

Progress report

The pathogenesis of cholera and some wider implications

Since this subject was last reviewed in *Gut*¹, there have been many publications devoted to the phenomenon of intestinal secretion of fluid and ions. The study of experimental cholera has undoubtedly provided the stimulus for intense interest in this field. Apart from its relevance to cholera itself, this work is also relevant to the mechanism of action of other bacterial enterotoxins, and perhaps to the production of diarrhoea by prostaglandins and gastrointestinal hormones. Indeed it is remarkable that the important review of intestinal secretion by Hendrix and Bayliss² was the first of its kind for nearly 30 years, and, as they point out, that the subject was scarcely mentioned in the five-volume 'Handbook of the American Physiological Society' devoted to the alimentary canal and published in 1968.

The Nature of Disturbed Movements of Fluid in Cholera Secretion versus Failure of Absorption

Direct perfusion studies in human cholera have demonstrated unequivocally that the proximal jejunum is in a secretory state in the acute phase of the disease and that this process reverses to normal absorption when the patients are studied during convalescence seven to 14 days later³. Secretion in the ileum can be demonstrated in many but not all patients during the acute stage, and stool volumes correlate best with the rate of jejunal secretion.³ By contrast the colon absorbs normally⁴. The composition of the fluid secreted is characteristic of the site of secretion, in that the bicarbonate concentration is low in jejunum and high in ileum³. Bidirectional flux measurements, using conventional perfusion techniques, have only been reported in one preliminary study and these suggested that there is an increased movement of water and sodium from plasma to lumen and that the reverse flux is unaffected⁵. This is the pattern of alteration in flux described in experimental cholera^{6,7}, and used as evidence that cholera is a secretory phenomenon without any primary effect on normal absorption.

There are difficulties involved in the interpretation of isotopic flux measurements. In a personal study, bolus⁸ and steady state⁹ isotope methods performed sequentially in the same subjects during the acute illness and in convalescence produced quite different results (unpublished observations). The most sophisticated approach to this problem is probably that of Love and his colleagues^{4,10} who use a double isotope bolus technique during whole bowel perfusion. This enables full correction to be made for the passage of absorbed isotope back into the lumen. Using this method, they found that both fluxes were depressed in acute cholera, and that the flux from lumen to plasma was depressed more than the reverse flux. These changes in flux were particularly marked in the duodenum and proximal jejunum and are in striking contrast to the previously mentioned evidence. It may be that these

observations reflect epiphenomena, such as changes in the villous microcirculation or submucosal tissue pressure, rather than the primary mucosal effects of the toxin. However, they do illustrate that observations based on experimental cholera in animals should not be applied unhesitatingly to the natural disease, and that the exact nature of the disturbed fluxes in man is still not certain.

Intestinal Secretion versus Ultrafiltration

Practically all recently published work has agreed that the abnormal movement of fluid into the jejunal lumen cannot be explained on the basis of filtration through a leaky membrane. Thus, clearance rates of mannitol from plasma to lumen are relatively low compared with stool flow rates in human cholera¹¹ and, using the technique of paired molecular diffusion rates from lumen to plasma, there is no evidence that membrane 'selectivity' is altered¹². This suggests that there is no change in the permeability or 'effective pore size' of the jejunal mucosa in the human disease. Contradictory evidence suggesting an increase in pore size was obtained in an earlier report on experimental rabbit cholera¹³ but this work has been criticized on account of the extremely long duration of the experiments. Moreover, increase in pore size would not necessarily result in intestinal secretion of fluid. In dog experiments pore size was increased by amphotericin B but this was associated with increased fluid absorption rather than the reverse¹².

