POSSIBLE FACTORS IN THE PATHOGENESIS OF CHOLERA

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CHOLERA is characterised by a great and sudden loss of fluid and salts from the intestine. Since *Vibrio cholerae* is confined to the lumen of the intestine and has been isolated only on very rare occasions from other parts of the body, it seems likely that the most important factors involved in the pathogenesis of the disease are those which cause gross changes in the permeability of the small intestine.

There have been reports of two different substances which may be implicated in these permeability changes. Burrows, Wagner and Mather (1944) claimed that a small dialysable toxin isolated by Burrows (1944) from *V. cholerae* caused changes in the permeability of rabbit intestine to water, *in vitro*. Burnet and Stone (1947) found that cultures of *V. cholerae* contained a mucinase enzyme distinct from the receptor destroying enzyme which caused desquamation of the lining epithelium of strips of guinea pig ileum. It has been further claimed that *V. cholerae* filtrates rich in mucinase activity alter the permeability of mouse small intestine to water, *in vivo* (Lam, Mandle and Goodner, 1955).

Due to the lack of an experimental animal in which the typical symptoms of human cholera can be reproduced, the significance of these factors in the pathogenesis of the disease is still obscure. In 1953 De and Chatterjee showed that cultures of *V. cholerae* injected into ligated loops of rabbit small intestine, resulted in a distension of the loops with fluid, and desquamation of the lining epithelium with haemorrhagic necrosis of the wall. This experimental technique offers a method by which bacterial fractions may be examined for their ability to reproduce similar changes to those seen when living cultures are injected.

In this paper, various toxic fractions isolated from cultures of *V. cholerae* (Jenkin and Rowley, 1959) have been tested by this technique, bearing in mind the possibility that the same determinant factors might be operating in this experimental test as in cases of human cholera.

MATERIAL AND METHODS

Bacterial strains.—The strains of *V. cholerae*, Ogawa 5596, Inaba 4693, and Hikojima 7270 were obtained from the National Collection of Type cultures. *Bacterium coli* strains 3884, 2712, 8004 and 2821 were kindly supplied by Dr. Roberts, Department of Pathology, Ruchill Hospital, Glasgow. The *Staphylococcus aureus* 524 was obtained from Dr. Rogers of the National Institute of Medical Research, Mill Hill.

Operative procedure.—A light anaesthesia was induced in the rabbit by intravenous injection of 0·7 ml. of a 5 per cent solution of sodium thiopentone. A small area of the abdomen was shaved and a deeper surgical anaesthesia induced with ether. With a scalpel a shallow incision about 2 in. long was made, slightly to the right of the mid line and the subcutaneous layers exposed. The skin flaps were dissected off and held apart by Spencer Wells artery forceps. A further incision was made, about 1 in. in length, through the muscle layers to the peritoneum. The peritoneum was opened with small scissors and the gut exposed. The

appendix was located manually and a loop of the ileum carefully withdrawn. Warm saline packs were placed around the incision covering the whole of the abdomen. About 3 ft. of the ileum was taken out and covered with warm saline packs, except for the small length chosen for the first experimental loop. Two ligatures were made approximately 3 in. apart with strong cotton, care being taken that none of the blood vessels supplying the loop were damaged or tied off. Coloured threads were used so that a particular loop could be easily identified. The cultures or fractions under test were introduced into the lumen of the ligated loop with a sterile syringe fitted with a gauge 22 needle and the loop then pushed back into the peritoneal cavity. In each animal 3–4 loops were made separated by at least 6 in. After tying off the loops and replacing the intestine, the muscle layers were sewn up with nylon thread, using a running stitch. The skin layer was sutured with 3–4 Michel clips and the abdomen gently manipulated to re-distribute the small intestine. The whole operation took 15–20 min. Most rabbits were making attempts to stand within 1 hr. and were always standing within 2 hr.

Consistent experimental results were obtained providing that young rabbits 12–24 weeks old were used and that the loops were situated in the ileum. The duodenum gave variable results but the ileum appeared to be more sensitive and gave consistent results. The strain of rabbit seems to be immaterial; six different breeds have been successfully used. The rabbits were always starved 24 hr. beforehand, but allowed water.

