reactions meat extract broth containing 1 per cent sugar
was used with phenol red as indicator. The pH of the medium was adjusted to 7.8
and sterilised fractionally.

Brom-cresol-purple milk was prepared, first by removing the cream from fresh
milk and then adding to one liter of the fat-free portion 40 cc of a 0.04 per cent
aqueous solution of brom-cresol-purple.

For the preparation of blood agar plates, the agar was melted and cooled down
to a temperature of $45^{\circ}C$.; next 10 per cent of sterile defibrinated rabbit's blood
was added and the mixture poured into sterile Petri dishes.

Voges-Proskauer reaction (117) was run on M.R.-V.P. medium. To 800 cc of
distilled water, 5 gm. of Proteose-peptone (Difco.), 5 gm. of glucose and 5 gm.
of dipotassium hydrogen phosphate were added. The mixture was heated over steam
for 20 min. with occasional stirring, filtered thru paper, cooled down to room
temperature, the volume brought up to one liter with distilled water and sterilised
by fractional method. The test was performed by adding 5 cc. of 10 per cent KOH
to 5 cc. of six days old culture of organisms and the mixture was aerated at
intervals of 30 min. by shaking. The test was considered positive by the

Strain	Time in hours	Fermentation Reactions					Milk	Liquef. Gelatin	Liquef. Loeffler's	Cholera Red		Hemo-digestion	V-P Test	Greig Test	Millon's Test	Agglut. Titer O-serum	Virulence to Mouse Killing Time
		Lactose	Sucrose	Dextrose	Mannite	Arabin.				Peptone-Nitrate	Trypto-Nitrate						
El-korein (Egypt)	18	−	+	+	+	−	−	−	±	−	++	+	−	−	S	1:1280	+ 8 hours
	36	+	+	+	+	−	A	−	+	−	++						
	60	+	+	+	+	−	AC	±	+	−	+++						
	96	+	+	+	+	−	AC	+	++	−	+++						
M-4 (Damascus)	18	−	+	+	+	−	−	−	±	−	+	+	−	−	S	1:640	+ 18 hours
	36	+	+	+	+	−	A	±	+	−	+						
	60	+	+	+	+	−	AC	+	++	−	++						
	96	+	+	+	+	−	AC	++	+++	−	++						

Table I_a. A study of differential characters of the El-korein and M-4 epidemic strains soon after their isolation.

Strain	Time in hours	Fermentation Reactions					Milk	Liquef. Gelatin	Liquef. Loeffler's	Cholera Red		Hemo-digestion	V-P Test	Greig Test	Millon's Test	Agglut. Titer O-serum	Virulence to Mouse Killing Time
		Lactose	Sucrose	Dextrose	Mannite	Arabin.				Peptone-Nitrate	Trypto-Nitrate						
...korein (Egypt)	18	−	+	+	+	−	−	−	+	−	±	+	−	−	S	1:640	+ 18 hours
	36	+	+	+	+	−	Sl. A	+	+	−	++						
	60	+ −	+	+	+	−	A	+	+	−	++						
	96	+ −	+	+	+ −	−	A	+	+	−	+++						
M-4 (Damascus)	18	−	+	+	+	−	−	−	±	−	±	+	−	−	S	1:320	+ 28 hours
	36	± −	+	+	+	−	−	+	+	−	+						
	60	+ −	+	+	+	−	Sl. A	++	++	−	++						
	96	+ −	+	+	+	−	A	++	+++	−	++						

Table I_b. A study of differential characters of the El-korein and M-4 epidemic strains one year after their isolation

+ − , Alkaline, starting from surface of medium and down.

± − , Alkaline on surface after only a slight acidity in the medium.

Strain	Time in hours	Fermentation Reactions					Milk	Liquef. Gelatin	Liquef. Loeffler's	Cholera Red Tryp-Nitr.	Hemo-digestion	Greig test	V-P test	Millon's test	Agglutination vs. O-serum	Virulence to Mouse Killing Time
		Lactose	Sucrose	Dextrose	Mannite	Arabin.										
Ogawa D-643	24	±	+	+	+	−	−	−	±	+	+	−	−	S	1:640	+ < 24 hours
	72	+	+	+	+	−	−	+	+	+++						
Ogawa C-487	24	±	+	+	±	−	−	−	±	±	+	−	−	S	1:320	+ < 24 hours
	72	+(−)	+	+	+−	A	++	+	++							
Ogawa C-601	24	±	+	+	+	−	−	−	±	+	+	−	−	S	1:320	+ < 24 hours
	72	+(−)	+	+	+	−	A	++	+++	+++						
Ogawa A-510	24	−	+	+	+	±	−	−	−	+	+	++	−	R	+ (titer not deter.)	+ < 24 hours
	72	+−	+	+	+(−)	alkal.	−	+	+	+++						
Ogawa E-593	24	−	±	+	±	−	−	+	+	+	+	−	−	R	1:20	+ < 24 hours
	72	±−	+	+	+−	−	− Coagul.	++	+++	+++						
Ogawa E-569	24	±	+	+	+	±	−	−	−	++	+	−	−	S	+ (titer not deter.)	+ 24 hours
	72	+ (−)	+	+	+	alkal.	−	++	+	++						
Inaba Stock	24		+	+	+	−	−	+	±	+	+	−	−	S	+ (titer not deter.)	+ 24 hours
	72	+(−)	+	+	+	−	−	++	++	++						
Hikojima Stock	24	±	+	+	+	−	−	±	±	+	+	−	−	S	1:640	+ 24 hours
	72	+ (−)	+	+	+	−	−	+	++	+++						

+(−) = less so than +
(See Table I b)

Table II. A study of differential characters of mouse-virulent V. cholerae stock strains

Strain	Time in hours	Fermentation Reactions					Milk	Liquef. Gelatin	Liquef. Loeffler's	Cholera Red Tryp-Nitr.	Hemo-digestion	Greig test	V-P test	Millon's test	Slide Agglutination vs. O-serum	Virulence to Mouse Killing Time
		Lactose	Sucrose	Dextrose	Mannite	Arabin.										
Ogawa Stock	24	−	+	+	+	−	−	−	±	−	+	−	−	S	+	O
	72	+−	+	+.	+−	−	−	++	+	±						
Ogawa D-556	24	−	+	+	+	−	−	±	−	−	+	−	−	S	+	O
	72	+−	+	+	+	−	−	++	+	++						
Ogawa E-521	24	−	+	+	+	±	−	−	±	−	+	−	−	S	+	O
	72	+−	+	+	+	alkal.	−	++	+	++						
Ogawa D-492	24	−	+	+	+	−	−	−	±	−	+	−	−	S	+	O
	72	+−	+	+	+	−	−	++	+	+						
Hooghly River	24	±	+	+	+	−	−	−	±	−	+	−	−	S	+	+ 36 hours
	72	+−	+	+	+	−	−	++	+	+++						
Inaba E-644	24	±	+	+	+·	−	−	−	±	−	+	−	−	S	+	+ 36 hours
	72	+−	+	+	+	−	−	++	+	+						
Jenkins	24	±	+	+	+	±	−	−	−	++	+	+	−	R	+	+ 36 hours
	72	alkal.	+	+	+−	alkal.	−	+	+	+++						
Inabo-Haffkins 168- Baghdad-508	24	−	+	+	+	−	−	−	−	+	+	−	−	R	+	O
	72	alkal.	+	+	+−	−	−	++	−	+++						

Table III. A study of differential characters of mouse-non-virulent
<u>V.cholerae</u> Stock strains

development of a eosin-pink color in 2, 12 or 24 hours.

