THE CHEMISTRY AND SEROLOGY OF THE VIBRIOS

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Philosophical ideas about disease causation, which at the end of the 18th Century had largely replaced the notions of a *contagium vivum*, received a rude shock with the first European outbreak of cholera in 1826. Thereafter cholera shared with anthrax the honor of stimulating much of the interest and research which culminated in establishing the bacterial theory of disease in the late 19th Century, and of becoming, as Beard (10) has stated, the world's most effective teacher of sanitation and public health. In more recent years, the study of cholera, like that of other diseases, has varied in interest and importance from time to time. During the last decade cholera research has gone through one of its more active periods. Extensive programs have been carried out in India, the Far East and Europe on many aspects both practical and theoretical, under the auspices of the Indian Research Fund Association and of the *Office International d'Hygiene Publique* in Paris, as well as under those of other bodies. Antigenic and chemical structure, variation, metabolism and the renewed study of the El Tor strains since their rediscovery at El Tor and in the Dutch East Indies, have all been the subject of informative research which it is proposed to review in this paper. It has been found necessary to omit consideration of the extensive reports on the experimental and therapeutic uses of bacteriophage, the epidemiology of cholera except as it is related to the source of some of the strains discussed, the production of cholera vaccine and its use in the field.

The main emphasis in recent cholera research has been to differentiate between pathogenic and non-pathogenic forms. The

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present idea is that the authentic cholera organism possesses the following combination of characteristics: fermentation of mannose and sucrose but not of arabinose; failure to hemolyze goat's blood; and agglutination with O-group I serum according to Gardner and Venkatraman (33) (see Table 3). As will be shown in the ensuing discussion, it is the combination of these characteristics which alone defines the authentic vibrio and none of them will do so by itself. This fact is, in the writer's view, difficult to reconcile with the orthodox teaching that the cause of cholera is a distinct entity utterly unrelated to other vibrios. The study of variation together with chemical data to be discussed below will indicate that the whole vibrio group is closely interrelated.

THE CHEMICAL STRUCTURE OF THE VIBRIOS

Carbohydrates. Landsteiner and Levine (62) extracted a substance having the characteristics of a polysaccharide from a vibrio strain. Fractions were extracted by hot 75 per cent alcohol which were antigenic, gave tests for protein and polysaccharide, and appeared to be formed from a combination of these two substances. Upon hydrolysis they showed strong reducing powers and yielded osazones. By dissolving one of the fractions in dilute alkali and reprecipitating with alcohol, another fraction was obtained which gave only faint protein reactions and an intense Molisch reaction; it was almost entirely non-antigenic, was not a protein, contained N and P, and when dissolved in acid solution and hydrolyzed gave rise to a substance of acid character. These findings have been entirely confirmed by later workers.

Linton (63) extracted a crude polysaccharide fraction by boiling vibrios from different sources with N/20 acetic acid until the suspension coagulated. The supernatant was worked up to free it from protein and the final material, which was biuret-negative and gave a Molisch test at a dilution of one million times, was used as antigen in precipitin tests. The conclusion was reached that agglutinating, non-agglutinating and water vibrios contained a common or closely related carbohydrate fraction

which differed from similarly prepared fractions of dysentery and typhoid organisms. Further work was then undertaken by Linton and his collaborators (80 to 83, 85) to see if specific polysaccharides could be extracted; and preparations by more exact methods were made from smooth and rough cholera vibrios and from water vibrios. This extensive chemical work led to the conclusion that at least two structurally different polysaccharides were present in the vibrio group as a whole. Each strain contained an aldobionic acid made up of galactose and glucuronic acid; in each a second readily hydrolyzable sugar was present as well; in some of the strains this sugar was galactose and in others it was arabinose. The galactose- and arabinose-containing carbohydrates were also identified in the "rice-water" stools of patients with cholera (84). By chemical methods similar to those used for isolation from vibrio cultures, biuret-negative substances were obtained from stools which after hydrolysis yielded reducing substances, and gave characteristic phenylosazones for galactose in seven cases, and for arabinose in three. At the same time it was shown (83) that polysaccharides from vibrios neutralized the antibacterial effect of immune sera.

Later studies by the same group of workers revealed a third type of polysaccharide in the vibrio group. In these strains, no aldobionic acid could be found in the hydrolysate in spite of repeated attempts and the use of various methods, and glucose was the only simple sugar identified, although the presence of non-amino nitrogen, amino nitrogen and phosphorus indicated a complex structure. The glucose type of polysaccharide was found in a strain which had dissociated from another strain having the galactose type of polysaccharide and this observation was the first which led to the study of the chemical basis for variation. The glucose-containing type of polysaccharide had been discovered previously by Jermoljewa and Bujanowskaja (52) in an old laboratory strain of *Vibrio cholerae*. When the study of the three polysaccharides had been carried thus far, a shorter method for identification was developed (72) in which a suspension of the whole organisms was directly hydrolyzed in dilute acid. The

manipulations necessary to obtain a phenylosazone were then carried out and comparative tests on a dozen strains indicated that the same monosaccharide was separated out whether the polysaccharide was first separated from the organisms, purified and then hydrolyzed, or was obtained directly by the hydrolysis of the whole organisms. This new method permitted the rapid identification of the characteristic polysaccharide.

White (159) had reported that a complex of polysaccharides with different ranges of serological reactivity existed in the vibrios. Since chemical work had indicated the presence of but a single polysaccharide in each vibrio, it was of interest to study this discrepancy along the lines taken by Avery and Goebel (6) when a somewhat similar situation had arisen in the study of pneumococcus. Linton and Mitra (68) showed that all three types of vibrio polysaccharide existed in the cell as acetyl compounds. Acetylation was of course readily destroyed by the usual method of extraction in which alkali was used. The relation of this finding to White's serological complex will be discussed later. The acetylated and deacetylated forms of each polysaccharide differed in their specific rotations from each other and from each of the two forms in the other two types. Type I (galactose) polysaccharide was almost completely extractable from the cell in the acetylated form; while with the Type II (arabinose) and Type III (glucose) a considerable proportion was not extractable in this form but could be subsequently removed in the deacetylated form by the use of dilute alkali. The possibility of contamination of these polysaccharides by agar was checked by preparing them from peptone water cultures and arriving at identical results (68). The highest degree of purity in the preparation of a vibrio polysaccharide was attained by Shrivastava and Seal (130), who based their method upon that of Heidelberger, Kendall and Scherp (47). Their product was a white stringy material resembling fibers of dried filter paper. It was difficultly soluble in water and gave a solution of high viscosity at concentrations of less than 3 mg. per ml. Its nitrogen content was 2.62 per cent and it had a specific rotation of +58.0°, and a positive Molisch test at 1:6 million dilution. During

hydrolysis a sudden rise in reducing power occurred between the 3rd and 4th hours, and during the same period the precipitin reaction with antisera against the whole organisms abruptly disappeared. This sudden rise appeared to indicate the presence of a second substance in the solution, whose rate of hydrolysis differed from that which hydrolyzed first, and recalled the hydrolysates in which an aldobionic acid was present (81). In spite of all attempts, however, Shrivastava and Seal could not isolate reducing substances from it, other than glucose, thus confirming earlier work on this type of polysaccharide (66, 86). The product was reactive in the precipitin test to 1:12 million dilution, and the reaction was specific for organisms having the same polysaccharide. Using the same chemical methods, Linton, Shrivastava and Seal (87) studied the character of the polysaccharide which a single strain produced when grown on eight different media, containing various combinations of peptone, infusion broth, buffers and glucose. The result was to show that the type of medium had a profound effect upon the specific polysaccharide, variation from 1.8 to 16.4 mg. per liter and of precipitin titres from 2.4×10^3 to 1.6×10^7 being observed. The less reactive polysaccharides were amorphous when dry and gave clear solutions of no obvious viscosity while those with high titres were like the polysaccharide described by Shrivastava and Seal. It was suggested that the physical appearance and characteristics as well as their serological reactivity was a function of the molecular size. The best medium consisted of 1 per cent peptone in infusion broth; the presence of buffers and especially of glucose in the medium gave lower yields and less reactive products. Both volatile and non-volatile organic acids were present after short periods of growth in sugar-containing media and these acids may have been responsible for the lower yields and lessened serological reactivity. It was also observed that older strains seemed to be less active producers of polysaccharides than those more recently isolated.

In connection with the purification of polysaccharides of the different types, Linton and his co-workers (88) found that they varied in specific rotation, percentage of acetylation, maximum

yield of reducing substances, ash and nitrogen, but there did not appear to be any constant differences which could be used to mark off one from the other, except of course the isolation and identification of the type sugar. They found that polysaccharides of case strains isolated from the first part of an epidemic developed a heavy opalescence when their aqueous solutions were treated with sulfuric acid, while strains isolated late in an epidemic, as well as water, carrier and old laboratory strains, did not do so. Study of the opalescent material indicated the probability that it was a carbohydrate-phospholipid complex. Checcacci (21) has recently reported the isolation from vibrios of a similar complex, which was antigenic and appeared to represent the O-antigen. The highly purified polysaccharides of Linton and Shrivastava and Seal were not found to be antigenic (130, 88). These were used in a series of cross-precipitin tests, and while there was a general agreement between chemical structures of the polysaccharides and their precipitin cross-reactions, the exceptions and discrepancies which were observed made the test useless in practice. It was considered possible that the degree of purification reached in the polysaccharides might have removed some factor essential for complete specificity.

Much information about vibrio polysaccharides, especially in their serological relations, has been obtained through the work of White. Yang and White (169) prepared biuret-negative and Molisch-positive substances by methods which White had previously used in his studies on Salmonella (153). When derived from smooth vibrio colonies, these substances absorbed 90 to 98 per cent of the O agglutinins from smooth antisera; but substances derived from rough strains by similar methods did not reduce the agglutinins from the same antisera. On the other hand, rough antisera had their agglutinins completely removed by both rough and smooth substances. This relationship is quite parallel to that found in R and S races of other bacteria (41). The R and S substances also gave a certain amount of specificity in cross-precipitin reactions with their homologous and heterologous sera. In further work, White (160) showed that the A-type phage was specifically inhibited by the poly-

saccharide substance of true cholera and El Tor vibrios. In this instance he prepared the polysaccharide substance by digesting the vibrio suspension with papain, precipitating the extracted material with alcohol and purifying the extract with picric acid and repeated precipitations with alcohol. Polysaccharides derived from non-cholerigenic vibrios and from rough and "rho" races, did not inhibit the A-type phage. This finding indicated that in the vibrios the smooth type polysaccharide bound the phage and led further to the conclusion that the resistance of the secondary cultures resided in the loss of this substance rather than in any positive modification of the vibrios. White also noted that some of the several types of phage were inhibited by the acetone-ether soluble part of the vibrios. Pandit, Maitra and Datta Roy (110) also studied the inhibiting effect of vibrio extracts upon phage, but they did not obtain any very definite correlation between the resistance of a strain to a phage and the inhibition of the phage by an extract of the strain, although in general the more phages a strain resisted, the fewer phage types its extract would inhibit. Gough and Burnet, who had first developed the method, demonstrated that the inhibiting agent of their extracts was a polysaccharide (38). It was not evident, however, that Pundit, Maitra and Datta Roy had actually extracted a polysaccharide for use in their tests, and hence their failure to obtain more definite results was perhaps not surprising. The extracts were classified into three groups according to their phage-type inhibition, but these groups were only partially similar to the groups found by chemical analysis of the polysaccharides. In an extension of this work, Maitra (93) was unable to show any correlation between the phage-inhibiting extracts and either Linton's classification of the vibrios or the serological classifications with O-antigens. Again it was not clear from the data presented just what portion of the vibrio was being used as the inhibiting agent, but the assumption was made that it was a polysaccharide complex.