If the permeability properties of the mucosa are not altered by the cholera toxin, then very large hydrostatic pressure gradients would be required to account for fluid secretion rates on the basis of ultrafiltration¹⁴. It is a well known clinical observation that profuse stooling continues even when the patient is grossly hypotensive and dehydrated. Experimentally, marked reduction in mesenteric arterial pressure does not materially reduce the rate of fluid accumulation in canine Thiry-Vella loops¹⁵, although there has been one contradictory report to the effect that experimental loop distension pressure is linearly related to mesenteric arterial pressure¹⁶. Most evidence suggests that fluid secretion is not greatly dependent on total intestinal blood flow. Nevertheless it must depend on an adequate supply of fluid and sodium from the extracellular fluid of the lamina propria and this is presumably dependent on subtle changes in the microvascular circulation. Vasoactive drugs can influence the rate of fluid secretion experimentally and such evidence has been used to support the ultrafiltration hypothesis^{17,18}. However, it is not necessary to postulate that the cholera toxin exerts its primary and major effect on the microvasculature, although this has been suggested by some histological evidence¹⁹. Recent immunofluorescent studies, using antibodies to cholera toxin, suggest that the toxin is localized initially to the mucosal brush border and then to the cytoplasm of the mucosal cell for several hours, during which time none can be detected in the lamina propria or blood vessels²⁰.

The theory that cholera toxin induces mucosal secretion of fluid rests in part on the absence of evidence that mucosal permeability is altered and on the likelihood that very large hydrostatic pressure gradients across the mucosa are not generated. It must be admitted that the mechanisms whereby the lamina propria remains adequately hydrated are obscure and that recent studies in man⁴ cannot easily be fitted into this hypothesis.

Site of Intestinal Secretion—Crypts versus Crests

Recent evidence, especially from the Johns Hopkins group of workers, has suggested that intestinal secretion in experimental cholera arises from cells of the mucosal crypts rather than the villous crests. Treatment with cycloheximide, an inhibitor of protein synthesis, produced morphological changes in the crypt epithelium of rabbits and diminished fluid secretion in response to the cholera toxin²¹. This has been confirmed by other workers²² but does not apparently apply to a rat cholera model²³. The rabbit intestine after cycloheximide continued to absorb glucose normally and this was attributed to the integrity of the villous crests²¹. By contrast, exposure of the mucosa to hypertonic sodium sulphate produced frank histological damage in the villous epithelium but not in the crypts and was associated with impaired glucose absorption but unimpaired fluid secretion in response to cholera toxin²⁴. These workers have suggested that fluid absorption and secretion are properties of different parts of the mucosa, occurring in parallel, and do not represent alterations of the direction of fluid transport by individual mucosal cells.

A difficulty in accepting the implications of this hypothesis is that the same group of workers has shown that cycloheximide partially inhibits the secretory response to hypertonic glucose solutions placed in the gut lumen²⁵ although the mechanisms of this response to cholera toxin and hypertonic solutions are undoubtedly different²⁶. It seems as though the integrity of the rapidly dividing cells of the crypts is essential for the loops to respond to two different stimuli, and therefore rather unlikely that the toxin is specifically altering crypt cell function. There is no evidence that the toxin binds specifically to crypt cells and, in one study using immunofluorescent techniques, the toxin appeared to bind equally to crypt and villous mucosal cells²⁰. Further proof of this hypothesis will depend on subtler methods of separating these two types of mucosal cell without the damaging effects of cycloheximide or hypertonic solutions.

Mechanisms of Intestinal Secretion of Fluid—Role of Cyclic AMP

Great interest in this field has been provoked by an increasingly impressive body of evidence that the cholera toxin stimulates a cyclic AMP-mediated intestinal secretory process. In rabbit ileal mucosa *in vitro* cyclic AMP and theophyllin inhibit sodium transport from mucosa to serosa and provoke chloride movement in the reverse direction, changes which mimic the effects of cholera toxin *in vitro*²⁷. In canine Thiry-Vella loops *in vivo* prostaglandins and theophyllin infused into the superior mesenteric artery produce intestinal secretion of fluid, which closely resembles that induced by perfusing cholera toxin through the lumen²⁸. There is some evidence in man that perfusion of prostaglandins through the intestinal lumen induces secretion of fluid and sodium²⁹.