Alkaline peptone water was prepared by adding 1 gm. of Bacto-peptone (granular) to 100 cc. of distilled water. The pH was adjusted to 8.4.

Peptone-nitrate medium was prepared by adding 200 mgm. of potassium nitrate to one liter of 1 per cent Bacto-peptone (granular) in alkaline distilled water. Such a medium after several trials proved to be unsatisfactory for "cholera-red" tests and was replaced by Tryptone nitrate broth in which Bacto-tryptone was used instead Bacto-peptone, keeping the amount of potassium nitrate the same. The latter medium proved to be very satisfactory and gave uniform results upon many trials.

"Cholera-red" test was performed by adding to seperate protions of 24 and 72 hours cultures of vibrios in tryptone-nitrate-broth few drops of concentrated sulfuric acid. A positive test was indicated by the presence of a pinkish-red color and was recorded as +, ++, +++ and ++++ according to the intensity of the color developed.

The Greig test was performed according to the method described by Gardner and Venkatraman (35). One cc. of a three days old broth culture of vibrios was added to one cc. of a 5 per cent suspension of washed goat erythrocytes, mixture was incubated at 37°C. for two hours and kept overnight in cold before reading. (See also Greig (46), (47), Read et al. (102).

For Millon's test the method followed was that described by Asheshov et al.(6) with a slight modification. One part of metallic mercury was added to two parts of 36°B nitric acid in a fume chamber. The liquid was diluted with two parts of water and the reagent was aerated for 24 hours in a large vessel until almost not trace of smell was left. A loopful of 24 hours growth of vibrios on agar was emulsified in two cc. of tap water, 0.2 cc. of the reagent was added and the tubes were placed in boiling water for five minutes. The results were recorded as S,

when the suspension remained turbid showing no flocculation, R when the flocculation was complete leaving the supernatent fluid clear, the intermediate results were recorded as S-R.

Virulence tests were carried out with mice using either a saline suspension from 24 hours growth of vibrios on agar containing one billion organisms, or a suspension from a same age culture in 5 per cent mucin containing eight million organisms. ‡ In both cases the dose was suspended in 0.5 cc. of the fluid and injected intraperitoneally. A strain was considered as virulent when it killed the animal under the test conditions in less than 24 hours (50), (98), (99).

O-agglutinating serum was prepared by injecting young rabbits intravenously with Inaba, Ogawa and Hikojima strains after heating the suspensions for two and a half hours in boiling water to destroy the H-portion of the vibrios. All the strains used were virulent to mice and smooth as tested by Millon's reagent. Two injections were given at weekly intervals, the first containing 4 billion and the second 8 billion organisms, making a total of 12 billion organisms.

Agglutination tests were performed by tube dilution and slide agglutination methods, using in both cases the same O-serum prepared on the rabbit. In the first the technic used was essentially the same as in Widal test. In running a slide agglutination test, the following procedure was adopted. A drop of a proper dilution of O-serum was placed on a depression slide and a bit of 24 hours growth of vibrios to be tested was carefully emulsified in it by means of a needle or a small size loop. The slide was left in an incubator for about five minutes and next a small drop of Löeffler's methylene blue was added to the serum-organism mixture, and left for another few minutes at room temperature . In presence of

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‡ The shreded mucin received from the Hebrew University in Jerusalem, Palestine, did not prove to be satisfactory in its property of protecting the vibrios to the extend claimed by Ranta and Dolman (loc.cit.). After several trials it was found that the number of organisms constituting the minimum lethal dose for mice when suspended in 5 per cent mucin we possessed, was eight million and not one million as found by these authors.

agglutination the clumps attained a blue color well visible at the margins of the depression, while a homogeneous light blue color was maintained in suspensions showing no such clumping. This method proved to be of great practical value specially in cases where clumps formed were too small to be visible by ordinary slide agglutination tests.

Analysis of Results.

The freshly isolated epidemic strains (El-Korein and M-4) were composed mainly of curved rods when preparations stained with Gram's method were examined. Straight forms were occasionally met, while filamentous or coccus forms were absent (111). Such forms, however, were quite abundant in the stock strains and further-more, the two epidemic strains after one year of transfer on artificial media, showed similar changes in shape and size quite comparable with those shown by stock strains in our possession, kept on same media for a prolonged period of time (7). Typical morphology of vibrios was restored when organisms were passed several times thru mice. It was interesting to note that in the tissues of the mouse the vibrios in most of the cases were endowed with a well developed capsule. The organisms in smooth stock cultures were in general, as motile as those in two epidemic strains, but those in rough stock cultures, were much less so probably due to clumping of vibrios during growth.

In all cases the colonies of epidemic strains and smooth stock strains on blood or agar plates were identical, while those of rough strains corresponded to classical description given for the Salmonellas. In no instance were "rugose" races met with and no such dramatic spontaneous colonial dissociation occurred in the cultures all thru the experimentation.