In summarizing his views at the time, White (159) concluded that each type of vibrio growth (S, R and rho) had its own characteristic complex of polysaccharides, the types differing from

one another by the loss of one member of the complex. The specific carbohydrates were four in number in the S form (α, β, γ, δ), three in the R form (β, γ, δ), and two in the rho form (γ, δ). Type α was dominant in the smooth form, β in the rough, and δ in the rho, and the loss of these dominant substances from the S and R forms led to the domination by another of the substances, which was previously present but masked. Chemical information about these fractions was scant, and White stated that referring to them as polysaccharides might be inaccurate, although his hypothesis assumed them to be such. All were biuret-negative, Molisch-positive substances which yielded reducing substances on hydrolysis. The crude fractions I and II had 3 to 4.5 per cent of nitrogen, irrespective of the type of strain from which they had been derived. The α substance was altered when treated with alkali, while the others were resistant. Precipitin tests were carried out not with the separated fractions, with the exception of α in one case, but with the crude fractions I and II; and from the S, R, and rho forms in each case. The results led White to the conclusion that his hypothesis of the distribution of this complex in the vibrios was correct, although he was admittedly working with impure fractions about whose composition little was known. The amount of evidence given in the paper does not seem adequate to support the rather complex interpretation of carbohydrate structure which it is made to bear. He emphasized the statement that more work was being undertaken upon this carbohydrate-complex, but there is no mention of it in any of his subsequent publications.

In a further contribution to the problem, White (162) noted that antisera prepared by injecting Inaba and Ogawa type vaccines (table 3) into rabbits varied greatly between high type-specificity in which they acted very selectively on the homologous type, and group specificity in which they acted to titre on other cholerigenic vibrios, although not appreciably upon vibrios from other sources. The difference appeared to lie entirely in the rabbit chosen for injection. He stated that these facts complicated the use of standard vaccines for the preparation of agglutinating antisera. In the hope that rabbits might respond

more uniformly to fractions than to whole vibrios, White injected "protein-free" specific substance. This material proved to be actively antigenic, but the same variation in specificity was obtained as with whole vibrios. It is worth noting in this connection that other attempts (Shrivastava and Seal, 130) to produce antibodies by the injection of highly purified polysaccharide were entirely unsuccessful, leading one to suppose that White's fractions were still contaminated with protein.

In view of the finding by Linton and Mitra (68) that the vibrio polysaccharides were acetylated and that treatment with alkali caused them to lose their acetyl groups, White suggested in the paper under consideration that the change in reactivity which he had previously attributed to the disappearance of the S polysaccharide after treatment with alkali was actually due to the removal of acetyl groups. If this were the case, the R polysaccharide would not be a separate entity "present but masked," in the smooth vibrio, but a deacetylated or non-acetylated smooth polysaccharide. But it would also follow that the smooth forms produced acetylated polysaccharide while in the rough ones non-acetylated polysaccharide would be found, and for this idea there is no evidence one way or the other since Linton and his co-workers unfortunately did not study acetylation in relation to smoothness and roughness, although they did find that the amount of acetylation appeared to vary a good deal from strain to strain (87, 88). White's idea of the smooth polysaccharide was that "it possesses a number of receptor groupings, some of which are type specific, some group specific, some alkali labile, some resisting alkali, and that in the rabbit now one, now another of these receptors plays the dominant rôle in stimulating antibodies." No view was expressed about the relation of this complex to that which he had described previously (159).

Two brief papers have concerned themselves with the preparation of residual antigens from the vibrios by the method of Boivin and Mesrobeanu (19). Damboviceanu (27) sought to find if true *V. cholerae* and non-agglutinating organisms from various sources differed in respect to the extracted portions. The specificity and range of these antigens in the precipitin reaction

was exactly the same as that of the whole organism in the agglutination test. Damboviceanu refrained from concluding, however, that in view of his results the fractions were the "cause" of the specificity. Reynal, Lieou and Feisolle (116) applied the same method and obtained an extract which in addition to being specifically precipitated by homologous antiserum, was also toxic, antigenic and caused anaphylactic shock when injected into guinea pigs previously sensitized with whole organisms.

Proteins. The chemistry and serology of the vibrio proteins have formed the subject of several papers. The methods used by Linton, Mitra and Shrivastava (77, 67) depended essentially on the observations of Kossel and Weiss (57) and Dakin (22) that proteins dissolved in weak alkaline solutions gradually altered their optical properties. This change took the form of a diminution in rotatory power, which was rapid at first but gradually lessened until after some days a constant value was reached. Lloyd (53) has discussed the chemical basis of this change. In the earlier use of the method of Kossel and Dakin, the proteins were allowed to stand in alkali until their rotation had become constant. They were then hydrolyzed by acid, the resulting amino acids isolated, and their optical properties determined. With the exception of glycine, the amino acids are ordinarily optically active after acid hydrolysis. The amino acids from the alkali-treated proteins were found to vary in this respect, some being active, some inactive, and some partially active. If the same amino acids in two proteins were found to have different optical properties after alkali treatment, it was assumed that they could not have occupied similar positions in the original proteins, since they had been acted upon differently by the alkali. A number of proteins were studied in this way by the earlier workers; the method was found to be dependable but technically not entirely satisfactory. The isolation and purification of the individual amino acids was a lengthy process and required much material. Woodman (168) showed that a simpler method would yield the same result. He followed the optical activity of the protein solution while it was dissolved in alkali, and plotted the specific rotation against time. The curves obtained in this way

were perfectly smooth and had concordant shapes in duplicate experiments.

Linton, Mitra and Shrivastava (77, 67) applied the method to globulin solutions obtained from a variety of vibrios. Altogether about 200 vibrios were studied and in the whole group only two kinds of curve were obtained. Data representative of both are shown in table 1. Carrying the reading up to 15 days gave no further changes. Proteins giving curves similar to 2027 were designated Protein I, while the other curve was called that of Protein II. With either kind of protein the agreement in the curves from one strain to another was extremely close, and the readings did not vary more than a degree and were usually identical at the same time period.

TABLE 1

Specific rotations of the pseudoglobulins of cholera vibrio 2027 and water vibrio W3075 in N/2 NaOH

HOURS	CHOLERA VIBRIO 2027 SPECIFIC ROTATION	WATER VIBRIO W3075 SPECIFIC ROTATION
1	−76°	−71°
5	−65°	−60°
24	−44°	−39°
48	−36°	−31°
96	−27°	−22°
120	−25°	−20°
145	−23°	−18°
196	−20°	−15°
217	−19°	−14°
264	−19°	−14°

Mitra (98, 99) undertook to study the two kinds of protein by examining the optical properties of the amino acids themselves after they had been released by acid hydrolysis from the racemized material. The diamino acids were prepared by the method of Kossel and Kutcher (56), and the melting points of the acids were in all cases similar to those found by other workers and the percentage of nitrogen was close to the theoretical. When the specific rotations of the diamino acids from the normal and alkali-treated proteins were compared it was found that histidine was optically inactive in both, while lysine was optically

active. On the other hand, arginine was partially active in Protein I and totally inactive in Protein II. This finding was taken to indicate that lysine and histidine occupied similar positions in the two proteins, while it was assumed that the position of arginine differed. Mitra concluded that structural differences did exist between the two protein types in respect to the configuration of arginine. Using the isobutyl alcohol method of Dakin (23 to 25), Mitra then investigated the monoamino acids in normal and alkali-treated proteins (99). Glycine was optically inactive, and the other amino acids which were optically inactive in both Protein I and Protein II were alanine, valine, tyrosine and aspartic acid. Leucine was optically inactive in Protein I, optically active in Protein II; glutamic acid active only in Protein I; proline partially active in Protein I and less active in Protein II; while hydroxyproline was optically active in the case of both proteins. Isoleucine, phenyl-alanine, serine and cystine were not found in either protein, probably because they were present in too small amount to allow their isolation. Mitra's general conclusion was that glutamic acid and leucine differed completely in their relative positions in the two proteins, while arginine and proline were also somewhat differently placed.

Mitra also studied the purified globulins of the two types of protein by means of the spectrograph (101), and observed that each yielded a concordant curve (in three specimens), which differed from the curve of the other protein type. The curves were distinct throughout the course of the experiment. The result was the same as that from racemization in indicating the existence of two proteins in the vibrio group.

Other studies have been made on protein fractions of vibrios, in contrast with the whole proteins just described. Linton and Mitra (65, 71) extracted a protein which they designated as fraction A by treatment with 0.025 N HCl-absolute alcohol. It had a specific rotation of about $-12.0°$ and a nitrogen distribution showing about twice the amount of amide nitrogen and half the amount of humin nitrogen as the whole protein. The A-fraction occurred in the globulin fractions of the protein, and it was destroyed when the above extraction was attempted

in 0.125 N HCl-absolute alcohol. The yield was about 1 to 2 per cent of the dry weight of the organisms. A second protein fraction, designated B, was also obtained but it did not differ significantly from the whole protein in its nitrogen distribution. Fraction A appeared to be chemically the same irrespective of the type of vibrio from which it was derived, and while it represented only a small part of the whole vibrio, it approached the latter in its serological activity and was as effective an antigen. It was also able to prepare the skin for reaction in the Shwartzman phenomenon (89), and it contained much of the vibrio polysaccharide. Since fraction A appeared to contain much of the serological reactivity of the vibrio, it was assumed that it represented the outermost portions of its surface, and the mode of extraction pointed in the same direction.