Cholera toxin stimulates the activity of mucosal adenyl cyclase^{30,31}, which converts ATP to cyclic AMP, and this is associated with increased tissue levels of cyclic AMP^{31,32}. In human cholera mucosal adenyl cyclase activity estimated on jejunal biopsy specimens is significantly higher during the acute phase of the illness than during convalescence^{33,34}. Experimentally, there is a close quantitative relationship between the time course of the onset of fluid secretion and increase of adenyl cyclase activity³⁵. It has also been shown that

prostaglandins stimulate jejunal adenylyl cyclase activity and increase tissue levels of cyclic AMP³¹. Neither cholera toxin nor prostaglandins affect the activity of the phosphodiesterase^{30,31}, which converts cyclic AMP to AMP and which is inhibited by theophyllin.

It has therefore been suggested that the primary action of cholera toxin is to activate mucosal adenylyl cyclase and that the cyclic AMP so formed is in some way responsible for the transport of fluid and ions across the cell and into the lumen. It now appears that adenylyl cyclase activity is associated with the lateral and basal membranes of mucosal cells and not appreciably with brush borders³⁶. On the other hand, the cholera toxin is a large molecule (M wt 84 000)³⁷ which appears to bind initially to brush borders²⁰. This suggests either that the toxin is in some way transported across the mucosal cells, or that it induces other changes which secondarily affect membrane-bound adenylyl cyclase. Perhaps these processes account for the characteristic delay of onset of frank secretion observed in most but not all³⁸ experimental situations. The precise way in which cholera toxin stimulates adenylyl cyclase activity is not known, and the mechanisms whereby cyclic AMP mediates its proposed effect on ion and fluid transport are quite obscure.

Much other evidence has suggested that sodium transport is intimately dependent on membrane-bound sodium, potassium-dependent ATPase³⁹. This is abundant in the intestinal mucosa and is also found mainly in lateral and basal membranes rather than in brush borders⁴⁰. Although there is some evidence that mucosal ATPase is depressed in acute human cholera, this does not appear to be a specific response and was observed in other diarrhoeal illnesses⁴¹. There has been no other experimental evidence to suggest a role for this ATPase in the pathogenesis of cholera and the effects of its inhibitor ouabain on experimental cholera have not been reported. As discussed elsewhere¹, the maintenance of normal glucose transport in natural and experimental cholera suggests that mucosal ATPase activity is intact, because active glucose transport is believed to depend on the maintenance of a low intracellular sodium concentration.

Parenteral Effects of Cholera Toxin

Evidence cited in the previous paragraphs suggests that the cholera toxin exerts its effect directly and perhaps exclusively on the intestinal mucosal cells. Clinically, the adverse effects of the illness can be entirely attributed to diarrhoea and, provided fluid replacement is adequate, the patient suffers no other untoward effects. On the other hand, parenterally administered toxin in dogs produces a complex variety of severe biochemical changes and often death, without associated diarrhoea⁴². Cholera toxin stimulates lipolysis in isolated fat cells⁴³ and glycogenolysis in liver homogenates probably by a direct effect on adenylyl cyclase⁴⁴. These observations suggest that in natural cholera absorption of toxin with its anticipated systemic effects is unlikely to play an appreciable role in the pathogenesis of the disease. On the other hand, Vaughan-Williams and his colleagues in a series of papers have shown that in infant rabbits cholera toxin can produce effects on adjacent loops not exposed luminally to the toxin⁴⁵, that parenteral toxin can induce diarrhoea⁴⁶, and that an absorbed toxic factor can be demonstrated by cross circulation experiments on paired animals⁴⁷. Somewhat similar observations on older rabbits have also been reported⁴⁸. It is difficult to reconcile these apparently dis-

crepant observations. They may be related to differences in the preparation and purity of the toxin used or to species or strain differences, and of course limited absorption of macromolecules is to be anticipated in neonatal rabbits. No recent work using purified toxin appears to have substantiated these claims and, as already stated, the relevance to the natural disease is probably minimal. However, the studies are of importance in relation to the possibility of using parenteral toxoid or antitoxin preparations prophylactically.

Pharmacological Modification of Altered Fluid Transport—a Drug Treatment for Cholera?

The effects of drugs on the altered fluid dynamics of cholera may shed light on pathophysiology and may have therapeutic relevance. Although the disease is self limiting and cure depends only on fluid replacement, a drug treatment aimed at stopping secretion could be invaluable in the practical management of large numbers of patients in an underdeveloped environment.