Preparation of cultures for injection.—This was found by experience to be the most important factor in ensuring consistent results. *V. cholerae* cultures were grown at pH 8·2 in nutrient broth, whilst *Bact. coli* strains and the *Staph. aureus* 524 were grown at pH 7·4. The cultures were planted in 200 ml. of broth in Erlenmeyer flasks and shaken to ensure good aeration at 37° overnight. The following morning a hundred-fold dilution of the culture was made in broth and shaken as before for a further 2 hr. at 37°. During the operation the culture was kept at 37° in a waterbath. These precautions ensured that a growing culture was injected into the loop. The pH of the broth appeared to be immaterial providing the strain grew well at that particular pH. All three cholera serotypes gave positive loops. (Fig. 1).

Bacterial counts on positive and negative loops.—Positive and negative loops were carefully removed and washed in sterile saline. The ligated loop was placed in a sterile petri dish, the ligatures removed and the contents washed out with sterile saline. Serial dilutions of the washout were made in saline and plated out on MacConkeys agar. After overnight incubation a hundred or more colonies selected at random were tested against a 1/500 dilution of the homologous antiserum. By this means an estimate of the total number of viable organisms of the specific type could be obtained.

Preparation of toxic fractions.—The protein toxin and lipopolysaccharide endotoxin were prepared as described by Jenkin and Rowley (1959).

Mucinase preparation and assay.—Filtrates containing high titre mucinase were prepared by the method of Chugh, Jensen and Kendrick (1956) and assayed according to Burnet (1949). For use in experiments with crude intestinal mucin, the filtrate was dialysed against 0·1 M phosphate buffer pH 7·3 containing 1 per cent $CaCl_2$.

Preparation of crude intestinal mucin.—The small intestine from rabbits which had been starved for 24 hr. was washed through with 0·1 M phosphate buffer at pH 7·3. It was then opened along its length and cut into a number of small segments which were suspended in the phosphate buffer washings and incubated at 37° with shaking for 2 hr. The suspension was centrifuged at 3,000 *g.* for 20 min. and the resulting supernatant precipitated with five volumes of absolute alcohol. The precipitate removed by centrifuging was dissolved in 0·1 M phosphate buffer pH 7·3 containing 1 per cent $CaCl_2$ and dialysed against the same with frequent changes for 48 hr. at 0°.

Estimation of Lactic acid.—Lactic acid was estimated by the method of Elsden and Gibson (1954).

Estimation of Hexosamine.—Hexosamine was estimated by the method of Rondle and Morgan (1955).

RESULTS

In a previous paper (1959) we reported our inability to isolate a small molecular toxin by the method used by Burrows (1944). Using his experimental techniques,

alcohol soluble, but non-toxic, fractions were screened for their ability to increase the permeability of rabbit intestine to water. The experimental results were negative; no increase in the permeability of the intestinal segments to water was ever observed.

The action of toxins and enzymes from V. cholerae *on ligated loops of rabbit ileum*

The lipopolysaccharide endotoxin and protein toxin were tested in ligated loops to see if they would produce a similar fluid distension to that observed when living cultures were injected. The amounts varied from 1–10 mg. per loop. The results obtained in these experiments were negative. Combination of the two fractions with a filtrate containing 500–1,000 uts/mucinase per ml. did not produce any changes in the ligated loop of ileum. Injecting large amounts of vibrio cells (20–40 mg. per loop) killed by heating at 75° for 30 min. or by the addition of 500 μg. streptomycin/ml. again gave negative results.