It is apparent that a certain amount of weakening in fermentation reactions do occur among cholera vibrios which have been passed for a long time on artificial culture media. A phenomenon well marked in the fermentative abilities of vibrios and to which curiously no attention has been directed by previous workers, except

probably by Panja (93), is the initial slight acidity formed from the medium by
the vibrios followed by an alkaline reaction starting from the surface of the
medium and down. The phenomenon is exhibited by the vibrios with weak fermentative
abilities and is more pronounced when meat infusion instead of peptone water is
used as a nutrient in the medium. The latent alkalinity is absent among strains
that have active saccharolytic properties, thus initiating extensive acid formation
with resulting sterility of the medium. However, those races with weak saccharoly-
tic properties bring about only a slight change in the pH of the medium that seems
not to interfere seriously with the multiplication of the organisms, which in turn,
attack the available proteins and produce alkaline products starting from the
surface of the medium where the free molecular oxygen is most abundant. This
reaction is well shown in lactose broth medium and less so with strains of very
poor fermentative activities, in mannite broth. That such changes are brought
about by vibrios which have lost partially their saccharolytic properties thru
passage on artificial medium for a long period of time, is shown by the fact that,
while the two epidemic strains early after their isolation fermented lactose
rendering the medium completely sterile, their ability to attack this sugar after
a year of cultivation on artificial medium was partially lost as shown by the
appearance of latent alkalinity on the surface of the medium. Furthermore, the
same reaction is manifested by all stock cultures which were still virulent to
mouse and with more intensity by those that had lost their virulence to this animal.
Thus, we can assume that probably all of these strains in their original form were
lactose fermenters. The same phenomenon observed in connection with lactose broth
was true to a less extend with mannite broth fermentation tubes (see Tables I_a , I_b,
II, III).

Dextrose and sucrose were fermented uniformly by all the strains and the
medium was rendered sterile at the end of 36 hours of incubation. Arabinose was
not fermented by any of the strains, although a slight lowering of pH in the medium

was observed on several occasions followed by alkalinity.

Both of our epidemic strains in their initial states produced acidity and coagulation in milk and these properties were found to be absent, at least partially, after a year of transfer on artificial media as in most of the stock strains. Such changes brought about in milk were, however, manifested with such irregularity, even by the same strain at different periods, that any possible conclusion on this line would be unwarrented. It seems probable that, excluding the ensymic action manifested by certain strains regardless to their fermentative abilities, the ability of a strain of V.cholerae to ferment lactose runs parallel with such changes occurring in milk.

Gelatin and Löeffler's serum medium were liquefied with more or less ease by all of our strains both epidemic and stock, except by two; the Jenkins and the Inaho-Haffkins 168, which were both Millon's positive. However, that Millon's positive strains are not necessarily devoid of liquefactive properties is shown by the fact that two Millon's positive strains, A-510 and E-593, were able to liquefy these media as readily as the Millon's negative strains.

Some irregularities, as in liquefaction of gelatin and Löeffler's serum medium, were encountered while dealing with "cholera-red" tests. An examination of Tables Ia, Ib, II and III might give the general impression that there is a weakening in the intensity of nitroso-indole reaction among old, degenerate and avirulent cultures, yet such a conclusions seems to be unduly warranted if one considers the reactions given by the two Millon's positive, avirulent strains - the Jenkins and the Inaho-Haffkins 168- which showed early and strongly positive "cholera-red" tests.

All strains have shown positive hemodigestion when streaked over blood agar plates, containing 10 per cent sterile defibrinated rabbit's blood. The Greig test has been negative in all strains except two - Jenkins and A-510. It is interesting to note that both of these strains have shown weak fermentation reactions, poor activity on milk, gelatin and Löeffler's coagulated serum and are marked by

strongly positive nitroso-indole reactions; both are rough as tested by Millon's reagent yet the first is avirulent while the second is virulent as tested on mice. A third strain - Inaho-Haffkins 168- exhibiting similar reactions as Jenkins and equally avirulent, was Greig negative; and a fourth strain - E-593- although differing in certain biochemical reactions from the first three, was rough, Greig negative, but highly virulent to mouse. Thus it seems reasonable to assume that virulence of a strain as tested on mice is independent of its physiological activities as manifested on artificial culture media. No difficulty was encountered in measuring the virulence of an organism as tested on mouse, by considering the length of its killing time. Thus, by subsequent mouse passages, keeping the dosage and the mode of injection the same in all cases, the killing time of a strain was brought down from 36 hours to 8 hours. Associated with such an extensive increase in virulence there has been only a slight enhancement in other physiological behaviours of the vibrios. Whether a mouse virulent strain is a true virulent V.cholerae as tested on human beings, is a problem that has to be answered. However, it must be confessed that a great diversity in susceptibility among different species of animals in response to injections of same strain of cholera vibrios was found and it can be assumed that among such group differences the human species would be included too. Finally, the fact that two agglutinable strains, Jenkins and A-510, though V-P negative, attained hemolytic properties as tested by Greig's method, suggests a close genetic relationship between a true V.cholerae and the El-Tor vibrio. Further discussion on this phase of variation will be indicated later in this paper.

If the possibility of a decrease in the agglutinin titer of the specific O-serum is excluded, then a reduced titer in the serum used would be entirely due to the type of antigen used in the test. Considering the fact that when such a serum tested with mouse passaged strains of El-Korein and M-4 showed titers identical to those obtained when epidemic strains in their original forms were

used instead, then the reduced titers obtained in tests when same races of organisms were used after one year of cultivation on artificial media, would indicate certain alterations or loss in the antigenic components of the cells (17).

II. Variations in Cholera Vibrios Acquired Thru 'Phage Action.

Cholera 'phage was received from Institut Pasteur, thru Faculté Française de Medicine in Beirut. No information was available about its type nor virulence; however, a preliminary experiment revealed that the strain was "omnivirulent" and was capable of lysing almost all of the strains of cholera vibrios both smooth and rough already described. Thus when 0.01 cc. of the 'phage suspension was added to each of 24 broth cultures of V.cholerae containing 250 million organisms per cc. and incubated at 37°C., there was complete clearing of the suspension at the end of 8-10 hours and no resistant or secondary races appeared upon further incubation.

There was some difficulty in getting races that were resistant to the "omni-virulent" 'phage. As d'Herelle suggests (57), V.cholerae having an optimum temperature of 36°C. and an optimum pH of 8.4 for its growth, it was not possible to obtain resistant races by varying these factors which are the same for the development of 'phage particles also. However, by weakening the virulence of the 'phage, i.e. leaving it in contact with a pooled, heavy suspension of V.cholerae for a variable length of time (5) and gradually raising its virulence by bringing it in contact with organisms which had developed a resistance comparable to its virulence and filtering thru bacteriological filters as soon as lysis had occurred, it was possible to develop races of V.cholerae which were completely refractory to the action of the original "omnivirulent" 'phage. In no instance was it intended to produce resistant strains against A, B or C 'phage seperately and no attention was paid as to whether the strains of V.cholerae at hand were lysogenic or not.

