

is more satisfactory than the alkaline peptone enrichment medium, even in the presence of cholera-phage. Hence in convalescent cholera cases when cholera-phage has appeared in the stool, the use of phenolphthalein starch medium would be a distinct advantage. Furthermore, the presence of the indicator serves not only for titration of the medium to a desired alkalinity but also as a growth index of the culture. Although its value must not be over-estimated until data from clinical cases of cholera are available, it seems worth while to record here the favorable results obtained under experimental conditions with the phenolphthalein starch medium and to recommend its trial in cholera cases.

6723

Rough Forms of *Vibrio Cholerae* from Convalescents.

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It has been observed¹ that dissociation of *Vibrio cholerae* could be enforced by bacteriophage *in vitro* and the resulting variants showed morphological and serological changes from the parent strain. Pasricha and his coworkers² reported that by subjecting a freshly-isolated phage-resistant cholera strain to repeated cultivation on laboratory media they could obtain certain colonies showing morphological, cultural and serological variation. They also produced evidence suggesting that the loss of agglutinability induced by a high titered cholera serum might occur in cholera vibrios normally during the course of the disease.

During the summer of 1932 a fairly widespread epidemic of cholera extended to Peiping where a limited number of cases not treated with bacteriophage was available for study. The purpose of the present investigation was to make a careful study of culturally-positive cases from the beginning to the end of the disease so as to isolate rough forms of *Vibrio cholerae* and cholera bacteriophage from the stool and vomitus specimens obtained on different days of the disease. As soon as a specimen reached the laboratory

¹ Chen, W. K., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1160.

² Pasricha, C. L., de Monte, A. J., and Gupta, S. K., *Ind. Med. Gaz.*, 1932, **67**, 64.

a few cc. were introduced into peptone water medium (pH 8.4) and incubated at 37°C. for 2-4 hours. Then plates of China-blue-rosalic-acid medium were inoculated and the peptone water cultures were filtered through Berkefeld N candles for detection of bacteriophage. If the suspicious colonies on the plates were numerous or more than 10, only 10 single colonies were picked up and inoculated on peptone water; otherwise all the suspicious colonies were picked for culture on various sugar media. Tests were carried out with every typical cholera strain isolated for its motility in semi-solid medium and checked by the hanging drop method, for "smoothness" or "roughness" by Millon's reaction,³ for agglutinability with known freshly prepared high titered serum from characteristic pure smooth strains, and for susceptibility to stock cholera phage⁴ and to the patient's own phage if it happened to be present in the filtrate of the stool cultures during convalescence. For detection of phage in the filtrate, the liquid culture method⁵ was used against a known pure smooth culture freshly isolated from a fatal dose and also against the patient's own primary smooth culture which was isolated from the first stool specimen after admission. Cases if diagnosed culturally after admission as cholera were followed to the end of the disease; daily specimens of fresh stool (in the acute stage even 2-3 stools per day) and vomitus were examined. The cholera colonies isolated from 195 stool and 16 vomitus specimens out of 21 positive cases were divisible into 3 distinct types according to their "smooth" or "rough" reactions, susceptibility to cholera-phage and agglutinability with high titered serum. Smooth type, lysible colonies by cholera-phage with full titer agglutination was always isolated in abundance during early acute stage of the disease when cholera-phage had not yet appeared. Among the 21 cases there were 13 such patients admitted early enough to give positive pure cultures of smooth type cholera vibrio during the first to third day of the disease.

As soon as cholera-phage appeared in the stool and the patient was convalescent, smooth type colonies became scanty or entirely absent, and intermediate and rough resistant variants of comparatively low titer agglutination (1:160-1:640) against known high titered serum took their place. Among the 21 cases the convalescence of 18 patients could be followed. From practically every convalescent

³ Gautier, R., League of Nations, Health Section Eastern Bureau: Singapore, 1930. (Note on Bacteriophage.)

⁴ Morison, J., and Vardon, A. C., *Ind. J. Med. Res.*, 1929, **17**, 48.