Similar acid alcohol soluble fractions were obtained by White (154). His extract was divided into two parts, Q_1 and Q_2, on the basis of differential solubility in dilute acid and alkali. Both were active antigens, giving rise to sera which agglutinated all types of vibrios, but which did not precipitate the soluble polysaccharides of S and R vibrios. Further work (157) showed that the Q_1 proteins from S and R strains were serologically the same. Anti-Q_1 sera agglutinated most living smooth vibrios only at a low dilution, while R strains were slightly more sensitive. On heating the vibrios at 100° for a few minutes, most of them became highly agglutinable with all Q_1 antisera. In accordance with his hypothesis of bacterial structure, White concluded that Q_1 substance was present in only a trace on the surface of living vibrios, but was more fully exposed and hence more reactive after heating. He also discovered one strain which was highly agglutinable with Q_1 antisera while alive, and concluded therefrom that Q_1 was not an artefact but was present as such in the living organism. Q_2 antisera were somewhat more specific for true cholera vibrios and they also showed the generalized reaction with heated vibrios which was found with Q_1 antisera. White indicated the possibility that the Q substances contributed to the non-specific O agglutination of heated vibrios, discovered by Gardner and Venkatraman (33). In addition, White (165) obtained antigenic

protein fractions whose antisera reacted with similar antigens from a wide variety of vibrios. These antisera did not agglutinate whole vibrios and the fractions were accordingly parts of the deeper structure of the organism, according to his hypothesis of bacterial structure. The use of NaOH in their preparation would also render it a distinct possibility that they were artefacts.

Linton, Mitra and Shrivastava (76) analyzed the protein portions of agglutinating and non-agglutinating vibrios by the van Slyke method and showed that they formed a homogeneous group in their nitrogen distribution. In comparison with the figures collected by Hirsch (50) for *Corynebacterium diphtheriae, Escherichia coli, Mycobacterium tuberculosis* and a strain of nitrobacteria, *Vibrio cholerae* had a relatively simple structure, which was marked off in its nitrogen distribution from that of other bacteria. The cholera vibrios had a relatively high content of the simpler amino acids and their basic amino acid content was definitely low, while the smallness of the amide nitrogen figure pointed to a comparative simplicity in their protein organization. Mitra (100) isolated the nucleic acid from vibrio protein and found both cytosine and uracil, while thymine was absent, and concluded that the acid had the pyrimidine constitution of a plant and not of an animal nucleic acid.

The elementary constituents of the vibrios were studied by Linton, Shrivastava and Mitra (85), who found that these organisms did not differ significantly among themselves; all contained about 0.5 per cent of phosphorus and slightly more sulphur, while the fat content of 2.5 per cent was that usually found in bacteria. Carbon, hydrogen and nitrogen showed the usual values for normal proteins. Damboviceanu and Barber (26) had shown that different kinds of bacteria grown under identical conditions differed both in the weight and in the composition of their ash. Continuing this work with vibrios, Barber (9) noted variations from 3.9 to 13.5 per cent in amount of ash present after growth under identical conditions. Strains having the smaller amounts of ash had the most calcium salts, while those having most ash had most salts of potassium, sodium and phosphorus. The vibrios were found to be poorer in calcium

salts than other microörganisms. Barber also showed that vibrio strains having the most ash are also those most strongly agglutinated by acids and trypflavine and having the highest surface potential; and in fact throughout her work there is the suggestion that the rough forms have more ash than the smooth. Her further observation that any strain might vary greatly in ash content from time to time in spite of the use of the same culture medium, might also find its explanation in the S-R transition, although Barber did not specifically study this change. Further evidence for the effect of dissociation upon ash content is found in the observation of Damboviceanu and Vasilesco (28) that bacteriophage derivatives, that is, rough forms, had a richer content of ash than the parent smooth strains.

TABLE 2

A chemical grouping of the vibrios

GROUP	PROTEIN TYPE	POLYSACCHARIDE TYPE
I	I	I
II	I	II
III	II	II
IV	II	I
V	II	III
VI	I	III

A classification of the vibrios based upon their chemical structure. Linton (64) outlined a classification of the vibrios based upon their protein and polysaccharide structures. Using the methods which have been reviewed, it was found that one polysaccharide and one protein was commonly obtainable from each strain of vibrio; when exceptions occurred it was invariably noted that the strain was undergoing dissociation. The study of numerous strains from all parts of India and from other places as far apart as Cairo, Tokyo, Basrah and Shanghai over a period of several years did not reveal the presence in them of any chemical structures other than those already described. Given a single protein and polysaccharide in each vibrio, it was possible to divide the strains into six groups, which were numbered in the order of their discovery as shown in table 2.

The first three groups were formed when the existence of two of the polysaccharides and the two proteins had been established. It was then possible to predict that a fourth group probably existed and when El Tor strains were studied they were found to have the expected structure (Linton, Mitra and Shrivastava, 77). Group V was formed when the glucose-containing polysaccharide was discovered and the existence of yet a sixth group, which would contain Protein I, was then predicted (Linton and Mitra, 67). In the study of the Japanese type strains the expected combination of constituents was found (Linton, Shrivastava and Mitra, 86). The protein-polysaccharide complex which forms the bacterial body can be broken down in many ways, limited only by the patience of the investigator and the number of methods and solvents he is prepared to use. In the vibrios, it appeared preferable to consider the protein and polysaccharide of each strain as a unit, and the resulting classification was far simpler than those obtained by purely serological methods, as will be shown in work to be reviewed later.

The classification shows that the vibrios form a closely allied group of organisms with interrelated chemical structures shared in an interesting and probably significant way. The strains of Groups I and II possess the same protein and different polysaccharides. These strains are derived from cases of cholera and have the serological and biochemical characteristics of the authentic O-group I *Vibrio cholerae.* Group I strains are far more common than those of Group II, which have, however, been isolated from epidemics with a high mortality in Assam. The phospholipoid fraction is common to both types when isolated in the early part of an epidemic (Linton, Shrivastava, Seal and Mookerji, 88), but it is not found in strains of other groups. The harmless water vibrios, which are so heterogeneous serologically (Taylor and Ahuja, 142), form a single chemical group with a homogeneous structure. They fall into Group III, which differs in its protein structure from the authentic cholera vibrios, and resembles Group II in its polysaccharide. The vibrios of Group IV, which came from El Tor and from chronic vibrio carriers in India, are believed on epidemiological grounds to be

harmless, although serologically the most refined methods have so far failed to distinguish them from the cholerigenic vibrios. The recent finding of hemolytic strains resembling El Tor strains in an epidemic in Celebes has once more cast doubt on the status of these strains in cholera epidemiology (de Moor, 102, 103). As will be pointed out later, when strains of Group I change their chemical structure, they usually vary to Group IV, and this may be a fact of some importance. Unfortunately, the Celebes strains have not yet been chemically classified. Group V, which, like III and IV, contains protein II, consists, like Group IV, of strains from chronic vibrio carriers. In cholera epidemiology a sharp distinction has been made between contact and chronic carriers. The latter carry vibrios which appear non-cholerigenic and these are generally of Group V. By contact carriers are meant those who have acquired vibrios from being in recent close contact with a case of cholera. The strains isolated from them are, as one would expect, of Groups I and II. Group VI strains are only rarely isolated in nature and representatives of this group are generally found among collections of old laboratory strains. They appear to be the result of polysaccharide variation from Group I after long-continued growth on artificial media.

SOME PRODUCTS OF VIBRIO GROWTH

Hirsch (48, 49) studied the metabolic activities of the vibrios. Under aerobic conditions, the metabolism of these organisms did not differ significantly from that of other intestinal organisms. Atmospheric oxygen acted as a hydrogen acceptor in the deamination of amino acids, and ammonia, acetic acid and carbon dioxide were formed. In the presence of oxygen and glucose, aspartic acid acted only as a source of nitrogen, and about 20 per cent of the sugar was oxidized while about 80 per cent was fermented; the vibrio appeared to prefer fermentation as a source of energy even under atmospheric oxygen tension, just as it preferred to use carbohydrate rather than protein generally in its metabolic activities. Under anaerobic conditions, vibrios could not grow in the presence of protein alone; when the oxygen

tension was that of the intestinal tract, carbohydrate was essential for growth. Under these conditions, glucose was broken down in two distinct ways, first to form lactic acid, and second to form acetic and formic acids and small amounts of ethyl alcohol. These studies by Hirsch laid a rational basis for the production of toxin by *V. cholerae*, to be described below.

Of considerable interest are the attempts which have been made to classify the vibrios on the basis of their acid production in various sugars. In the hands of Heiberg and Taylor this study has yielded valuable information regarding the interrelations of the vibrio groups. Heiberg first showed (45) that acid production in media containing mannose, arabinose and sucrose defined six vibrio groups. His first type, which produced acid from mannose and sucrose but not from arabinose, contained all vibrios of the serological type of O-group I of Gardner and Venkatraman (33), which are considered the true cholerigenic vibrios (table 3). He worked with 384 vibrios from varied sources, and of these 287 fell into the first group and 75 into the second, while the remaining four groups each contained only a few strains. Combiesco-Popesco and Cocioba (20) classified 107 strains from different sources into agglutinable and non-agglutinable types on the basis of their reaction with O-group I antiserum. They found that all the agglutinable vibrios fell into Heiberg's Group I; inagglutinable vibrios derived from cases of clinical cholera into his first two groups; while the El Tor strains of the 1930 pilgrimage were scattered throughout the first four groups. It was apparent that some discrepancies existed between O-group I agglutinability and group I fermentation, and the evidence that the purely biochemical classification of Heiberg would alone not lead to strict accuracy in diagnosis was completed by the work of Taylor, Read and Pandit in India (145). They found that 117 case strains and eight carrier strains, all O-group I agglutinable, fell into Heiberg's first group. This set of 125 strains had been freshly isolated in field studies. On the other hand, over a quarter of the inagglutinable strains from human sources gave the same reaction, and 11 per cent of the water vibrios as well showed the fermentation reaction of Type

I. This result was confirmed by Mertens and Mochtar in Java (97). It was clear that the biochemical information was less exact than that furnished by serological methods, but at the same time it was evident that where large numbers of strains had to be dealt with, as in quarantine stations, those strains not giving the Type I reaction would not have to be examined serologically, thus eliminating a great deal of work. In a later study, based on 558 strains which were non-agglutinable with O-group I serum, Taylor, Pandit and Read (144) found that 18 per cent fell into Heiberg's group I, again indicating that positive results in this group were of little value, although negative results excluded a very large proportion, if not all, of the strains having no serological relationship to the cholerigenic vibrios.