To increase the comparative scale of the studies, mouse virulent strains of V.cholerae, (see Table II) namely, D-643, C-487, C-601, A-510, E-593, E-569,

Inaba Stock, Hikojima Stock, El-Korein and M-4 were selected. Transfers of these organisms were made in peptone water and to 18 hours growth, 0.01 cc. of the "omnivirulent 'phage was added. All flasks, containing organism - 'phage mixture were incubated at 37°C. At the end of 10 hours complete lysis was observed in all except in the culture flask containing strain A-510, and no secondary growth appeared in lysed cultures upon further incubation. At the end of 48 hours more of the organisms were added to the lysed cultures and the flasks again placed in the incubator. This process was repeated until the medium, after an initial clearance, developed secondary cultures as indicated by the turbidity of the medium. Immediate transfers were made from the new growth on fresh peptone water and rest of the culture passed thru bacteriological filters. To 18 hours growth of new races, few drops of the filtered 'phage was added, increasing the amount if necessary, until complete lysis of organisms was brought about, followed by secondary growth; after which, subcultures were again made and the contents of the flasks filtered and used for lysis of new races obtained from the second transfer. This process was continued for 8-10 times, until the races obtained on the last transfer were completely refractory to the original omnivirulent 'phage.

To check the actual acquired resistance of such refractory races, a loopful of 18 hours broth culture of original and resistant strains, was added to corresponding tubes each containing one cc. of peptone water. Next a loopful of the "omnivirulent" 'phage was added equally to all the tubes and the mixture incubated at 37°C. Control tubes containing peptone water and inoculated with individual strains from both series were kept. At the end of 24 hours of incubation, the tubes containing resistant races in presence of 'phage showed growth quite comparable with the controls, while those containing the original strains were clear and remained so upon further incubation.

It was not found possible to precipitate dramatic changes in the strains studied as claimed by Morison (86) and Pasricha et al.(95). While variations induced in

physiological activities and serological behaviour of these organisms were
relatively trivial, yet, they do explain a number of related phenomena, manifested
as a result of alterations in the bacterial cell brought about under certain
environmental conditions. The results of such studies are shown on Table IV.

Analysis of Results.

Examination of the findings on Table IV, shows that the 'phage variants of
cholera vibrios studied can be divided into three groups: (a) strains D-643, C-487,
C-601, Hikojima stock and El-Korein, that have developed resistance to the
"omnivirulent" 'phage, but still have kept their original smooth characters, (b)
strain E-593, which, by becoming resistant to the action of 'phage has lost its
rough character and turned out to a smooth form and (c) strain M-4, while smooth
originally, by gaining resistance against 'phage has been rendered rough as judged
by Millon's test and agglutination reactions. It is very probable that smooth-
rough variations in the last two groups are due to the dissolution of the smooth
or rough elements by specific 'phage action, but it is also true that, all the
strains subjected to the action of such 'phages, regardless of their final dissoci-
ative changes, have developed a certain degree of resistance to the action of the
"omnivirulent" 'phage, in presence of which, in their original state, they were
readily dissolved.

No appreciable difference in motility could be detected among individual
races of each of these groups when compared with those of the original strains,
except perhaps strain E-593, which after turning smooth was more typically motile
than the original rough form.

There was much pleomorphism present in almost all of the variants tested.
The tendency of organisms was to lose their typical vibrio forms and attain
straight, spiral cocco-bacillary and filamentous forms. Such changes in mor-
phology were more marked in old agar cultures. The M-4 'phage variant was devoid
of spiral or cocco-bacillary forms but was composed mainly of thick straight rods
a characteristic of rough strains to be discussed later in this paper.

| Strain | Time in hours | Fermentation Reactions | | | | | Milk | Liquef. Gelatin | Liquef. Loeffler's | Cholera Red Tryp. Nitr | Hemo-digestion | Greig test | V-P test | Millon's test | Agglut. titer O-Serum | Virulence to mouse killing Time |
		Lactose	Sucrose	Dextrose	Mannite	Arabin.										
Ogawa D-643	24	−	+	+	+	−	−	−	−	±	+	−	−	S	1:1280	0
	72	alkal.	+	+	+	−	−	+	+	++						
Ogawa C-487	24	−	+	+	−	−	−	−	+	±	+	−	−	S	1:1280	0
	72	+ −	+	+	+ −	−	A	−	++	+						
Ogawa C-601	24	−	+	+	+	−	−	+	−	±	+	−	−	S	1:640	0
	72	+ −	+	+	+	−	A	++	+	++						
Ogawa E-583	24	±	+	+	+	−	−	+	−	±	+	−	−	S	1:320	0·
	72	+ −	+	+	+	−	A	+++	+	++						
Hikojima Stock	24	±	+	+	+	−	−	−	+	±	+	−	−	S	1:2560	+ 24 hours
	72	+ −	+	+	+	−	A	+	+++	+						
El-Korein (epidemic)	24	±	+	+	+	−	A	−	−	+	+	−	−	S	1:1280	+ 24 hours
	72	+ −	+ ·	+	+	−	A	+	+	+						
M-4 (epidemic)	24	±	+	+	+	−	Sl. A	−	±	±	+	−	−	R	1:40	0
	72	+ −	+	+	+ (−)	±	A	+	++	+						

Table II. A study of differential characters of the 'phage treated Variants of <u>V.cholerae</u> stock and epidemic strains

Considering the fermentation reactions, changes, as compared with their original activities, seem to be trivial in group (a) organisms. However, it can be clearly seen that conversion from rough to smooth form of strain E-593, has brought about a marked increase in fermentative abilities, specially in the ability to ferment lactose and mannite. It is interesting to note that during the course of experimentation, a degenerate race of this strain was obtained, which was extremely rough, inagglutinable and was unable to ferment lactose and mannite, but fermented sucrose and dextrose to a certain extend followed by alkalinity. That a smooth strain after reversion into rough form loses some of its fermentative abilities is once again shown in strain M-4; the sugars that escaped mostly the enzymic attack of such degenerate races, were lactose and mannite.

The only appreciable change in milk has been brought about by the smooth 'phage variant of the originally rough strain E-593. The fact that milk was clotted with no acid formation by the rough original form and was acidified without clotting when inoculated with the same organism in its smooth variant form, clearly indicates that, besides acid clotting, an ability possessed by smooth organisms with high fermentative capacities, the vibrios are endowed with certain enzymic activities that are capable of bringing about similar changes in the milk independent of, and not associated with such variations. Probably the irregularities encountered with liquefaction of gelatin and Löeffler's serum medium when inoculated with cholera vibrios in their different antigenic phases and the non-correlative changes manifested in the nitroso-indole reactions under the same conditions, can be attributed to similar factors in the organisms as defined for milk.

In no instance any change was observed in blood agar hemolyses nor in the Greig tests. The results obtained under both these tests were quite comparable with those shown by the original strains.