Effect of injecting fluid from an infected loop into a fresh loop

It is possible that *V. cholerae* like the anthrax bacillus (Smith, Keppie and Stanley, 1955) produces a substance which causes the pathological changes seen in the ileum, only under conditions of *in vivo* growth. One might expect to find such a toxin in the fluid of an infected loop. The loop fluid contains large amounts of the mucinase enzyme 1,000–2,000 uts/ml. as well as a heavy growth of *V. cholerae*, so that all the factors which might cause damage and alteration of the permeability of the ileum are likely to be present. The fluid contents of three positive loops were withdrawn and 1 mg. streptomycin/ml. added to prevent further bacterial growth. This material was injected in 5 ml. amounts into 2 ligated loops in each of 3 other rabbits within 2 hr. after removal. The animals were killed 24 hr. later and once again all loops were negative. From these results one may conclude that none of the toxins or enzymes present in the loop fluid produced by *V. cholerae* singly or in combination could be responsible for the pathological changes in the loops.

Since positive results were obtained only in the presence of a growing culture, various simple metabolic products which might be present during growth of the organism were tested for their ability to produce a positive loop.

Effect of sodium salts of organic acids

Sodium salts of a number of organic acids such as acetic, succinic, propionic, pyruvic and lactic which are known to be breakdown products of bacterial glycolysis, were injected in various quantities into experimental loops. It was found that only in those loops injected with 20 mg. or more of sodium lactate were positive results obtained (Table I). The gross appearance of these postive loops closely resembled that seen in loops injected with living cultures. Distension with fluid was marked, haemorrhagic necrosis of the intestinal epithelium had occurred and in the majority of these positive loops sloughing of the lining epithelium was apparent.

Growth of injected bacteria in the ligated loop and production of lactic acid

Since various people (De, Bhattacharya and Sarkar, 1956; Taylor, Maltby and Payne, 1958 and McNaught and Roberts, 1958) have reported that some

Bact. coli strains will also cause changes in ligated loops of rabbit intestine similar to those seen when *V. cholerae* is injected, a number of *Bact. coli* strains, a *Staph. aureus* 524 and *V. cholerae* Ogawa 5596, were tested in ligated loops. In all cases, positive and negative loops were analysed for lactic acid content and the number

TABLE I.—*Effect of Injecting Sodium Salts of Organic Acids into Ligated Loops of Rabbit Ileum*

	50 mg.	25 mg.	12 mg.
Sodium lactate	+++	+++	—
Sodium pyruvate	—	—	—
Sodium propionate	—	—	—
Sodiu acetate	—	—	—
Sodium succinate	—	—	—

+++ fluid distension of the loop with haemorrhagic necrosis of the epithelium.

of specific bacteria determined in each loop. The different results obtained between positive and negative loops were striking. In all the positive loops the specific bacterial count was very high and the loop fluid contained significant amounts of lactic acid. In the negative loops, little or no growth had taken place and much smaller quantities of lactic acid could be demonstrated (Table II). These results confirm what had been previously found, that only in the presence of growing cultures could positive loops be obtained.

TABLE II.—*Specific Bacterial Count and Lactic Acid Content of Positive and Negative Loops of Rabbit Ileum.* 10^8 *Bacteria Injected into Each Loop.*

Strain	Total specific bacteria in loop	Lactic acid mg./loop	State of Loop
V. cholerae			
Ogawa 5596	2×10^{11}	27·4	+++*
	$4{\cdot}5 \times 10^{11}$	24·2	+++
Bact. coli			
8004	$2{\cdot}8 \times 10^{10}$	31	+++
	$3{\cdot}2 \times 10^{10}$	41·5	+++
3884	$3{\cdot}0 \times 10^{10}$	24·3	+++
Staph. aureus			
524	$5{\cdot}5 \times 10^{9}$	23·4	+++
Bact. coli			
2712	5×10^{8}	7·0	—
	$2{\cdot}25 \times 10^{8}$	2·5	—
2821	$3{\cdot}2 \times 10^{8}$	8·4	—
	$2{\cdot}8 \times 10^{8}$	5·8	—

* Key as in Table III.

The role of mucinase enzyme and lipopolysaccharide endotoxin

During bacterial growth both mucinase and endotoxin will be released, the latter due to cell lysis. It seemed important to test whether these substances whilst being individually incapable of producing a positive loop, might " sensitize " the intestine to the action of sodium lactate.