Ethacrynic acid inhibits fluid secretion in experimental canine cholera⁴⁹. This is of no therapeutic relevance, because the massive diuresis induced would further threaten the patient's fluid balance, and the mechanism of action is obscure. However, there is evidence of a 'second sodium pump' in the kidney, and perhaps in the colon, which is inhibited by ethacrynic acid but not by ouabain⁵⁰. It may be that this sodium pump is in some way involved in the altered movement of sodium and fluid across the mucosal cell in response to cholera toxin but its role in small gut sodium transfer has not been elucidated. Ethacrynic acid also inhibits toxin-induced lipolysis by isolated fat cells⁴³.

Actazolamide inhibits the secretion of sodium, bicarbonate, and fluid produced by cholera toxin in rabbit ileum *in vivo*⁵¹. This suggests that carbonic anhydrase may be involved in the generation and secretion of bicarbonate by the mucosal cells⁵² and, as already mentioned, accumulating ileal fluid in cholera is characteristically rich in bicarbonate. Earlier evidence in rats has shown that acetazolamide may also inhibit sodium and water transport by the ileum in addition to depressing bicarbonate secretion⁵³. The mechanism of these effects is obscure and may not even depend on the presence of carbonic anhydrase⁵⁴. Indeed activity of this enzyme is low in the small intestinal mucosa, at least of the guinea pig, and high in the colon⁵⁵, whereas the colon is apparently insensitive to the action of cholera toxin. In a rat cholera model, acetazolamide had no effect on fluid accumulation²³.

Antiinflammatory agents such as aspirin and indomethacin inhibit fluid accumulation in rat ileal loops exposed to cholera toxin⁵⁶. There is evidence that salicylates and indomethacin inhibit prostaglandin synthesis *in vivo*⁵⁷, and it has been suggested that cholera toxin may induce prostaglandin synthesis in the intestinal mucosa with secondary effects on adenyl cyclase⁵⁸. No therapeutic trial of these drugs in natural cholera has yet been reported.

Wider Implications of the Pathogenesis of Cholera

OTHER BACTERIAL ENTEROTOXINS

Escherichia coli

In regions where cholera is prevalent, many patients have an acute but milder

illness in which *Vibrio cholerae* is never isolated from the stools. In some but not all of such patients, certain strains of *E. coli* can be isolated in almost pure culture from intestinal contents⁵⁹. The alterations in small bowel fluid movements in these patients resemble closely those observed in cholera when studied by perfusion techniques⁶⁰. It appears that these strains of *E. coli* can produce one or more enterotoxin with effects on animal intestinal preparations similar but not identical to those produced by cholera toxin^{61,62,63}. The detailed properties and structure of *E. coli* enterotoxin(s) have not yet been clarified and, although it is speculated that they may also stimulate adenyl cyclase and act on the same final common path as cholera toxin, there is as yet no firm evidence that this is the case.

It should be remembered that these toxin-producing strains are not included among those normally regarded as pathogenic. Patients infected with other strains may show evidence of mucosal invasion and damage both in the small and large bowel⁶⁴. There are therefore at least two distinct ways in which pathogenic strains of *E. coli* can produce an acute diarrhoeal illness. The toxin-producing strains are closely analogous to cholera in the absence of frank mucosal invasion and the nature of the fluid secretory response.

*Other bacteria*⁶⁵

Other filtrable toxins active on the gut are produced by *Staphylococcus aureus* and *Clostridium perfringens*. There is evidence that staphylococcal enterotoxin B can impair fluid absorption in canine intestinal loops *in vivo*⁶⁶ and a toxin produced by several strains of Cl-perfringens induced ileal secretion of fluid in rabbits⁶⁷. There are no detailed reports available about the effects of these or other related toxins on ion transport by intestinal preparations.