The agglutination tests closely define the changes brought about in the antigenic structure of the variants. While comparing the group (a) resistant smooth races with the corresponding original strains, a definite rise in the agglutination

titer was found when tested on the same O typeI serum. Presumably, such a gain
in titer was due to lysis of the rough portion in the stock strains by the
corresponding 'phage races, resulting in a new highly smooth race that was quite
comparable in its antigenic structure with those of the mouse passaged forms,
against which the anti-serum was prepared originally. Thus, the titer obtained
with the 'phage variant of El-Korein was double that of the stock strain when
tested on the same O-serum and quite comparable in this aspect with the titer
obtained in the original epidemic race. Again, while there is a gain in titer
as strain E-593 is converted into its smooth form, the loss is apparent in strain
M-4 which has undergone a variation in the reverse direction.

That virulence of cholera vibrios as tested on mice, might be an independent
factor not related to any such dissociative changes, was pointed out in the
previous discussion. While some of the strains in group (a) have maintained their
mouse killing capacity, others in the same group have lost it completely; further-
more, curiously enough, strain E-593, after attaining smooth characters becomes
completely avirulent to mouse and strain M-4, originally smooth and virulent,
loses the latter property upon roughening. In 1932, Linton (70) commenting on
the possible relationship between virulence and carbohydrate content of vibrios
states the following possibilities: (1) carbohydrates are present in virulent
organisms and absent in non-virulent ones, (2) virulence exists as an independent
factor not related to the carbohydrate content of vibrios but quite related to a
protein like constituent of the cell similar perhaps to the M-factor of virulent
hemolytic streptococci described by Lancefield (69) or (3) that this property is
not related to any particular cellular constutient, but is a function or result
of the activity of the cell as a whole.

If one considers that smooth or rough forms of cholera vibrios, in one
phase or the other of their life cycle, might be both virulent or avirulent to
mouse, as shown clearly in the present experiments, then the first two assumptions

forwarded by Linton should collapse and the only hypothesis which might still hold true would be the third. It seems probable that choleraphage plays an important role in such fluctuations of virulence without interfering seriously at times in other phases of vibrionic activities, but as to what the exact nature of such changes is, is a question that has to be answered.

III. Variability of Cholera Vibrios in Presence of Immune Bodies.

Attempts to produce variants of V.cholerae by exposing cultures to the action of immune bodies were directed along two main lines: (A)- exposure of cultures to the action of a homologous anti-serum in vitro in the presence of complement and (B)- passing organisms thru the peritoneal cavity of guinea pigs previously immunised with killed vaccines.

A. Variants Produced in Vitro Under the Influence of Anti-Cholera Serum.

The method followed in dealing with the first part of the experiments, was essentially the same as described by White (127). Strains of V.cholerae, D-643, C-487, C-601, E-593, Hikojima stock, El-Korein and M-4, were transferred on agar slants and incubated at 37°C. for 48 hours, after which the growth was washed off with alkaline peptone water and the concentration of the vibrios brought down to one billion organisms per cc. Pooled complement was obtained from several well nourished guinea pigs, showing a titer of 1:30 when tested with the hemolytic system of cells. The anti-serum was the same as the one used in running agglutination tests, prepared in the rabbit against smooth mouse passaged strains of Ogawa, Inaba and Hikojima and was free from any preservative.

Doubling dilutions of the antiserum, ranging from 1:10 to 1:320, were made and 0.5 cc. of each dilution was added to a corresponding small tube. Next, 0.5 cc. of vibrios suspended in peptone water and 0.5 cc. of the pooled guinea pig serum diluted to 1:15 in peptone water were added to each of the tubes uniformly. The mixture was then incubated at 37°C. for about 8 hours. At the end of this period, all the strains, except E-593, showed an almost clear supernatent fluid and a

heavy sediment in the first three dilutions while a turbidity was marked in the fourth tube and was more accentuated in rest of the tubes, where the serum dilutions were higher

Preliminary observations revealed that dissociation obtained among the vibrios treated by this method was only slight, so the process was repeated using the anti-serum in its first three dilutions and a complement four times as strong as the one used in the first trial. Although the races obtained in this second experiment possessed some of the characteristics of rough vibrios, yet a complete dissociation into rough forms was not attained until a method essentially the same as described by Goyle (43) in 1932 was adopted. The races obtained from the second experiment were grown on peptone water containing 10 per cent fresh serum from an immune rabbit which still retained a large portion of its complement and transfers made from corresponding tubes on similar media at intervals of 8 hours, taking the inoculum from the surface of the medium where organisms resistant to the lytic action of the serum were more often found. After 6-7 such transfers, the susceptible smooth vibrios disappeared from the medium, leaving behind rough races which were completely refractory to the action of smooth O-serum and grew well on the surface of peptone tubes. These new rough races were carried on agar slants and studied. The results are shown on Table V.

Analysis of Results.

Table V is self explanatory. It can be seen clearly that all of the originally smooth strains have turned out to be rough, as judged by Millon's test and simultan-eous with this change the fermentative abilities have been distinctly weakened as manifested by a marked loss in the ability to attack lactose and mannite. Such variations, however, have not affected in any way the Voges-Proskauer reaction and blood agar hemolysis; while the ability to liquefy gelatin is increased, that of producing nitroso-indole reaction is weakened to a certain extend. The most signif-icant changes are brought about in the agglutinating titers, which are markedly

		Fermentation Reactions					Milk	Liquef. Gelatin	Liquef. Loeffler's	Cholera Red Tryp-Nitr.	Hemo-digestion	Greig test	V-P test	Millon's test	Agglutin. titer O serum	Virulence to Mouse killing time
Strain	Time in hours	Lactose	Sucrose	Dextrose	Mannite	Arabin.										
Ogawa D-643	24	−	+	+	+	−	−	+	±	+	+	−	−	R	1:40	0
	72	± −	+	+	+ −	−	−	+++	++	++						
Ogawa C-487	24	−	+	+	+	−	−	+	+	−	+	−	−	R	1:20	0
	72	± −	+	+	+ −	−	−	+++	++	±						
Ogawa C-601	24	−	+	+	+	−	−	+	−	+	+	−	−	R	1:20	0
	72	± −	+	+	+ −	−	−	+++	+	++						
Ogawa E-593	24	−	+	+	+	−	−	+	+	+	+	−	−	R	1:20	+ < 24 hours
	72	± −	+ −	+	+ −	−	−C̄	+++	++	++						
Hikojima stock	24	−	+	+	+	−	−	+	±	−	+	−	−	R	1:20	0
	72	± −	+	+	+ −	−	−	++	+	±						
El. Korein (epidemic)	24	−	+	+	+	−	−	+	+	±	+	−	−	R	1:20	0
	72	± −	+	+	+ −	−	−	+++	++	+						
M-4 (epidemic)	24	−	+	+	+	−	−	+	+	±	+	−	−	R	1:10	0
	72	± −	+	+	+ −	−	−	++	++	+						

Table V. A study of differential characters of races of _V. cholerae_ developed in vitro in presence of immune bodies

reduced as tested against smooth antiserum, and in the virulence of the organism to mouse which is completely lost. In short the variants thus produced, present all the characteristics of a strain undergoing S-R dissociation as observed in Salmonellas.