Absorption of endotoxin from ligated loops

Two ligated loops in each of 4 rabbits were injected with 1 ml. of a rapidly growing culture of *V. cholerae*. After 18 hr., 2 of the rabbits presumed to contain

positive loops were given 25 μg. of lipopolysaccharide from the same organism intravenously; the other 2 animals were given 1 ml. of pyrogen-free saline. When killed 7 hr. later, the presence of positive loops was confirmed and a bilateral haemorrhagic necrosis of the kidney cortex, the beginning of the generalised Schwartzman phenomenon, could be seen in the rabbits given endotoxin whilst the controls were negative. This suggests that bacterial endotoxin has been absorbed from the gut.

Increase in sensitivity to sodium lactate in endotoxin treated rabbits

Two rabbits were given intravenously 1 ml. of saline containing 25 μg. of Ogawa 5596 lipopolysaccharide. A third rabbit acting as a control was given 1 ml. of saline only. Three hours later ligated loops were prepared in all the animals and injected with varying amounts of sodium lactate. The rabbits were killed 6 hr. later and it was found that amounts of sodium lactate gave positive loops in the endotoxin treated animals which in the control animal failed to produce any effect (Table III).

TABLE III.—*Increase in Sensitivity to Sodium Lactate in Rabbits given 25 μg. Endotoxin i.v. 3 hr. before Operation.*

Rabbit	Amount of sodium lactate injected		
	25 mg.	10 mg.	5 mg.
a	+++	++	+
b	+++	++	++
c*	+++	−	−

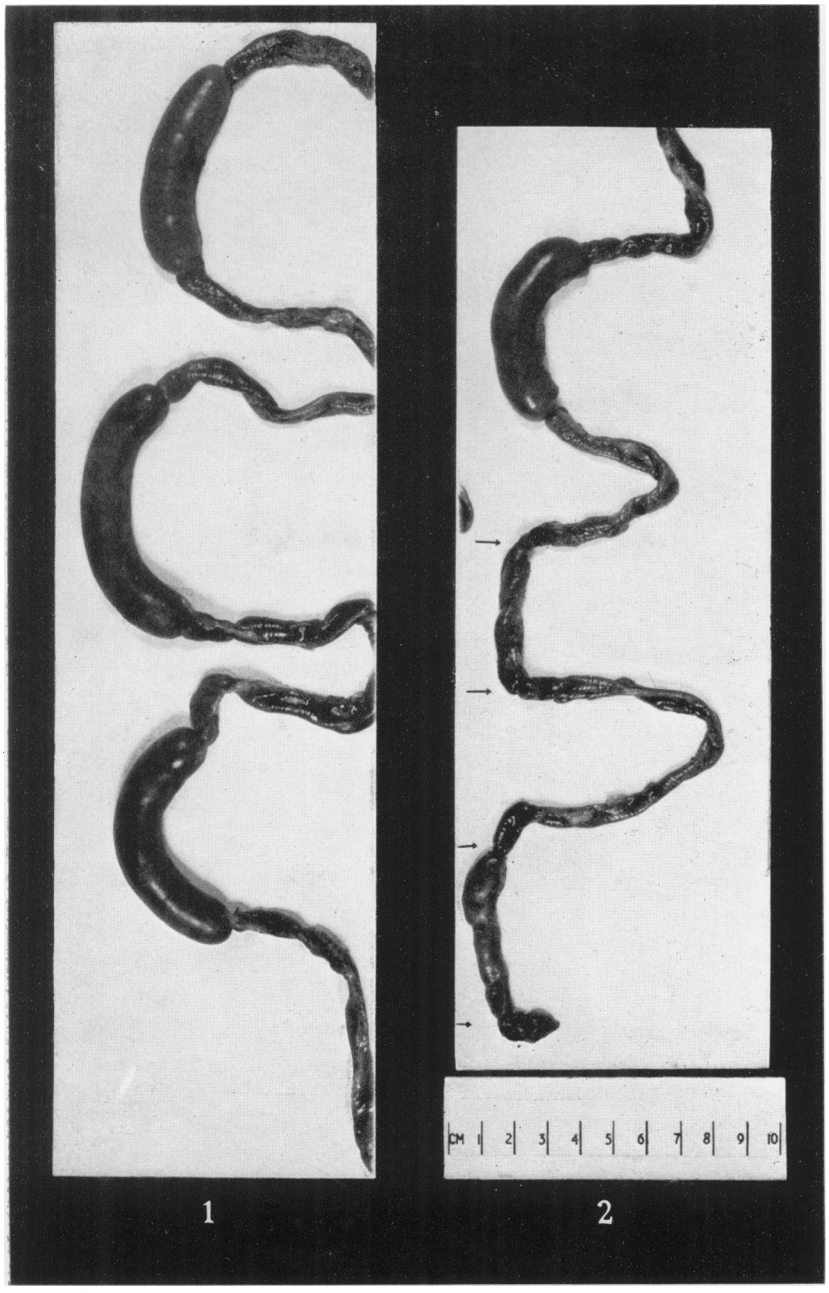
c* control animal injected with saline only.
+++ fluid distension of the loop and haemorrhagic necrosis of the wall.
++ fluid distension of the loop, few scattered haemorrhages.
+ fluid in loop, not distended and no haemorrhagic changes.