An interesting extension of the work on fermentation is represented by the studies of Read (117) and Seal (126) on new differential media for vibrio isolation. It has long been a problem in cholera to demonstrate the vibrio in all clinically characteristic cases of the disease, and it has often occurred that no vibrios were obtainable in a large proportion of undoubted cholera cases. For example, Pasricha (51) found that of 502 patients suffering from clinical cholera, in the Campbell Hospital, Calcutta, no vibrios could be isolated in 62 per cent even when the most painstaking and continued efforts were made and a variety of methods and media employed. In 1936, when the annual cholera epidemic in Calcutta was more severe than usual, the same worker found that out of 1380 patients with clinical cholera only 61 per cent yielded vibrios of which 82 per cent were agglutinable with the diagnostic serum. The first thought in these instances must be that the technical methods are deficient, and accordingly steps were taken to improve the media. It was shown by Taylor, Pandit and Read (144) that the fermentation of mannose appeared to be a characteristic of the serologically authentic cholera vibrios. Goyle, in unpublished work quoted by Read (117), found that "the agglutinating vibrio could be isolated in almost pure form from an (artificial) inoculum containing that vibrio and a non-mannose fermenting vibrio, when mannose was added to simple peptone water and other media." Read (117)

demonstrated that under the usual conditions of isolation in liquid media the authentic agglutinable organism was rapidly overgrown, not only by coliform organisms, but by mannose-fermenting, non-agglutinable organisms. On the other hand, in the presence of non-mannose-fermenting vibrios the mannose-fermenting, agglutinable type could be isolated in pure culture even after 24 hours' incubation. After a large number of experiments, Read developed a bismuth-sulphite medium, based on one of those of Wilson and Blair (166), containing mannose as the only sugar. This medium in the laboratory was highly differential for all mannose-fermenting vibrios, while its pH of 9.2 kept down the growth of coliform organisms. As we have already pointed out, not all such fermenters are serologically of the O-group I type, and it was not certain how far the delicately growing vibrios of this kind would survive in face of competition with mannose-fermenting non-agglutinable vibrios.

The investigation of this point was undertaken by Seal (126), who compared Read's medium with the usual alkaline peptone water, the pH of which had been raised from 8.0 to 9.2. He found that a considerable increase in the number of agglutinable vibrios isolated from cases of clinical cholera occurred in the new medium in comparison with peptone water, and that isolations from water samples were also improved. However, the medium did not succeed in every case of clinical cholera in bringing out the agglutinating vibrio and this result was ascribed to the large number of non-agglutinating mannose fermenters present in these patients. In short, the medium, while a long step in the right direction, still left room for improvement in its specificity as a completely differential means of isolating O-group I vibrios from every case of cholera. Wilson and Reilly (167) confirmed the results of Read and Seal. Using a collection of old laboratory strains of vibrios and with some further modifications of the medium, they obtained profuse growth of true cholera vibrios and complete suppression of *Escherichia coli* and *Bacterium lactis aerogenes* from artificial emulsions in feces. The suppression of *Streptococcus faecalis* was less complete, but this organism appeared not to interfere with the growth of the choleri-

genic vibrios. Proteus organisms from cases of suspected dysentery and typhoid also grew on the medium and had to be differentiated from the vibrios by stained films. The medium did not differentiate completely between cholerigenic and cholera-like vibrios; of 25 strains of the latter, six grew well and 19 showed scanty growth or none at all; nor was it differential for El Tor vibrios.

Metabolism and the chemical classification. In a study of the respiration and the aerobic and anaerobic glycolysis of 67 vibrio strains, Linton, Mitra and Mullick (69) found that metabolism was most active in vibrios isolated from cases of cholera and belonging to chemical Group I. In Groups II and III the metabolism was less active and in Group IV it was sharply marked off in that aerobic glycolysis was practically negative. Strains of the rugose or "Medusa-head" type (Linton, 64) showed less active metabolism in every respect, notably in anaerobic glycolysis. In none of the other groups was there any difference in respect to this activity, but among the rugose strains the value for anaerobic glycolysis was only 10 per cent of that found in the others, indicating a profound modification in their growth habit. Group VI was almost as active metabolically as Group I, and Group V occupied an intermediate position. In general there occurred a correlation between metabolism, chemical structure and the sources of the strains. The most active vibrios were derived from cases of cholera, followed by Groups II and VI, which have the Protein I structure. The least active strains were of the rugose type. Such strains as these, which make but slight demands upon their environment, would appear to be the best adapted to survive under unfavorable conditions, and some evidence was found (Linton, Mitra and Seal, 72) that they were in fact more resistant to direct tropical sunlight than smooth vibrios were. This suggestion was later renewed by White (164) but it has never been submitted to any very thorough examination, and the place of these strains in cholera epidemiology is quite unknown. Later work by Linton, Mitra and Mullick (70) on the metabolism of 210 vibrio strains gave much the same result, and in a further series of 33 strains whose chemical changes

during variation were studied (Linton, Mitra and Seal, 75) the types of metabolism were also determined and found to coincide with the chemical groups. The chief interest in this series lay in the chemical and metabolic changes occurring during dissociation, and this paper will accordingly be referred to in more detail below. In general it may be said of the work on metabolism that while it could not stand alone as the sole means of separating the vibrios into significant groups, yet used in connection with chemical analysis it supported the chemical classification. Baars (7) grew authentic cholera vibrios and El Tor vibrios in a medium containing 2 per cent glucose, peptone, salts and chalk, and found that both kinds of organisms gave CO_2, organic acids and ethyl alcohol after 24 hours' incubation. In commenting on Baars' work, Gispen (36) stated: "By using another method, Baars obtained results the opposite of Linton's, in showing that the El Tor vibrios produced more CO_2 than did the cholera vibrios. However, he used a peptone medium, not containing $NaHCO_3$, and read his results after 24 hours. Thus the two authors have not examined the same property and their results cannot be compared." Linton, Mitra and Mullick worked with a Barcroft manometer and with media containing 0.1 per cent glucose and recorded the results after 40 minutes.

Seal and Mitra (127), studying the oxidation-reduction potentials of 37 strains of known chemical composition, found that the curves obtained for individual organisms in each chemical group were distributed over ranges which merged into one another and made it impossible to differentiate one group from another. By taking the averages of the curves of the various groups it could be shown that the organisms containing Protein I (Groups I, II and VI) has a higher final potential at 72 hours than those containing Protein II (Groups III, IV and V), just as they had a higher metabolic rate. On the other hand, the changes in pH in the media during growth were the same for all the chemical groups.

Hemolysins and the problem of the El Tor Strains. Interest in the hemolytic properties of the vibrios arose with the isolation of the El Tor strains in 1905. Two points of view developed

about these strains, some bacteriologists maintaining that freshly isolated vibrios from clinically proven cases of cholera were not hemolytic, while others sought to prove that the isolation of hemolytic vibrios from cholera was possible. It was early recognized that differences in hemolytic power might occur with bloods from different kinds of animals and with growth on liquid or solid media. It was also demonstrated that variations in hemolytic power in the same strain might occur from time to time. Some at least of these difficulties were cleared up by van Loghem (90), who showed in 1913 that the apparent hemolysis of blood by *V. cholerae* was actually a hemodigestion, and differed essentially from the hemolysis produced by El Tor strains. The hemolysin was a true exo- or hemotoxin. Pollitzer (115) noted that vibrios which possessed both hemodigestive and hemolytic powers had a tendency to lose the former rather readily and that this change was sometimes permanent. The additional loss of hemolytic activity was rare and in his experience always transitory. As the result of continued work, technical advances toward uniformity and understanding of the hemolytic tests were made, but at the same time the question became unimportant for practical purposes, until the rediscovery of hemolytic vibrios in the Mecca Pilgrimage of 1930 and succeeding years, at the quarantine camp at El Tor, and especially the Celebes epidemic of 1937–38, brought the subject to the fore again. The new vibrios from El Tor resembled *V. cholerae* in all respects except that they were actively hemolytic and apparently not cholerigenic. Van Loghem (91) studied these strains and concluded that like the vibrios of the 1905 isolation they hemolyzed blood rather than digested it. Van Loghem summed up the problem which then arose in the form of two questions: Can a non-hemolytic true vibrio become hemolytic? And can a hemolytic strain cause cholera?

The answer to the first question is complicated by the fact that hemolysis itself is quite a variable property. Heiberg found, for example (45), that rabbit's blood was extremely susceptible to vibrio hemolysin, while goat's blood was resistant. Human, horse, and sheep blood occupied intermediate positions. He

accordingly confined his experiments to the use of goat's blood, and showed that in numerous strains a first hemolytic test would be positive and a second negative; a few strains acted in just the opposite way. He further found that a comparative test with single colonies picked from platings of the same strain had various hemolytic powers, ranging from active to negative. He concluded that "the power of hemolysis is hardly related to simple exogenous or hereditary factors, but that it seems to depend upon laws which only more complicated investigations may be able to reveal"; and that accordingly no satisfactory classification based solely on hemotoxin production was possible. The answer to the first question would thus appear to be a possible affirmative, although with many qualifications.

The second question has been brought into prominence by the Celebes epidemic (de Moor, 102, 103) and appears to have been answered in the affirmative. Cases of clinical cholera occurred in South Celebes in September and October 1937, and again in the first three months of 1938. Like cases of true cholera they showed great variation in their mortality and symptomatology. There were 18 cases with 11 deaths in 1937 and 21 cases with 19 deaths in 1938, and all of the latter were examined bacteriologically. The agent of disease appeared to extend by contact, a method of extension which has been stated by Taylor (141) to be a characteristic only of the non-hemolytic vibrios of O-group I, but this opinion appears to require modification in view of de Moor's findings. The Celebes vibrios were serologically O-group I and biochemically Heiberg's Group I, but at the same time they were strongly hemolytic and hence de Moor (102) did not consider them true cholera vibrios, although he stated that they were the cause of the highly fatal epidemic from which they were isolated. De Moor concluded that Asiatic cholera should be considered a disease in the same sense as bacillary dysentery, in which the same or a very similar disease may be due to bacteriologically different organisms. His epidemiological observations led him to conclude that the disease had not been imported into Celebes but was indigenous and endemic there.

Van Loghem (92) took a somewhat different view of these strains. He stated: "For the first time, vibrios agglutinable, hemolytic and toxic—that is to say, El Tor vibrios—have been isolated from patients suffering from an acute enteritis." He did not agree, however, that the disease was truly cholera, since it was due to a different kind of vibrio, and he accordingly proposed to call it "enteritis choleriformis El Tor," or more simply "enteritis—El Tor." It remains to be seen whether de Moor's or van Loghem's conception of this new organism and disease will prevail. Read (51) has recently reported a case of cholera in Bengal from which a hemolytic O-group I vibrio was isolated; it appears likely that at times a vibrio of this type may be cholerigenic.

The question of hemolysis in cholera vibrios, El Tor and Celebes strains was further studied by Otten (108). He pointed out the three factors which must be considered in the study of hemolysis and the interpretation of the results, viz., the age of the culture at the time it is tested for hemolysins, the method of incubation of the mixture and the way in which the blood is subjected to the hemolytic action. When compared with authentic cholera vibrios and El Tor vibrios in respect to these three factors, Otten showed that the Celebes strains occupied an intermediate position in hemolytic power. He also observed, in confirmation of Doorenbos (30), that when conditions were suitably adjusted *V. cholerae* itself constantly produced hemolysin after short periods of growth. Otten left it an open question whether the Celebes vibrios were El Tor vibrios with slightly hemolytic powers, or strongly hemolytic cholera vibrios, and confined himself to drawing attention to the fact that early hemolysis cannot be used to differentiate various types of vibrios derived from cases of cholera.