Strain E-593, while originally rough, was rendered more so after treatment with smooth antiserum, possibly losing the small portion of smooth elements in its possession thru lysis as suggested by its further weakened ability to attack sugars and by a positive Millon's test. Curiously enough, in spite of all these degenerative changes the new variant kept its mouse killing ability and after several passages thru this animal, was still holding its original rough character, although after such passages saccharolytic properties were regained and was better agglutinable than the original rough race.

B.- Variants Produced in Vivo in Tissues of Immune Mice.

After producing variants in vitro, it was deemed of interest to determine whether similar changes in vibrios would occur if they were brought in contact with tissues of mice previously immunised with a smooth vaccine strain. The El-Korein strain, after six passages thru mice, was seeded on meat extract agar, the growth collected in physiological salt solution, heated in boiling water for 2½ hours to destroy the "H" portion of the organisms, phenol was added as preservative and the concentration of the suspension was brought down to two billion organisms per cc. To immunise mice, a total of 3 billion organisms were injected intraperitoneally, in 0.5 cc. and 1 cc. amounts, leaving an interval of 10 days between the first and the second injections.

While death, among some of the test animals, occurred 10-12 hours after the first injection due to unknown reasons, a curious anaphylactoid type of a reaction developed invariably in all the mice few minutes after the second injection. However, in no instance the reaction was fatal and the delayed mortality that occurred in some of the mice with this second series of injections was probably

due to the lysis of the vibrios in the peritoneal cavity of these animals liberating a large amount of endotoxins thus bringing about a fatal toxemia. Our observations on the anaphylaxis in mice were essentially the same as described by Weiser (123). Two minutes after the injection the animals clung together and started a mild shivering all over the body that was intensified in the succeeding few minutes. Then, they fell on their side or stood on the front legs, the hind legs being mostly effected, fur ruffled, eyes were partly or fully closed, nose dropped and the respiration was appreciably quicker than before; in general the animals were hyperexcitable, as shown when a mild stimulus was applied to the body or when an object with a noise was dropped on the ground. This state continued for about 10 minutes, after which the symptoms became milder; the animals could stand on their hind legs, eyes were opened, shivering and irritability gradually disappeared. The normal state was restored 25 minutes after the start of the anaphylactic symptoms.

Twenty days after the second injection of the immunizing dose, 12 mice still survived and these were divided equally into six groups. To groups of two mice each, one M.L.D. in saline of the following strains of V.cholerae was injected intraperitoneally : El-Korein, El-Korein (rough), E-593, E-593 (rough), E-593 and M-4 (last two 'phage variants). Cultures on peptone water were made from the blood and from the peritoneal fluid of the animals 24 hours after the injections. In all instances the blood of all the killed animals was sterile and the peritoneal fluid showed no typical vibrios, although in some instances it was found to be contaminated with Gram-positive cocci or rods. Whether such contaminations, in spite of strict aseptic technic, was due to external sources or that organisms migrated from the intestinal canal into the peritoneal cavity during this period, is impossible to state in the present study. Similar observations have also been made by White (132).

After such negative observations, the cultures were about to be discarded, when on several of the plates streaked with material from the peritoneal cavity of immune mice injected with El-Korein and M-4 ('phage variant) strains, few colonies of Gram-negative organisms were found that were quite fastidious in their growth requirements. Upon further analysis two distinct colonies were differentiated each representing a different organism. The first, isolated from the peritoneal fluid of mice injected with El-Korein strain was a non-motile plump rod, fermented dextrose and sucrose but not lactose nor mannite, was not agglutinated by 0 type I serum but was virulent as tested on mice. Growth on nutrient agar was slow and mucoid in character. After several transfers on this medium the colonies appeared earlier than before, the organisms attained a sluggish motility and took a more elongated form, but in no instance vibrio forms were observed. Subsequent passages of the organisms through a number of mice did not change much of its original characters. However, after successive subcultures on mannite broth, containing killed organisms of smooth El-Korein vibrios, they gradually became mannite fermenters. The second organism isolated from the peritoneal fluid of mice injected with M-4 ('phage variant), was a moderately motile straight rod, S-R as tested by Millon's reagent, fermented dextrose in 24 hours, sucrose and lactose in 72 hours, did not ferment mannite, was inagglutinable, virulent to mouse and after three passages thru this animal kept its former characters.

Due to unfortunate mistake both of these strains were lost and no further investigation on this line was possible; however it can be assumed in spite of these inconclusive results, that such mutations might possibly occur in the immune tissues, where the forces directing the organisms to undergo such dramatic changes are much more dominating than those existing in vitro experiments. Further work on this line is required.

IV. Cross Protection Tests on Mice.

The strains El-Korein (epidemic), El-Korein (rough), E-593 (stock), E-593 ('phage variant-smooth), E-593 (rough) and M-4 ('phage variant-rough) were seeded on meat infusion agar in large Sabouraud tubes and incubated at 37°C. for 24 hours. At the end of this period, growth was washed in 0.5 per cent phenol-saline, the concentration of the suspensions was brought down to 2 billion organisms per cc. and kept in the cold for 48 hours before use. In cases where the growth was rough, sterile glass beads were added to tubes and the suspension homogenised by shaking for 5-10 minutes. 0.5 cc. and 1 cc. amounts, from each lot of vaccine prepared, making a total dose of 3 billion organisms, were injected intraperitoneally at one week interval, to corresponding groups of mice, each group containing 40 white Swiss mice varying from 20-30 grams in weight.

Fifteen days after the second injection, the survivals in each group, were further sub-divided into two equal batches and 5M.L.D.s in mucin of strains, El-Korein (epidemic) and E-593 (stock), were injected to corresponding batches of mice in each group. Control mice received 1 M.L.D. and 5 M.L.D.s from each of the test organisms. Mortality among the animals was recorded at the end of 5,10 and 24 hours periods and in each case the organisms were isolated from the blood, otherwise the death of the animal was attributed to some other cause. The results are shown on Table VIa.

The cross protection tests were repeated under the same procedure and experimental conditions, except on a narrower scale, using only two strains of V.cholerae - El-Korein (epidemic) and E-593 (stock) - for both immunizing and test purposes and injecting 2 M.L.D.s to each series of mice from each of the strains, instead of 5 M.L.D.s as in previous tests. The results of these latter observations are given on Table VI$_b$.