Role of mucinase

The initial studies by Burnet *et al.* (1947) on the desquamating action of the mucinase enzyme on the intestinal mucosa has been independently confirmed by a number of research workers (Singer, Wei and Hoa, 1948; Gurkipal Singh and Ahuja, 1953). However, no quantitative studies have been made on the rate of splitting of intestinal mucin by the mucinase enzyme. The assay of the mucinase enzyme using ovomucin as the artificial substrate is unsatisfactory, as it may bear little relationship to the splitting of the natural substrate. Crude intestinal mucin was prepared as described and the action of a *V. cholerae* culture filtrate on this substrate was studied by following the release of hexosamine. The results given in Table IV show that at pH 7·4, 30 per cent of the hexosamine in the substrate had been released whilst at pH 6·4 and pH 8·4 the amount of amino sugar liberated was much less. These preliminary studies suggest that the optimum

EXPLANATION OF PLATE.

FIG. 1.—Positive loops of rabbit ileum 24 hr. after injection of *V. cholerae.*
FIG. 2.—The effect of aluminium oxide on the production of positive loops by *V. cholerae.* The 2 loops at the bottom received 1 g. aluminium oxide in addition to *V. cholerae.*



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activity of the enzyme lies between pH 7·0 and pH 8·0. The pH of the fluid from positive loops varied between pH 7·4 and pH 8·0, and would thus give optimum conditions for this enzymic activity.

TABLE IV.—*Release of Hexosamine from Crude Intestinal Mucin by an Enzyme Filtrate from* V. cholerae.

Time in hr. at 37°	Per cent of total hexosamine released		
	pH 6·4	pH 7·4	pH 8·4
1	0	7	0
2	7	11	0
3	14	14	0
4	14	18	7
5	14	30	7

Effect of adding adsorbents to the loops injected with V. cholerae 5596

If the mucinase enzyme and/or the lipolysaccharide endotoxin could be removed then it might be possible to prevent damage to the injected loop, the lactic acid being removed as rapidly as it is formed under relatively normal physiological conditions. A number of adsorbents such as kaolin, magnesium trisilicate, aluminium phosphate and aluminium oxide in 1 g. amounts were introduced into the ligated loop immediately after injecting 1 ml. of the growing culture. The animals were killed 24 hr. later and it was found that those loops containing aluminium oxide were negative. Control loops injected with *V. cholerae* alone were all positive (Fig. 2). The other adsorbents did not seem to have any effect in preventing a positive loop. Counts made on the aluminium oxide loops and control loops showed that the organism had grown to the same extent in each. (Table V).

TABLE V.—*The Effect of Aluminium Oxide on the Production of Positive Loops of Rabbit Ileum by* V. cholerae

Rabbit	Total *V. cholerae* in loop at time of death	ml. of fluid in loop
All loops injected with 5×10^8 *V. cholerae.*		
a	$2 \times 10^{10} \times 8$	8
b	$3 \times 10^{10} \times 12$	12
c	$2 \times 10^{10} \times 30$	30
d	$4 \times 10^{10} \times 15$	15
Loops receiving 1 g. of aluminium oxide in addition to bacteria.		
a	2×10^{10}	0
b	2×10^{10}	0
c	7×10^{10}	0
d	2×10^{10}	0

DISCUSSION

In the present study three factors have been implicated in the production of a positive loop, these are lactic acid, bacterial endotoxin and a mucinase enzyme.