The fact that the conditions for hemolysin production need such delicate adjustment lessens the value of several observations made on large collections of strains when little or nothing is revealed about the technique which was followed. For example, Genevray and Bruneau (35), using more than 500 freshly isolated strains, found that most of them showed some hemolysin pro-

duction at 24 hours, while Kabeshima (54) found that 91 per cent of his 206 strains were hemolytic. On the other hand, de Vogel (151) reported that none of his 400 freshly isolated strains was hemolytic.

The study of the Celebes strains was taken up by Mertens and Beeuwkes (96). When very thick suspensions were incubated for one or two days, the supernatant fluid of the El Tor and Celebes strains practically always showed hemolytic properties, while only a few authentic cholera vibrios did so. Thus a strict differentiation could not be made in this way, but further work revealed two better methods. Extracting the three kinds of vibrios with acetone-alcohol, they obtained a thermostable, hemolytic agent from the El Tor and Celebes strains which was present only in small amount in two of their cholera strains. Secondly, by growing the three vibrio types on a synthetic glutamic acid medium to which goat red cells had been added, a complete differentiation was found possible. All their experiments were in accord in showing the identity of hemolytic properties of the Celebes and El Tor strains and their difference in this respect from the true cholerigenic vibrios.

Several studies attempted to correlate hemolysins with other vibrio secretions. Goyle (39) concluded that the hemolysins were true exotoxins, since they were thermolabile, antigenic and filterable. An immune serum against a given hemolytic strain neutralized the hemolysins of other strains in a fairly quantitative way, indicating that they were homologous if not identical. It is of interest to note that Vassiliadis (146) reported that the injection of non-hemolytic vibrios into rabbits gave rise to a hemolysin neutralizing antibody. Goyle found that bloods from various animals varied in susceptibility to hemolysins, but not in the same order as Heiberg had reported. Gohar (37) showed that hemolysin and exotoxin ran parallel in the El Tor vibrios, and suggested that they were identical, although his evidence was not conclusive; and the lack of toxic symptoms in patients from whom the El Tor vibrios are isolated appears to make the correlation doubtful. Bernard (12) extracted an enzyme from solid and liquid media after a strain of proteolytic vibrios had

grown on them; the enzyme was regarded as the source of the hemodigestive activity of the strain but it was not hemolytic (16). Beeuwkes (11) confirmed the method, but showed that two enzymes were present, a proteolytic and a hemolytic. Bernard later found (13) that the enzyme could be separated and concentrated with ammonium sulphate. The enzyme resembled the papainases, and was active against denatured and normal protein (14) as well as against heat-killed vibrios (15). Bernard also found (17) that by modifying his technique a hemolysin could be separated from El Tor strains but not from true cholera vibrios. Among the characteristics of this substance (18) was its thermolability at 56°, its susceptibility to ether and formalin and its resistance to toluene. Like other plant and animal hemolysins it was neutralized by cholesterol. Bernard suggested that differences in hemolysin content in different strains might be explained by assuming a free hemolysin in the El Tor group and a partially neutralized hemolysin in the true cholera vibrios. In addition he showed that both types of vibrios yielded an acetone-soluble substance, thermostable and hemolytic, which lysed living vibrios. It was quite distinct from the true hemolysins.

Doorenbos (29) discovered that the addition of a few drops of sheep blood to a saline suspension of a newly isolated vibrio led to the rapid appearance of a violet color and to the flocculation of the red cells; at the same time, the hemoagglutinin was absorbed from the culture. This phenomenon of hemoagglutination appeared suddenly in some strains and lasted only a few hours. Doorenbos also treated 70 non-hemolytic strains of vibrio with bacteriophage and found that 14 of the secondary cultures became hemolytic, concluding that the phage action caused the change. This conclusion is weakened by the observation of Heiberg (45) that strains which are free from phage will also vary in the same way. Scholtens (121) confirmed Doorenbos' work and found that the strains with hemolytic properties showed spontaneous agglutination (i.e. roughening) in normal saline suspensions and were phage-resistant and non-lysogenic. Doorenbos (31) stated that the 52 El Tor strains found between

1930 and 1936 appeared to be less hemolytic with each year's isolations; he suggested that some of these strains may be weakly hemolytic and at the same time somewhat pathogenic. Gispen (36) in a summary of much previous work on the relationship of the El Tor and the cholerigenic vibrios, included a list of 14 characters in which the two differed and concluded that the El Tor organisms occupied a position intermediate between the pathogenic and non-pathogenic strains. His own differential test in which he sought to show that cholerigenic vibrios became non-agglutinable after being heated while the El Tor vibrios did not change, could not be confirmed by Taylor (51), and de Moor (103) found it less clear-cut than Gispen had stated. Marras (94, 95) summarized his extensive work on the El Tor strains isolated between 1931 and 1938 in stating that they were found in healthy pilgrims and in pilgrims suffering from common maladies; that they possess a non-specific O group which is identical with the non-specific O group of the cholerigenic vibrios; that they are cholera-red and Voges-Proskauer positive while authentic cholera vibrios are cholera-red positive and Voges-Proskauer negative; that since the Celebes strains are pathogenic they therefore cannot be El Tor strains which are non-pathogenic and do not give rise to epidemics.

Toxin and antitoxin. The most complete work on this subject is that of Hahn and Hirsch (43, 44), which arose as mentioned above from the work of Hirsch on vibrio metabolism (48, 49). By adding glucose and NaOH continuously to the anaerobic cultures of certain strains, they were able after a few days to obtain toxic filtrates which could be concentrated and dried and against which an antibody (antitoxin) could be formed. The M.L.D. of the stable dry toxin was 1 to 2 mg. for guinea pigs. It was interesting to note that only the hemolytic strains were toxic and that the antitoxic sera were also antihemolytic. The toxin was very sensitive to acids, not dialyzable through parchment, moderately heat-stable, and very labile in solution. It was present as early as after 8 hours' incubation and reached its maximum concentration after three days. This work was confirmed by Andu and van Niekerk (3) in so far as the production

of the exotoxin was concerned, although they believed that an endotoxin was also present. Soeleiman and van Niekerk (131) returned to this work and concluded that the toxin of Hahn and Hirsch was largely an endotoxin. An antitoxin which they prepared by injecting the toxin into rabbits had little protective effect. On the other hand, Hahn (42) used the toxin of Hahn and Hirsch to produce an antitoxin in a goat and a horse. This antitoxin was used in British India in a series of 145 cases of cholera, in which the death rate averaged 25 per cent. This mortality is higher than that found in Calcutta cholera hospitals, where intravenous saline is being administered, but the figures given in Hahn's paper mean little in any event since the time in the epidemic at which the cases were treated is not given. The serum was not used in India beyond this series of cases reported by Hahn.

Kraus and Kovacs (59) treated centrifuged supernatants of 10-day old bouillon cultures of El Tor strains with formalin and after allowing these "toxoids" to stand for eight days injected them into rabbits. In contrast to the untreated centrifugates which were highly toxic, the "toxoids" proved non-toxic and antigenic as well, and animals so treated were protected against the toxin as well as against infection with living vibrios. These workers maintained that the toxins of paracholera and El Tor vibrios are qualitatively the same as those of the authentic cholera vibrios. Kovacs (58) in continuation of this work found, however, that the toxin of El Tor vibrios gave a necrotizing reaction in the skin of man, rabbit and guinea pig, in contrast to the toxin of cholerigenic vibrios. The reaction could be prevented by mixing the toxin with its antiserum before injection. Takita (137) found, as others had done, that not all strains of vibrio are toxigenic. His method of growth in aerobiosis with large exposed surfaces was in complete contrast to that of Hahn and Hirsch. After two to seven days the filtrates were lethal for mice and rabbits. Takita produced an antibody to this toxin which he considered a true antitoxin. He stated that the neutralization of his toxin by antitoxin took place according to the multiple law, but his data indicated rather that the amounts of

antitoxin required were greatly increased with small multiples of toxin, a result which is characteristic of the endotoxins. Animals could be immunized both actively and passively and the toxin appeared not to be the same as the hemotoxin of the El Tor strains. Takita was able to differentiate his antitoxin from the toxin-neutralizing antibody which occurs irregularly in certain animals and in man. This antibody was thermolabile in contrast to the antitoxin, and did not protect animals although it neutralized the toxin *in vitro*.

The Shwartzman phenomenon with vibrio filtrates has been studied with contradictory results. Vassiliadis (147) found that two out of three rabbits gave the reaction with a filtrate of a five-day old culture of an authentic cholera vibrio, while none of six rabbits reacted with filtrates from two El Tor strains. Linton, Harwant Singh and Seal (89), on the other hand, produced complete cross-reactions with filtrates from 20-hour cultures of the first four chemical groups, including El Tor strains, and hence concluded that the factors responsible for the reaction were not specific, as Vassiliadis had supposed.

THE ANTIGENIC STRUCTURE OF THE VIBRIOS

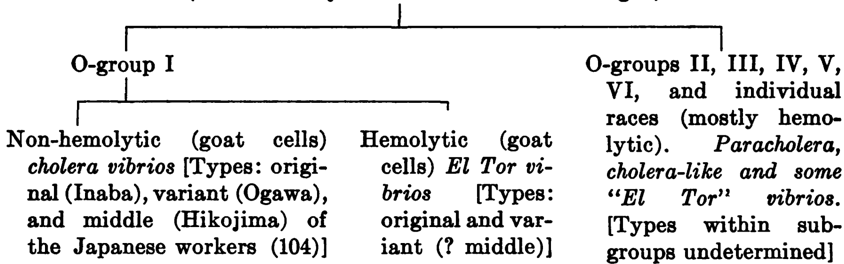
The fundamental work on the receptor structures of the cholera group was done by Balteanu (8), who showed that the vibrios, like other intestinal organisms, contained two components. In accordance with accepted terminology he named the heat-labile, flocculating portion "H" and the heat-stable granulating portion "O". Balteanu noted that the contrast between the two types of clumping was not so sharp as in other intestinal bacteria, and he attributed this difference to the monoflagellate condition of the vibrios. A suspension of flagella, prepared by shaking and centrifuging a culture, showed floccular agglutination with an antiserum against whole vibrios. The flagella were heat-labile, and when injected into rabbits yielded an antiserum which reacted with flagellar suspensions to give floccular clumps. A number of workers have filled out the picture of antigenic structure which Balteanu outlined. Aoki and Oshiro (4) studied the three types of vibrios (Inaba, Hikojima and Ogawa) originally

described by the Japanese workers (Nobechi, 104). These types arose from the same original strain and were differentiated by absorption experiments, and Aoki and Oshiro found that variation from one type to another would occur. Abdoosh (1) confirmed Balteanu's work in showing that both H and O agglutinogens were present in the vibrios, and in determining the distribution of these antigens in his collections of strains, he found that the vibrio group contained several heat-stable antigens; that all his cholerigenic vibrios had the same O antigen, and shared their H antigen with non-cholera vibrios; and that the hemolytic El Tor strains he worked with had the same H and O components as the cholerigenic vibrios. In 1931 Shousha

TABLE 3

Working scheme of cholera group

Cholera group of vibrios
(Biochemically similar. Common H antigen)



recommended (129) that agglutinating sera against heated strains be used in cholera diagnosis to avoid H-group reactions.