Test Strains 5 M.L.D.s in Mucin	El-Korein (epidemic)			El-Korein (rough)			E-593 (Stock)			E-593 (rough)			E-593 (phage variant)			M-4 (phage variant)			Controls 1 M.L.D. - Mucin			Controls 5 M.L.D - Mucin		
	Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours		
	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24
El-Korein (epidemic)	0/14	2/14	8/14	0/12	10/12	12/12	0/16	8/16	16/16	0/12	4/12	12/12	0/14	6/14	14/14	0/12	6/12	12/12	0/16	2/16	16/16	4/16	16/16	
E-593 (Stock)	2/14	4/14	8/14	6/14	8/14	12/14	6/18	10/18	15/18	4/18	10/18	10/18	4/14	10/14	12/14	0/10	2/10	6/10	1/16	4/16	16/16	5/16	16/16	

Table **VI** a. Cross protection tests on mice immunized with phenol-treated Vaccines of _V. cholerae_ and its Variants

Test Strains 2 M.L.D.s in Mucin	Vaccine strain El-Korein-(epid) Immune mice			Controls - 1 M.L.D - Mucin			Controls - 2 M.L.D.s - Mucin		
	Deaths and Survivals Hours			Deaths and Survivals Hours			Deaths and Survivals Hours		
	5	10	24	5	10	24	5	10	24
El-Korein (epidemic)	0/20	0/20	0/20	0/20	3/20	18/20	2/20	8/20	20/20
E-593 (Stock)	0/20	0/20	0/20	2/20	7/20	20/20	3/20	12/20	20/20

Table **VI** b. Cross protection tests on mice immunized with a phenol-treated Vaccine prepared from El-Korein (epidemic) strain of _V. cholerae_

Deaths / Total number inoculated

Analysis of Results.

By examining the contests of Tables VI$_a$ and VI$_b$ it can be seen that the mortality rate at the end of 24 hours has been 100 per cent uniformly in all the groups of control mice regardless of the size of the inoculum employed. However, if one considers the results obtained at regular time intervals, there seems to be a relationship between the size of the dose used and the time taken to kill the animals, the latter becoming shorter within certain limits, as the number of organisms used in the test is increased. That similar variation in the killing time of cholera vibrios occur as a result of fluctuations in its virulence, was indicated in our previous discussions.

While groups of mice immunized with vaccines prepared from El-Korein (epidemic) and E-593 (stock) have been fully protected equally against 2 M.L.D.s of both of these strains, the results have been different when 5 M.L.D.s were used instead. Thus, the groups of mice immunized with a vaccine prepared from El-Korein (epidemic) strain, were partially protected against five lethal doses of its homologous strain and E-593 (stock) as well, yet those immunized with a vaccine prepared from the latter strain, were not protected at all against El-Korein (epidemic) strain and only partially against the homologous strain when same lethal doses were used in the test; indicating that, strain E-593 (stock), in spite of its high pathogenecity to mice, does not seem to possess parallel antigenic properties as shown in immunization experiments. This fact becomes more apparent when the results obtained with its homologous degenerate strain is examined. (see Table VI$_a$ column 4).

The fact that smoothness of a culture as such is not a complete index of its protective capacities when used for immunization purposes, is clearly shown in results obtained under E-593 'phage treated smooth variant. Groups of mice protected with suspensions of this race seem not to possess any extra resistance over the groups of mice protected with its original rough parent form, when five lethal doses of El-Korein (epidemic) or E-595 (stock) were used as test organisms.

In the final analysis, it seems justifiable to conclude that, strains picked up for vaccine preparations should be smooth and virulent to susceptible animals. Of course the advangage of including different antigenic types - Inabla, Hikejima and Ogawa - in such preparations is understood, but as to what extend such results hold true when applied to human beings, is a question that can hot be answered in this paper.

GENERAL DISCUSSION.

It seems to be well established that organisms, isolated from an epidemic of cholera in its most spreading form, are typically vibrios in morphology (111) and that any alteration from this characteristic form is suggestive of some degeneration brought about under the deleterious influences of certain environmental changes. That actually such pleomorphic or involution forms do occur when typical vibrios are transferred on laboratory media for a long time or subjected to the influence of 'phage or immune bodies, is clearly demonstated by the present described experiments. Similar observations are described by Balteanu (7) and others (580, (39), (10), (55) and (79).

Further, it is mostly agreed upon that such epidemic strains, when freshly isolated from acute cases or cadavers, are capable of fermenting dextrose, sucrose and mannite but not arabinose, are smooth in colony characters and agglutinable with O-serum prepared against epidemic strains of the same species (31), (111), (52), (16) (115) and (92). That fermentation of lactose is a further characteristic of such strains is shown by Gohar (38) and confirmed in the present studies on the El-Kerein epidemic strain. While such characters seem to be the constant properties of a true epidemic strain, extensive variations in one phase or the other of this organism has been reported by a number of workers (136), (71), (73), (113), (114), (86), (89), (135), (43), (127), (129), (93), (35), (134), (133) and at times erroneously used as basis of classification for this group of organisms (82), (35).

It seems apparent that organisms, after being subjected to the influence of 'phage, immune bodies or certain other deleterious actions of environmental conditions, which have wrought basic changes in the cell and effected its metabolic processes to a certain extend, are by no means to be classified as typical cholera vibrics and conclusions based on studies of such variants seem not to be justifiable when applied to organisms freshly isolated from frank cases of epidemic cholera.

It is quite possible that similar or more extensive vibrionic variations occur in the intestines of immune individuals and probably the extend of such alterations is quantitavely determined by the effectiveness of influences simulating such changes on one side and the number or resistance of vibrics exposed to the action of these antagonistic forces on the other side. The fact that agglutinable vibrics disappear from the stools of patients soon after the convalescent period, strongly suggests that similar variations do occur also in the organisms under the influence of a specific 'phage or immune bodies found in the intestines of the individuals and bring about either complete lysis or extensive deep seated alterations in the vibrics, thus rendering them unidentifiable by ordinary cultural or serological procedures (124), (67), (19), (34), (107), (59), (45), (97), (23), (76) and (104).

It is reasonable to assume that typical strains of cholera vibrics after their disposal from the body, undergo similar variations in nature, which may effect their cultural or serological characters (120), and that under certain conditions such variants give rise to serious outbreaks of diseases resembling in certain respects to cholera without attaining the true antigenic properties of an epidemic strain of V.cholerae (78), (61), or that, as a result of some stimulating factors, these non-agglutinable vibrics revert into agglutinable forms, gain virulence and give rise to frank cases of cholera.(91). If ever such changes occure, they do occur under the influence of specific 'phages and other unknown factors or thru passages in the body of unknown reservoir hosts where reversion of vibrics into their

original virulent form takes place.