If one can draw any analogy between the ligated loops and human cholera, it seems possible that these three substances may play an important role in the aetiology of this and similar enteric diseases.

From the literature it is apparent that a wide variety of bacteria both Gram-negative and Gram-positive once established in the gut may give rise to diarrhoeal enteritis. De *et al.* (1956) have reported the isolation of *Bact. coli* strains from cases that had been clinically diagnosed as cholera, whilst from many cases of cholera non-agglutinable vibrios have been isolated (Taylor, 1938). Pseudo-membranous enterocolitis, a condition in which rice-water stools are frequently passed, may be caused by *Staphylococci* (Gardner, 1953) or by *Clostridium welchii* type D (Brumfitt and Wright, 1957). The *Bact. coli* strains isolated by De *et al.* (1956) were found to cause damage to ligated loops of rabbit intestine, similar to that seen of *V. cholerae*. The ability of some *Bact. coli* strains to produce positive loops has been confirmed by Taylor *et al.* (1958) and McNaught and Roberts (1958). It is apparent from their results that there is no correlation between the serotype of the organism and its ability to produce the fluid distension and haemorrhagic necrosis.

The causation of enteric diseases by a variety of antigenically dissimilar organisms suggests that a common factor of metabolism may be involved. It is unlikely that these different bacteria possess the same endotoxins, though the mucinase enzyme appears to be widespread (McClean, 1936) so that one must look for a factor produced by these bacteria during growth. It has been shown in the present work that lactic acid or sodium lactate, if injected into ligated loops, causes fluid distension and a haemorrhagic necrosis of the epithelium. Lactic acid has been implicated in the tissue damage seen in the local and generalised Schwartzman reaction and the role it plays discussed by Thomas and Stetson (1949) and Stetson (1951). It is possible that the haemorrhagic necrosis seen in the ligated loops may be brought about by a similar mechanism. The work of Bullen and Scarisbrick (1957) on enterotoxaemia in sheep suggests that lactic acid production may be involved in altering the gut permeability thus allowing absorption of the ϵ toxin of *Cl. welchii* type D. In order to establish a toxaemia in the sheep the organism had to be multiplying rapidly in the gut of an animal on a rich carbohydrate diet. In the present study, all the bacteria giving rise to positive loops were capable of multiplying rapidly in the gut and producing lactic acid. Bacteria that had no apparent pathogenic effect multiplied slowly or not at all and only very small amounts of lactic acid were produced. From the present work a tentative hypothesis may be advanced as to the sequence of events giving rise to a positive loop. Growth of the bacteria in the loop produces concurrently mucinase, sodium lactate and bacterial endotoxin by autolysis. The mucinase enzyme destroys the intestinal mucin exposing the epithelium to the action of sodium lactate and allowing absorption of the endotoxin. Lipopolysaccharide endotoxin is known to alter the carbohydrate metabolism of the rabbit, such that there is a rise in the circulating lactic acid and accumulation of this substance in the tissues (Kun & Miller, 1948). The accumulation of sodium lactate causes tissue damage leading to gross changes in the permeability of the gut and subsequent fluid loss.

The protection afforded by aluminium oxide may be due to the absorption of mucinase enzyme and/or endotoxin, thus allowing the lactic acid produced to be taken up from the gut and broken down normally.

SUMMARY

A study has been made of factors which may be implicated in the pathogenesis of cholera and similar enteric disease using the ligated rabbit intestinal loop technique of De and Chatterjee (1953). Three factors have been found, lactic acid, mucinase enzyme and lipopolysaccharide endotoxin, the most important of these being production of lactic acid by the bacteria during growth. The possible role of these three factors in the pathogenesis of cholera is discussed.

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