These ideas were further extended by Gardner and Venkatraman (33), who were the first to study an adequate number of strains. They defined the cholera group as those vibrios which were biochemically and bacteriologically similar to *V. cholerae* and possessed a common "H" antigen. The scheme in table 3 taken slightly modified from their paper summarizes their findings.

Within the cholera group as thus defined they discovered a number of specific heat-stable O antigens, of which the first was the most important, as it was found only in strains known to cause cholera, and in some of the El Tor vibrios, which could be

separated, according to Gardner and Venkatraman, by the hemolytic test. We have already discussed the El Tor and Celebes vibrios and may conclude from the evidence given that they can occasionally prove pathogenic, although as these also fall into O-group I the value of the classification is only slightly lessened. Gardner and Venkatraman adopted the useful suggestion of Shousha (129) that the term "El Tor" be limited to hemolytic strains having the same specific component as the true cholera vibrios. The practical outcome of this work was to confirm the conclusion of Shousha (129) that the usual antisera for identification of cholera vibrios were useless, since their H components would agglutinate a wide range of vibrios having no connection with the disease; and Gardner and Venkatraman accordingly recommended that such sera should no longer be used in diagnostic work. This conclusion was fully confirmed by Russo (118). Otsubo (107) confirmed the serological differentiation of the Inaba and Ogawa types within O-group I.

Additional information about vibrio antigens has been given by Scholtens (119, 120), who found two serological groups in the authentic cholera vibrios. Exhaustively adsorbing the agglutinins of one cholera strain by another led to the disappearance of its agglutinating power for half of his strains and left only very slightly impaired the agglutinins for the rest. These vibrios were thus divisible into two groups: those giving rise to two kinds of agglutinins and those forming only a single agglutinin. Further work by Scholtens (122, 123) showed that the phenomenon was a general one. All strains contained agglutinogen A_1 and some contained B_1 in addition, the latter never appearing alone and probably existing in the cell as a haptene. Scholtens' work was entirely confirmed by Heiberg (46), who showed in addition that A_1 and B_1 agglutinogens made up the thermostable somatic antigen of O-group I of Gardner and Venkatraman. Strains having either A_1 alone or A_1 together with B_1 agglutinogens were both found to occur in cases of cholera.

Antisera prepared against dried O-group I antigens of the Inaba and Ogawa types were used in diagnostic work on a large scale in India under the auspices of the *Office International*

d'Hygiene Publique. The results of this work, as reported to the *Office* (34) were not published in any detail, but the conclusion was reached that these O-group I antisera specifically agglutinated the causative organisms of cholera and did not agglutinate a large group having a common H antigen, whose causal connection with cholera was doubtful. Taylor (140), reporting on the same study, felt justified in taking up the position that no series of cholera cases could be attributed to a vibrio of fixed serological type other than that of the non-hemolytic O-group I of Gardner and Venkatraman. The weight of evidence appeared to support this conclusion in general, although a number of facts have made its truth in this absolute form somewhat doubtful. In a group of 828 vibrio strains isolated from cases of cholera, Taylor (141) reported that 86.5 per cent were O-group I agglutinable, non-hemolytic and had typical biochemical characteristics, while 13.5 per cent were inagglutinable and serologically and biochemically diverse, like inagglutinable vibrios from nature (Taylor and Ahuja, 142). The vibrios which were not O-group I showed a remarkable heterogeneity, both serological and biochemical, and Gardner and Venkatraman's original five O-groups of this kind could apparently be extended almost indefinitely, besides leaving a large number of strains with individual O antigens. Taylor, Pandit and Read (144) studied 558 strains which were not O-group I agglutinable and had been isolated from clinical cases of cholera, chronic carriers and water. These were set up against 33 antisera to selected strains which were not O-group I, including O-groups II and VI of Gardner and Venkatraman. Only 311 strains (56 per cent) could be classified and these fell into 31 groups, the three largest each containing between 30 and 40 strains, and the remaining 247 strains having individual O antigens. Only 57 of the strains fell into Gardner and Venkatraman's groups II–VI. Similar results with a large collection of strains were obtained in Java by Mertens and Mochtar (97). Taylor and his colleagues believed that there was little evidence to connect any of these strains with cholera. They stated, "The heterogeneity of species actually isolated from cases, the absence of a series of cases due

to one serological type, the multiplicity of types that occur in one case, the actual identity of types in all three sources, even when no connection with cholera can be demonstrated, and the presence of these vibrios in healthy carriers without any symptoms of disease, all indicate a *chance* rather than a *causal* relationship with disease." They were careful, however, to point out two sets of observations which prevent a definite conclusion. They obtained a series of 30 strains of a type known as Rangoon Rough-1, of which 26 were from patients with cholera and on two occasions had been isolated from the same plate as the authentic cholera vibrios. They apparently hesitated to discard this group, although if it has a causal connection, which would appear to be a possibility at least, their hypothesis of the exclusive causative rôle of O-group I strains becomes untenable. A second source of doubt lay in the observation that sera prepared against strains from cases had agglutinated a higher percentage of case strains, than of carrier and water strains, while the opposite was observed with sera against carrier and water strains. If these strains had all been of the same origin, the same percentage of each should have been agglutinable with the three types of sera, and accordingly their finding pointed to a dissimilarity of origin; the authors were inclined to think that the result was an error of random sampling, although this was not proven. Taylor and Ahuja (142) later studied 90 water vibrio strains not of O-group I, from sources from which cholera contamination could be excluded, and again found that these strains were serologically diverse. A high percentage of them agglutinated to 50 per cent or over with Inaba H + O serum, indicating how confusing the use of H antiserum could be and what an advance the exclusive use of O antisera had brought about in diagnosis. These workers pointed out that this universal distribution of vibrios in water would lead to their establishment in the human intestine, and hence to their appearance in the stools of healthy individuals who subsequently acquired cholera. They did not bring any proof of this suggestion. A similar study, but in an area endemic for cholera, was made by Pasricha, Chatterjee and Das (112), who reported that none of the vibrios isolated from water, flies or

cockroaches agglutinated with O-group I serum. Lahiri and Das (60) made a similar observation on vibrios isolated from domestic animals in an endemic area, and as further evidence of the wide distribution of these vibrios, Pandit and Maitra (111) found them in 90 per cent of 105 water sources; these were not tested serologically, but biochemically they were diverse, as indeed similar vibrios were found to be in all the papers just cited.

While Gardner and Venkatraman had believed that a common H antigen was possessed by vibrios having distinct O antigens, it remained for Ahuja and Gurkirpal Singh (2) to investigate more thoroughly the H antigens of the cholera group as a whole. They worked with 219 strains not in O-group I and found that 35 per cent of them were agglutinable with H + O serum and if this serum were alone being relied upon "would have been normally diagnosed as *Vibrio cholerae.*" They confirmed Vassiliadis' finding (149) of the increased sensitivity to H agglutination which results from shaking suspensions with chloroform, 53 per cent of their strains being agglutinated after this treatment and to much higher titres, as against the 35 per cent that agglutinated without the treatment. Using such chloroform-treated suspensions, they showed that some strains had H antigen identical with that of *V. cholerae,* and in others either major or minor portions were identical, while the remaining strains were individual. Finally, a high degree of flagellar heterogeneity and individuality was exhibited by strains which had no H relationship with the choleringenic vibrios, just as Taylor and his co-workers had shown for the O antigens of the same group.

Careful perusal of the reports reviewed in this section brings out the following lines of evidence which were relied upon to show that vibrios not of O-group I did not cause cholera: first, their heterogeneity, both serological and biochemical; second, their existence in nature in places where no cholera was occurring; and third, the fact that *in vitro* they will overgrow the authentic cholera vibrios, and by analogy would do the same *in vivo,* thus accounting for the cases of clinical cholera in which the true vibrios cannot be found. While the evidence in favor of the exclusive rôle of O-group I vibrios is suggestive, it is not wholly

satisfactory, as we have already indicated. In Taylor's large series of vibrios from cholera cases, 13.5 per cent were ruled out as the causal organisms on what are, after all, wholly arbitrary serological grounds; the high degree of serological diversity which they exhibit cannot of itself be taken as proof of non-pathogenicity, and in fact the reports do show that series of these vibrios actually exist, which, if this hypothesis were not being maintained, would undoubtedly be considered as the causes of the disease in which they were found. Streptococci and dysentery bacilli are serologically diverse, but one does not on that account alone exclude some of them and include others among the pathogens. Much the same may be said for the second point: pathogenic organisms whose growth requirements are not strict are widespread even in the absence of their specific diseases, and the areas from which Taylor and his collaborators obtained these inagglutinable vibrios had been severely visited by cholera in times past. In regard to the third point, we can only state that there is no published evidence to show that the overgrowth of agglutinable by inagglutinable vibrios which occurs *in vitro* also takes place *in vivo*; this is a pure speculation. The large number of cases of cholera in which only non-agglutinable vibrios can be found is considered by Taylor to be the result of contamination of the intestine from the environment and hence the "isolation, apart from the stools of a case of cholera, of vibrios agglutinable with H serum, does not permit (us) to consider them as being *Vibrio cholerae*." This statement brings to mind the early dictum of Koch that without the isolation of a vibrio with certain arbitrarily defined characters from clinical cases, cholera could not be present. After some lives had been sacrificed to this dogma, the ban was lifted and the basis for diagnosis broadened. Taylor himself appeared to recognize this danger, for he wrote that "the typical vibrio cannot be isolated in all cases, and when a suspected case occurs, the necessary preventive measures should be instituted without awaiting such isolation." In short, cases which necessitate these measures occurred without the type of vibrio upon which Taylor and his colleagues had laid so much stress, while other vibrios did occur but because of the narrow

basis for diagnosis they are excluded as possible causal agents. It would seem wiser to find a diagnostic basis which would include these cases rather than to labor to exclude them and thus leave open the possibility of error.