It is objectionable to make the generalized statement that all agglutinable vibrios are virulent and all non-agglutinable forms are not. Almost all experiments, planned by various workers to determine the virulence of cholera vibrios, have been carried on laboratory animals, in which the disease takes a septicemic form without exhibiting any of the symptoms manifested in man in which the disease is a localized intestinal infection with no tendency to spread to other parts of the body. The fact that, a strain which is virulent to a laboratory animal is not necessarily agglutinable to a high titer when brought in contact with an O-serum prepared against an epidemic strain, nor it is typical in its biochemical activities, and that, similar strains subjected to the influence of specific 'phages might be rendered to be smooth but avirulent as tested on mice; and finally stock strains of cholera vibrios after prolonged cultivation might still show high agglutination titers when tested with serum prepared against epidemic strains but may have completely lost their virulence to mice, suggests that the term "virulence" as applied to denote disease producing ability of an organism, might be completely misleading in such instances. A strain labeled "virulent" after being tested on laboratory animals may actually cause no disease when tested on human beings.

If one accepts the contention that such virulent-inagglutinable strains, not detected thru ordinary cultural or serological methods, could be reverted into their original forms in the tissues of a susceptible animal however, then the assumption made by Ouchi (91), as to the presence of an unkown intermediate host in nature playing a part in the epedemiology of cholera, seems to be justifiable at least for interepidemic periods. The main mode of transmission of cholera is undoubtedly from the patient or "acute carrier" to the susceptible individual as suggested by Khan (103), but the possibility of introducing the infection in a community thru such vibrionic reversions cannot be totally ignored. No definite solution of this problem could be postulated unless attempts are directed in

reproducing the disease in experimental animals in the same typical form as it occurs in man.

There is much experimental evidence to indicate that the El-Tor vibrio is a hemolytic variant of a true V.cholerae (94), (37), (24), (32), (25). During these studies two cholera vibrios were encountered which had attained hemolytic properties as shown by the Greig test. It is interesting to note that one of these strains was virulent to mouse while the other had completely lost this property.

Although in some instances, the possession of a hemolytic power in an organism is associated with its disease producing properties as in certain Streptococci, yet in others, as in H.influenzae, is indicative of degenerative changes in the organism; but in no case we can state with certainty that the loss or gain of this characteristic in an organism brings about basic fluctuations in its virulence. Thus it is reasonable to assume that an outbreak of a cholera like disease in Celebes was either due to a non-hemolytic V.cholerae which after passing thru individuals attained hemolytic properties or that originally they possessed this property together with a certain degree of virulence to cause a serious outbreak among non-immune individual. The inagglutinability of certain strains of El-Tor vibrios in presence of O type I serum is a further evidence of a deviation from the parent form as manifested in alterations of its antigenic structure. Thus it seems probable that loss of agglutinability and gain of hemolytic properties might occur simultaneously or independently in nature or in the body, as a result of certain stimulating factors which interfer in the normal metabolism of the cell and at times become so deep-seated that render the organism completely avirulent.

Finally, it seems justifiable to assume that Inaba, Ogawa and Hikojima types of V.cholerae are only variants of the same ancesters which have undergone certain trivial changes as manifested in their serological reactions. The antigenic analysis of certain groups of vibrios by Burrows et al. (17) clearly shows the existence of a common antigenic factor (A) characteristic of only cholera group of organisms and demonstration of (B) and (C) factors, in addition to the (A) factor, associated

with the Japanese types is a further indication of a correlation to the parent
organisms; while the presence of other common antigenic components distributed at
random among the true cholera group, of vibrios, El-Tor vibrios and other cholera
like organisms strongly suggest the genetic interrelationship existing among these
groups of vibrios, which would be impossible to detect by any ordinary cultural or
serological methods.

SUMMARY AND CONCLUSION.

Different phases of variations among cholera group of organisms, as they occur
spontaneously or under the influence of certain agents, were studied in the laboratory
and the results lead to the following conclusions:

1. Typical epidemic forms of V.cholerae undergo spontaneously thru certain
changes when transferred for long periods of time on laboratory media. Such changes
might effect almost all phases of the organism; the morphology, which loses its
characteristic vibrio shape; the colony consistency, which might undergo an S-R
dissociation; the antigenic structure, which suffers a loss or alterations in its
components parts; the virulence, which might be retained when tested on mice or lost
completely to this animal, the biochemical activities, which are definitely weakened
and the hemolytic power, which might be gained as tested by Greig's method.

2. Similar variations occur in the same group of organisms, when subjected
to the influence of a specific 'phage or immune bodies, except that chnages induced
thru such drastic measures, are more extensive and lead the organism to undergo thru
a series of profound alterations effecting deeply its basic cellular activities.

3. It is argued that a freshly isolated epidemic strain of V.cholerae is a
lactose fermenter, besides being a dextrose, sucrose and a mannite fermenter, is
non-hemolytic, highly antigenic and virulent when tested on mice. Any deviation
from these characteristic features, suggests a degenerative change in the cell,
brought about spontaneously or thru certain agents acting on the organisms in vitro

or in vivo.

4. Due to the fact that both smooth and rough forms of cholera vibrios, as obtained under the conditions mentioned under 1., 2., and 3., might cause death in mice when injected intraperitoneally or might be equally harmless to this animal under the same experimental conditions, it is claimed that the term "virulence", as applied to indicate the disease producing capacity of these organisms, would not seem to represent the true property of cholera vibrios when such tests were to run on human beings under natural conditions.

5. The results obtained by former workers that, a smooth fresh epidemic strains have more protective powers when used as vaccines for immunization purposes, are confirmed.

6. It is suggested that hemolytic properties in an organism might exist as a separate entity, totally independent of its virulence and that the El-Tor vibrio is probably a variant of a true cholera vibrio which has undergone certain alterations in its cellular structure and virulence, the extend of such changes depending upon the effectiveness of interfering environmental conditions. A possible relationship between cholera vibrios and other allied organisms is discussed.

7. It is suggested that the main mode of spread of cholera is probably from the patient or "acute carrier" to the susceptible individual, but the possibility of occurence of cholera like outbreaks at certain interepidemic periods as a result of vibrionic reversion, can not be completely ignored, That problems in epidemiology of cholera could be enormously clarified by finding a possible method of reproducing the disease in its typical form in experimental animals, is indicated.

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