HORMONAL EFFECTS—DIARRHOEA-PRODUCING TUMOURS

The concept that cyclic AMP may stimulate intestinal secretion raises the possibility that intestinal fluid transport may be regulated hormonally. Although a number of reports have appeared suggesting that various gastrointestinal hormones inhibit fluid absorption or even provoke secretion, the physiological relevance of these findings remains unknown and there is no definite evidence that they are mediated by mucosal cyclic AMP. The evidence will be briefly reviewed here, particularly in view of the probable relevance of these observations to the production of diarrhoea by certain tumours.

In studies *in vitro* in the hamster pentagastrin, secretin, and cholecystokinin inhibited sodium transport by jejunal and ileal everted sacs⁶⁸. In dogs pentagastrin *in vivo* inhibited ileal⁶⁹ and jejunal⁷⁰ transport of sodium, whereas secretin had no effect⁷⁰. No comparable effect of pentagastrin was found in rats using an *in-vivo* technique⁷¹ and perfusion studies in man have so far yielded conflicting information. Thus infusions of pentagastrin in man have been shown to inhibit⁷² and have no effect⁷³ on sodium and water absorption by the jejunum. In an abstract, inhibition of transport has been produced by secretin and glucagon in man⁷⁴, whereas glucagon was found to enhance absorption in the rat⁷⁵. Infusions of pentagastrin in man do not evidently produce diarrhoea, whereas a combination of gastrin and glucagon has produced diarrhoea in a small number of subjects⁷⁶. Recent interest has centred on another gastrointestinal polypeptide hormone—gastric inhibitory peptide (GIP)—which inhibits gastric acid secretion and promotes intestinal secretion of fluid by empty jejunal loops of dogs *in vivo*⁷⁷. It appears that several

gastrointestinal polypeptide hormones may modify intestinal fluid transport and even produce diarrhoea in man, but much of the present information is confusing and contradictory and has only been published in brief reports or in abstract.

In relation to tumour-associated diarrhoea it has been suggested that raised levels of circulating gastrin in the Zollinger-Ellison syndrome may be partly responsible for diarrhoea by some direct action on the intestinal mucosa, irrespective of luminal pH changes. Intestinal perfusion studies in patients after total gastrectomy have suggested impaired jejunal fluid absorption⁷⁸. However, the diarrhoea usually ceases after this operation; comparably raised gastrin levels in pernicious anaemia are not associated with diarrhoea and infusions of pentagastrin in normal subjects do not produce diarrhoea⁷⁶. In relation to the less well understood syndrome of pancreatic cholera, Grossman has suggested that the tumours may produce combinations of gastrin and glucagon⁷⁹ but there is no evidence to support this concept in the patients so far reported. Zollinger and his colleagues have provided some further evidence that the 'diarrhoeagenic hormone' is secretin or a secretin-like substance⁸⁰, but the detection of 'secretinomas' probably awaits the development of a sensitive radioimmunoassay technique. In one recently reported patient, the tumour appeared to be producing GIP⁸¹, referred to above. It may be that there are several 'diarrhoeagenic' hormones or combinations of hormones in patients of this type, and this is an area of very active current interest.

Summary and Conclusions

Much evidence suggests that the cholera toxin stimulates a specific mucosal secretory mechanism, especially in the proximal small intestine. It is possible that secretion arises from mucosal crypts rather than the villous epithelium. The toxin stimulates mucosal adenylyl cyclase activity either directly or indirectly and raised tissue levels of cyclic AMP are believed to mediate the altered movements of fluid and ions across the mucosa into the lumen. It is probable that other enterotoxins, especially those produced by some strains of *E. coli*, act in a similar fashion. There is fragmentary evidence that certain gastrointestinal polypeptide hormones influence the small intestinal handling of fluid and ions. This may be of physiological significance and may be relevant to the production of diarrhoea by certain tumours. There is no direct evidence as yet that these hormonal effects are mediated by cyclic AMP.

It is tempting in 1973 to accept that the cholera toxin is but one of several luminal and humoral factors which produce intestinal secretion and diarrhoea via a single final common path mechanism in the mucosal cell. However, there is evidence to suggest that this is not the sole pathophysiological disturbance, especially in the acute illness in man. It is difficult to separate primary from secondary disturbances and much still remains to be learnt about the detailed mechanism of action of the cholera toxin.

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