Further illustrations of the difficulties which attend the attempts to incriminate a single serological type as the sole cause of cholera were given in the paper by Gardner and White (34). This included a report by Anderson on the use of O antisera in India; he found that in sporadic cases or small epidemics in Assam, strains were isolated which agglutinated with H + O sera but not with O sera of O-group I. In practically all of these cases, however, when a sufficient and often a large number of colonies had been examined, one could find at least one colony which agglutinated with O sera. According to the hypothesis it was then necessary to assume that this one strain was the cause of the condition, while the vibrios isolated in large numbers at the same time were to be considered only as casual contaminants of the bowel. In the same paper, Anderson reported a certain number of clinically undoubted cases of cholera in which the most thorough search would not reveal the "true" vibrio, although other vibrios were abundantly present. In commenting on this finding, Gardner stated that while it seemed to throw doubt upon the exclusive etiological role of O-group I vibrios, still one should consider the fact that the case was clinically typical as indicating that it had been derived from a patient with typical O-group I vibrios. Quite aside from the fact that Gardner and White presented no evidence to show that such a derivation had occurred, Anderson's findings would appear to indicate equally well that O-group I sera alone would not pick out all cholerigenic vibrios. That Anderson's findings were the result of the treatment of these cases by choleraphage was the opinion expressed by White in the same paper. While it may be true that some strains of cholerigenic vibrios after treatment *in vitro* show lessened or no agglutinability, it is purely an assumption to suppose that such changes would take place in the entirely different conditions in the intestinal tract, and no proof of this suggestion is given by White. In this connection it is interesting

to note that Taylor (143) has shown that bacteriophage treatment does not have any effect upon clinical cholera.

It would seem a fair summary of the evidence to state that while the majority of cases of cholera are probably due to a single type of organism, cases do occur in which other types are concerned, and that at times these may assume epidemic proportions, as the Celebes epidemic indicates. It is possible that if the authors who have reported on the discovery and uses of the O-group I antigen had considered the question of variation in the vibrios, a more useful basis for classification and diagnosis would have been found, but the importance of this factor has been consistently denied in their papers. In this connection the ideas of Doorenbos (32) may be briefly noted. In his view every vibrio isolated in the presence of clinical cholera may be complete or incomplete in its serological and biochemical reactions. No matter how incomplete it may be, however, it still has some potential relationship to cholera and to the complete type of vibrio. Atypical vibrios are more or less modified typical vibrios; for example, the non-agglutinable vibrio is the organism of sporadic cholera just as the agglutinable vibrio is of epidemic cholera. While much detail is given to support this view, it must still remain somewhat speculative, although it forms an interesting contrast to the rigid classification into pathogenic and nonpathogenic forms attempted by the workers already cited.

The actual distribution of the subtypes Inaba and Ogawa (table 3) has been studied by Pasricha (113), who found that 60 per cent of 438 strains from clinical cholera in Bengal were Inaba while 26 per cent were Ogawa, 3 per cent Inaba both H and O, and 11 per cent inagglutinable with all three sera. In a virulent epidemic in South India, on the other hand, Venkatraman and Pandit (150) showed that all of the 84 strains examined were of the Ogawa type. Pasricha (51) observed variation in the agglutination reaction from one type to the other, and de Moor (103) discovered in the Celebes epidemic a series of six strains from one case in which both Inaba and Ogawa O antigens as well as a common component were present, and suggested the practical usefulness of an antiserum containing both kinds of type-

specific antibodies, such as these strains would yield. It is evident that the relationship between the Inaba and Ogawa types is of the closest, as indeed one would expect from their origin.

Gardner and Venkatraman (33) had found that a few minutes' exposure of a vibrio suspension to a temperature of 100° sufficed to remove the H agglutinability, but if the organisms were to be used to prepare O antisera it was necessary to heat them in a boiling water bath for 2 hours. The chemical basis of these changes were investigated by Linton, Mitra and Seal (73). The data obtained suggested that destruction of the H antigen involved first a rapid change in the surface of the organism which was reflected in the removal of amino nitrogen and in increased surface potential, and did not progress much with long-continued heating; and second, by a progressive series of changes which involved the gradual loss of total nitrogen and amide nitrogen and a gradual disappearance of the A-fraction (p. 272); in short, a continuous mild hydrolysis of the vibrios. The first change destroyed the H antigen in the sense that the heated organisms with their heightened surface charge had lessened or no agglutinability, while the progressive changes so altered the structure that antisera prepared against it had a new specificity and range of reactivity. The rapid and slow series of changes appeared to explain Gardner and Venkatraman's findings. The amount of change which occurred during the destruction of the H antigen is shown by the following figures: 10 per cent of the total nitrogen, 12 per cent of the amino-nitrogen, between 10 and 13 per cent of the total substance of the organisms, and from 45 to 65 per cent of the polysaccharide must be removed before the organisms are in a suitable condition to yield O antisera on injection. The size of these figures appeared to discredit the view that H antigen is exclusively found in the flagellum.

Continuing this work, Linton, Mitra and Seal (74) studied the relation between surface charge (potential difference or P.D.) and agglutinability in heated and unheated suspensions. Heating for one hour at 60° greatly increased the P.D., especially at the higher salt concentrations, with a consequent lowering of agglutinability, since the P.D. then fell outside the critical zone

found by Northrop and DeKruif (105, 106). Heating for long periods at 100° did not cause much more increase in the surface charge. Vibrios of the various chemical groups could not be differentiated on the basis of their surface charges, nor could Soru (134) find any relationship between rate of migration in an electric field and the agglutination reaction. Linton, Mitra and Seal noted that the charge-reducing effect of immune serum played a dominant role, since with a high agglutinin titre flocculation would occur even when the saline was so dilute that the charge on the unsensitized organisms was of the order of −30.0 millivolts. With heterologous antisera even of the same chemical group, the charge-reducing effect was much less, and might not bring the strain into the zone where agglutination would occur, even though the antiserum had combined with the vibrios to some extent, as shown by cataphoresis. Soru (132) found that vibrios had a negative sign of charge between pH 1.19 and 10.5, whereas sensitized vibrios had a similar charge between pH 4.5 and 10.5, an isoelectric zone between pH 3.6 and 4.5, and a positive charge when the acidity was greater than pH 3.6. The isoelectric range was not the same for all sera, and from Soru's data it seemed as if the higher the titre of the serum the less acid the isoelectric range. Soru concluded that agglutination was due to a modification of surface tension which came about through the adsorption of agglutinin on the cell surface. She then showed (133) that in fact such a modification in surface tension did occur, the sensitized vibrios being much lower in this respect, while normal rabbit serum and anti-typhoid serum lowered the surface tension only slightly.

DISSOCIATION AND ANTIGENIC STRUCTURE

Balteanu's paper (8) laid the foundation for future work not only in the serology but in the dissociation of the vibrios as well. He summarized the earlier observations which had dealt almost exclusively with questions of colony form and vibrio morphology, both of which have been more recently studied and illustrated by Seal (124). More thorough work had to await the general studies of Arkwright (5) on smooth and rough forms and of Weil and

Felix (152) on the H and O types. The first clear differentiation of smooth and rough forms in the vibrios was made by Shousha (128) in 1924, when he isolated typical representatives of each kind. He found that the rough strains were spontaneously agglutinable in normal saline and stable in more dilute saline. They were identical biochemically but otherwise exhibited the typical S and R differences.

Linton, Shrivastava and Mitra (86) found that variations in metabolism, biochemistry, serology and colony form were accompanied by changes in chemical structure as shown by an analysis of the proteins and carbohydrates. This study of the chemical basis of variation was carried out with a considerable number of strains, and was then repeated with single-cell cultures by Linton, Seal and Mitra (79). They stated that beginning with a culture descended from a single cell and having one set of characteristics and a certain chemical structure, it was possible to produce from it a new strain having another set of characteristics and a different chemical structure than its parent. The new strain accordingly fell into another chemical group than the original, and now shared the same characteristics as other strains, isolated from any source, of the same chemical structure. They emphasized that the variations which occurred always remained within the six chemical groups. In this frame-work of two proteins and three polysaccharides, the powers of synthesis and variation appeared considerable, but no other chemical constituents were found at any time. They concluded that the vibrios possessed a strictly limited capacity for variation. Linton (64) presented a report on this work to the *Office International d'Hygiene Publique*. Linton, Shrivastava and Mitra (86) also discovered another type of variation occurring in a series of strains which exhibited the well known phenomenon of variable agglutinability. These Basrah strains, so-called from their place of origin, had attracted much attention (Panayotatou, 109; Doorenbos, 29), and Linton and his co-workers correlated their variability with the fact that each strain was mixed, some of the constituent organisms having one type of polysaccharide and others another type. During growth and subculture, first

organisms containing the one type of polysaccharide would predominate and determine the agglutination reaction, and again the other type would be in the majority. Highly variable mixed strains of this kind are not common, and depending as they do upon the mechanical mixture of two kinds of vibrios, the variability which they exhibit is quite distinct from the already described change in synthesis of polysaccharides and proteins. Strains apparently of the same type as the Basrah strains were reported on by Taylor, Pandit and Read (144), who studied them serologically.

In comparing the agglutination reaction in vibrios of various chemical constitutions, Taylor and Ahuja (138) showed that antiserum against a vibrio of one chemical group would not agglutinate another member of the same chemical group in some instances. They found O-group I agglutinable strains to occur in five of the six chemical groups, the exception being the water vibrios of Group III, and drew attention to the fact that serological methods might fail to show differences in chemical structure. They also isolated from water in an area far from any case of cholera, a non-agglutinating, hemolytic vibrio which on subculturing for eight months became agglutinable with O-group I serum and non-hemolytic without at the same time undergoing any apparent variation in chemical constitution. In continuing this line of work, Taylor and Ahuja (139) found that three inagglutinable vibrios became agglutinable with antisera against both H and O fractions of true cholera vibrios after serial passages through mice. Two of the strains exhibited changes in chemical structure at the same time, while the third remained fixed. White (161) criticized these findings and showed that the presumed mutants contained a type of phage (LL) which was absent in the parent strains. He believed that if mutation had actually occurred "it did so by profound catastrophe" but he preferred to think that the "alleged mutant cultures are not derived from the parents presented." He did not give any indication of his opinion as to their actual origin.

Also in line with the evidence that the vibrio agglutination reaction is mutable, Takano (136) produced immunological

varieties by subculturing in immune sera while at the same time biochemical, hemolytic and other characteristics were not altered. Some of his originally agglutinable strains acquired new immunological characteristics, and entirely resembled atypical strains found in cases of cholera. He also studied a strain which was originally atypical but acquired the serological characteristics of the cholera vibrio, although absorption tests showed that it still retained its original receptors. A third variation was noted in a strain which underwent no alteration in its agglutination reaction, but by absorption was found to have acquired the receptors of atypical strains. Lal, Ghosal and Mukerji (61) found that three to ten serial passages lasting from six hours to six days of vibrios of various kinds through house flies resulted in changes in chemical structure and in metabolic activity, but not in changes in fermentation reaction nor in the O-group agglutination reaction. These results, which were not due to bacteriophage contamination, recall those of Taylor and Ahuja (138) on the inability of agglutination to show differences in chemical structure. In view of the difficulties of interpretation, Lal and his co-workers did not feel justified in drawing any conclusions from their work. In attempting to carry out similar experiments on flies, Shortt (51) and Soparkar (51) could not obtain survival of vibrios in the fly longer than a few hours, and they found that extracts made from the intestine or abdomen of the fly had a vibriocidal effect. No attempt to correlate these contradictory observations on the house-fly has been published. Goyle and Sen Gupta (40) produced rough strains with the agglutinative type of growth by aging smooth strains in peptone-water cultures and also by growing them in 10 per cent immune serum in peptone-water. While their growth habit in liquid media changed, these strains did not show the characteristic rough forms of other intestinal bacteria when grown on agar. Serologically the new forms were identical with vibrios found in clinical cholera and quite distinct from smooth forms. In view of the fact that inagglutinable or less agglutinable vibrios are more common in cases toward the end of an epidemic and during convalescence than at the height of the disease, Goyle and Sen Gupta suggested

that advancing immunization was the cause and that at least some of these vibrios were identical with those which had appeared in immune serum. Their experiments lent support to the view that *V. cholerae* produced variants under natural as well as under artificial conditions.