⁵ d'Herelle, F., Malone, R. H., and Lahiri, M. N., *Ind. J. Med. Res. Mem.*, 1930, No. 14.

case the rough type of resistant variants was isolated at the same time with the appearance of cholera-phage. Motility of the rough variants was also sometimes impaired and non-motile colonies of rough cholera vibrio were isolated in 4 cases. The motility, however, could be restored on subsequent transfers in laboratory media. The rough type of cholera vibrio besides giving different degrees of "roughness" to Millon's reagent also showed granular growth in broth, spontaneous agglutination in saline and sometimes rough morphology of surface as well as deep colonies on ordinary laboratory media. From the convalescent cases few colonies of absolutely non-agglutinable ("NAg" type) cholera-like vibrios appeared in 5 cases out of the 18 recovered patients. These "NAg" strains of cholera-like vibrios were rough to Millon's reagent, resistant to our stock cholera-phage and also to freshly isolated cholera-phage mixtures from convalescent cases. It is also of interest to point out here that in severe cases of cholera-phage appeared comparatively later than that in mild cases which also gave earlier cultures of predominating rough type cholera vibrio. Cholera-phage in the stool lasted much longer after recovery than did the cholera variants. Cultures of the stool from convalescent cases were repeatedly negative for cholera variants but cholera-phage was isolated up to the time of each patient's discharge. Cholera-phage was not found in any specimen of the vomitus although cholera vibrio was positive in one of the 16 specimens. This is to be expected since patients usually had vomiting only during the acute stage of the disease when phage had not yet developed.

To determine the serological relationship of the rough colonies anti-sera were prepared by immunizing rabbits with each of the following strains: 4 strains of smooth type cholera vibrio ("Ag"), 4 strains of slightly agglutinable rough variants and 4 non-agglutinable cholera-like vibrios ("NAg"). Cross agglutination and absorption tests among the "Ag" and "NAg" forms brought out the antigenic independency of the "Ag" type of true cholera vibrios from the "NAg" forms of cholera-like vibrios. Anti-sera of the 4 strains of "NAg" cholera-like vibrios which did not constitute a serological homogeneous group were antigenically different from each other in contrast to the "Ag" strains of true cholera vibrios which were antigenically similar. Rough resistant variants (some non-motile) which appeared at the same time during convalescence with the "NAg" cholera-like vibrios were agglutinable only to a low titer (1:160-1:640) against the true cholera serum and had absolutely no agglutination with any anti-serum of the 4 "NAg" cholera-like

vibrios. Anti-sera prepared from the non-motile, slightly agglutinable rough variants gave a low titer (1:640) agglutination only, but could agglutinate each other and the smooth cholera vibrios to the full titer but not the "NAg" cholera-like vibrios. The above serological reactions do not suggest that the slightly agglutinable variants are intermediate types between the "Ag" type of true cholera vibrio and the "NAg" types of cholera-like vibrios. The "NAg" cholera-like vibrios may be "mutants" of true cholera vibrio possibly resulting from phage action *in vivo*. But the question has to be considered also in the light of the "NAg" cholera-like vibrios being of no etiological significance and mixed with the rough variants of true cholera vibrio during convalescence when the activity of the true smooth cholera vibrio is checked by cholera-phage.

To determine whether the variants are reversible, all the non-motile rough variants of true cholera vibrio and the "NAg" cholera-like vibrios were subjected to daily repeated transfers in peptone water medium (pH 8.4) for 90 days. Only the rough variants of true cholera vibrios showed a tendency to revert to better agglutinable "S" or "SR" motile forms. "NAg" cholera-like vibrios remained stable as to their in-agglutinability against the true cholera serum. Animal passages also failed to have any effect upon the agglutinability of the "NAg" cholera-like vibrios. Reversion by bacteriophagic action as described by Pasricha had been tried, but as all the "NAg" cholera-like vibrios were absolutely resistant to all our cholera-phage mixtures isolated so far, no positive findings have as yet been obtained.

Conclusions. Within a limited number of 21 cholera cases proven by positive cultures carefully followed up from the beginning to the end of the disease, it has been found that dissociation of cholera vibrio as determined by its chemical, morphological and serological changes was found to occur naturally during convalescence with the development of bacteriophage against the pure smooth cholera cultures.

Occasional non-agglutinable strains of cholera-like vibrios mixed with the slightly agglutinable rough variants of true cholera vibrio have also been isolated during convalescence. But serological reactions do not bring the "NAg" forms into any relationship with either the pure smooth type or the slightly agglutinable rough variants of true cholera vibrio. The "NAg" cholera-like vibrios are not reversible and at present the writer has no means to conclude that they are positively the mutants of true cholera vibrio.

On the other hand, the slightly agglutinable rough variants, including the non-motile ones, have a weak somatic antigen in common with the true smooth cholera vibrio. They are reversible and are actually the true rough variants of cholera vibrio apparently resulting from the bacteriophage-action during the natural course of the disease.