The sum of this work is to show plainly the lability of the agglutination reaction in the vibrios, and accordingly to make one cautious about accepting complex serological classifications which have been formed upon the assumption that immunological variation will not occur.

As already described, the study of aerobic glycolysis and respiration by Linton and his collaborators had given confirmatory evidence for the existence of the six chemical groups. In a study of metabolic changes during variation, Linton, Mitra and Mullick (69) found that changes occurred in metabolism concurrent with changes in chemical structure and that in general as strains deviated further from the smooth agglutinable type, their metabolic activity lessened. Derived strains with a new chemical structure exhibited metabolic activities similar to those of the chemical group into which they now fell. More evidence for this statement was obtained in studying a large series of parent vibrios and the rough strains derived from them (Linton, Mitra and Seal, 75). These had been obtained by White (156), using his method of exposing young smooth cultures to their homologous activated antisera. Most of the organisms were lysed by this treatment, but there were some of the rough type among the survivors, and these were compared in chemical structure, metabolic activity and electrophoresis with the parent strains. The parent case strains in this series all belonged to chemical group I and it was found that the rough strains derived from them all fell into group IV, showing at the same time no change in the value for respiration, while the aerobic glycolysis, in consonance with previous findings for Group IV strains, had fallen practically to zero. The metabolic and chemical groupings were accordingly identical, and the variants resembled in these respects the typical Group IV strains found in nature. The changes of agglutinability which White recorded were not surprising in view of the fundamental nature of the chemical changes.

In continuation of studies already reviewed on physical aspects of the agglutination reaction in the vibrios, variation was found to be accompanied by changes in surface charge. In every case the change brought about by treatment with activated antisera led to an increased surface potential in the survivors (Linton, Mitra and Seal, 75). Organisms which were quite distinct in electrophoresis when in the smooth state often became similar or identical in this respect when growing in the rough state. This result may supply the physical basis for White's finding (156) that the rough vibrio strains are more generalized serologically than the smooth strains; the phenomenon of bacterial convergence, of which this supplies one instance, has long been recognized (41). In rho strains, which White derived from rough strains by the same method, the potential is higher than in the rough parent strains and much higher than in smooth strains. The rho strains were stated by White to be even more generalized serologically than the rough strains, a result which may again be related to their higher potential. The practical application of surface potentials in the agglutination reaction was brought out by Linton and Seal (78), who showed that strains might be classed as agglutinable when living and as inagglutinable or of low agglutinability when heat-killed, the difference apparently depending upon raised surface potential due to the heating. This factor had to be taken into account when diagnostic tests were being carried out.

Sulmann (135) could not isolate variants by selective subculturing or by raying the cultures with ultra-violet. Heating them at 48° for periods from 8 to 28 minutes in saline he obtained a variant which he thought had increased virulence for guinea pigs, although the differences were slight and might have been due to chance. The heating appeared to cause no other visible change in the cultures. More pronounced variation was induced by Vassiliadis (148) who shook saline suspensions of vibrios with chloroform and found that their agglutinability with O-sera was greatly reduced or had actually disappeared, while their H agglutination was now active at titres four to eight times as high as before treatment. Chloroform-extracted strains injected into rabbits gave O agglutinins but no H agglutinins, indicating that

the O antigen had not been destroyed. Vassiliadis also showed that the chloroform extraction of non-agglutinable strains led to their becoming agglutinable with anticholera sera to titres of 1:16,000. Of considerable interest was his finding that sera against inagglutinable vibrios agglutinated cholera vibrios which had been treated with chloroform; absorption tests gave similar results. The upshot of this work was to indicate the fineness of the distinction between the agglutinable and inagglutinable types of vibrios.

Yang and White (169) found that roughening in the vibrios involved loss of the specific soluble substance. The extreme rough variants were identical either when produced by treatment of smooth strains with A-type choleraphage or when colonies were picked. They believed that the change was not a modification in the fundamental architecture of the strain but that the rough forms were present in the original strain. By treating rough cultures with activated immune serum a further variant was obtained. In a subsequent communication White stated (155) that this variant had lost most of the rough receptors and would agglutinate to only about 25 per cent of titre with rough antisera. In his view this form represented "a grade of degeneracy below that of the R form," since it had lost more receptors and so departed further from the normal S type. Efforts to obtain still further degradation by exposing this so-called "rho" form to its own activated immune serum were unsuccessful. In another study (156) White prepared rough variants by choleraphage action on smooth strains or by treatment of smooth cultures with homologous activated antisera. The essential finding was that rough forms often converged and were of the same serological type irrespective of the differences which existed between the smooth parent strains. But while the rough groups were broader and more inclusive than the smooth groups, there was no general merging, and some strains were just as specific serologically in the rough as in the smooth form. The antisera against the rho forms, however, agglutinated all of his 19 rough vibrios with one exception to titre, irrespective of their source in nature, indicating that these forms possessed a still more generalized serological structure.

On the basis of chemical studies outlined above, Linton and his co-workers had put forward the view (86) that variation from smooth to rough involved a loss of polysaccharide. Their results had also indicated the existence of a second type of variation in which the actual chemical constituents changed from one structure to another. While agreeing that the first type of change did occur, White (158) denied that any positive transformation took place. He stated: "To the student of bacterial change the issue is important. On this point of fact rests the decision whether roughening is a variation by inhibition, suppression or loss, or is a positive change, a transformation. After long study of roughening in this and other groups I take my stand by the former hypothesis." The sole question at issue appeared to be whether the "characteristic" rough carbohydrate was already present but masked in the smooth forms, and was exposed as a new surface during the smooth-rough transition, or whether the organism could synthesize an entirely new polysaccharide during variation. The lines of evidence upon which White depended to prove the preexistence of a characteristic R polysaccharide in vibrios with smooth growth habit appeared to be three in number: (a) On precipitating smooth carbohydrate from solution with alcohol, small amounts of rough carbohydrate remained behind in the supernatant fluid, especially in the presence of acid, and this material reacted exclusively with rough antisera; (b) Upon exposing smooth polysaccharide to N/10 NaOH and heat for a few minutes, it no longer reacted with smooth cholera antiserum. Rough polysaccharide, similarly treated, did not change its reactivity with R antisera. The smooth polysaccharide was alkali-sensitive, the rough insensitive; (c) The rough and smooth polysaccharides were different in their cross-precipitation reactions. He concluded that "What is clear is that in roughening one of these receptors or substances, that serologically active in the S vibrio, is lost, while the other hitherto masked, intervenes in the (possibly complex) serological reactions of the variant."

In a study of these points, Linton and Mitra (68) found that technical manipulations of the polysaccharide in White's work could account for some of his results, without the necessity of

assuming the presence of two polysaccharides in the same organism. The differential precipitation of S and R polysaccharide was found to be the result of deacetylation; when working with acetylated polysaccharide some of it always became deacetylated during purification and this portion remained dissolved in acid solution in the presence of alcohol, but precipitated when the solution was made alkaline. The second point could similarly be accounted for as the result of deacetylation leading to changed serological reactivity. That R and S polysaccharide differed in their serological reactions also appeared to depend first upon the state of acetylation and second upon the transformation in type of polysaccharide during variation such as chemical analysis revealed. These results appeared to throw some doubt on the above-quoted conclusion of White.

It does not seem necessary to review the details of the controversy between Linton and White regarding the nature of variation in the vibrios, since the two views have been made clear already. References to further work may be found in papers by White (161, 163 to 165) and by Linton and his co-workers (79, 68).

Seal (125) compared cell division in rough, rugose and smooth vibrios, beginning with single cells and continuing the observations under the microscope until micro-colonies had been formed, and found that the essential difference lay in the degree to which the cells adhered to each other after division. A colony had a smooth appearance because after division the new cells slipped past each other and separated and then came to lie in a compact mass, while in the rough strains this slipping tendency was practically absent, the cells remaining in contact and forming chains, branches and bands with many open spaces and irregularities. This mode of division led in turn to the formation of angles and projections which stuck out from the colony and penetrated the agar surface. Seal ascribed the consistency of the rough colony to the binding together of the bacterial masses by these chains and irregular off-shoots which also gave the rough appearance to the growth. White (163), in a study of the finer structure of the vibrio colony, showed that either S or R forms could give rise to the rugose type of growth. Pasricha

(114), in his study of this form, had found an intracellular capsular substance and White (163) reported that the rugose condition was due to this gelatinous intercellular substance and that in some cases definite capsules were present as well. He later reported (164) the separation of the intercellular substance and showed that it was a haptene which reacted exclusively with antisera against rugose races.

The work reviewed in which single-cell cultures were found to produce strains of new chemical structure and serological reactivity make it profitable to ask whether changes in enzyme constitution may lie at the basis of these variations. Knight (55) has shown that the training of bacteria to grow on various media "is a distinct response to the chemical stimulus of the changed nutrient conditions." The changes in chemical structure were likewise a response to different types of growth media, and it seems a possibility that the adaptive enzymatic change for nutrition might carry with it in some instances the production of enzymes to synthesize new cell constituents as well. Where the cultures have descended from a single cell it is difficult to account for variation on any other basis. Linton, Seal and Mitra (79) agreed with Knight's statement that "there must be limits to the scope of adaptation which a given bacterium can undergo experimentally, since if there were not, 'specific' characters could hardly exist." The former workers pointed out that while almost nothing was known about the enzyme system of the vibrios, it might be suggested that each of the chemical types possessed the potentiality of producing all of the chemical structures of the whole group and that under various external stimuli, such as changes in the media, sometimes one and sometimes another of the constituents would be formed as an adaptive response.

CONCLUSION

This review shows that the study of the vibrios has proved no exception to the rule that fertile research opens up as many problems as it solves. Studies in chemical structure and serology have made considerable advances, but the correlation between

them awaits further work. The application of the concepts of somatic and flagellar antigens has defined rather than solved the problem of cholera etiology. The results obtained in the study of dissociation may prove of value when applied to epidemiology, or it may be found that the variants are merely abnormalities produced in the laboratory. These larger problems as well as lesser ones in the study of hemolytic power, metabolism and toxigenicity urgently call for study to the end that cholera may be still further reduced and confined in its Asiatic home.

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