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EFFECT OF CHOLERA TOXIN ON PHOSPHORYLATION AND KINASE ACTIVITY OF INTESTINAL EPITHELIAL CELLS AND THEIR BRUSH BORDERS

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EFFECT OF CHOLERA TOXIN ON PHOSPHORYLATION AND KINASE ACTIVITY OF INTESTINAL EPITHELIAL CELLS AND THEIR BRUSH BORDERS

APPROVED BY

DISSERTATION COMMITTEE

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ABBREVIATIONS USED IN THIS WORK

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ATP - adenosine triphosphate

ATPase - adenosine triphosphatase

BSA - bovine serum albumin

cAMP - adenosine-3':5'-phosphate

Ci - curies

cm - centimeters

EDTA - (ethylenedinitrilo) tetraacetic acid, disodium salt

g - gram

g - gravity

l - liter

μ - micro

m - milli

M - molar

mg - milligrams

ml - milliliters

mm - millimeter

mol - mole

n - nano

NAD - nicotinamide adenine dinucleotide

TCA - trichloroacetic acid

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CHAPTER I

INTRODUCTION

Cholera is a terrifying disease. It strikes suddenly and massively, sweeping through entire families and villages. Typically the symptoms begin with a vague feeling of fullness in the abdomen. Then comes a feeling of giddiness and the victim begins to pass copious quantities of liquid stool--at first brown and then opalescent, the "rice water" stool of acute cholera. Excruciating cramps seize the victim's abdomen and limbs, and he goes into deep shock. If treatment is not started promptly, one-half to three-fourths of the people acutely stricken will die from dehydration within a few hours or after a few agonizing days. Treatment consists of monitoring the quantity of fluid and replacing, either intravenously or orally, this quantity, with fluid of the proper composition.

The volume of fluid lost is devastating. Over a period of three to four days, the total infusion of fluid to replace that which has been lost, may equal twice the patient's body weight. The fluid that is lost is essentially the same composition as that of blood plasma. The body cannot tolerate such massive ion losses. Potassium depletion can cause disturbances to heart rhythm, or even heart stoppage, profound weakness and possibly paralysis. Bicarbonate losses lead to acidosis which in turn causes a constriction of the blood vessels and a weakening of the heart action (1).

Evidence to date suggests that this massive fluid loss is mediated by a single enterotoxin produced by the bacterium, Vibro cholerae. This heat and acid labile toxin has been extensively purified by LoSpalluto and Finkelstein (2). They have found it to be a protein, essentially free of any lipid or carbohydrate components, with a molecular weight above 80,000. This toxin acts upon the entire bowel to produce the characteristic fluid loss (3) but produces no discernible difference in mucosal morphology (4).

In the normal intestine both absorption and secretion occur simultaneously. The fact that absorption and secretion are two separate processes accounts for what seems to be striking species differences to the secretory stimulus of cholera enterotoxin. Different species have very different rates of absorption from the intestine (5).

Suggested in the literature are two possible driving forces for choleric intestinal secretion: active transport by the epithelium and a hydrostatic pressure differential from the interstitial tissue to lumen (6). Gordon (7) compared intestinal clearance of sodium and mannitol in two patients with acute cholera. The difference indicated that more resistance to mannitol was present than to sodium. This was not the case for excess serosal pressure, since Hakin and Lifson (8) found the clearance for mannitol and sodium to be identical when secretion was induced by increased serosal pressure. On the basis of Gordon's observation it seems highly probable that the fluid loss results from an active secretory process of the intestinal epithelial cells and not from a pressure induced flow.

There is direct evidence for active secretion in cholera. The short circuit technique of Ussing and Zerahn (9) permits study of electrolyte transfer across biological membranes in the absence of electrical and chemical gradients. Using this technique, it was found that there was net absorptive flux of both Na^+ and Cl^- in isolated ileal mucosa and that adding glucose increases the Na^+ absorption. Field (10, 11, 12, 13) found that added cAMP or theophylline caused a large increase in the short circuit current. The net Na^+ flux was reduced to zero; the Cl^- flux was reversed, and Cl^- secretion occured. Cholera toxin, after a suitable lag period, produces the same effect (14).

The increased resistance across the luminal border of the individual cells after exposure to theophylline, cAMP or cholera toxin supports the concept that the restriction of sodium entry rather than inhibition of pumping is the more likely explanation for the zero Na^+ flux seen after exposure to these compounds (15). Added glucose restores the Na^+ absorption, indicating that the active transport of the cell can be subdivided into substrate dependent and substrate independent components (16).

The resultant net Cl^- secretion in the absence of electrochemical gradients (i.e. short circuited) suggests the presence of active Cl^- transport from the serosal to luminal surfaces of the intestine (17). This was not just an in vitro phenomenon, because increased Cl^- secretion against an electro-chemical gradient has also been demonstrated in the isolated perfused ileal loop after exposure to cholera toxin (18).

Certain prostaglandins mimicked the secretory effect of cholera toxin when infused into the superior mesenteric artery of dogs (19). There were two major differences between cholera toxin and other compounds that increase cellular levels of cAMP. Secretion only occured as long as the compounds were being infused. When they were removed secretion stopped. Mucosa exposed to cholera toxin continued to secrete even after extensive washing. It only stopped when the cells exposed to cholera toxin were shed from the villi.

The other difference was the time interval between exposure to the stimulating compound and the start of secretion. With prostaglandins, theophylline, and di-butyryl cAMP, secretion started immediately after exposure. With cholera toxin, there was a lag time of two to three hours between the initial exposure and the beginning of secretion (20).

Other investigators indicated that the link between cholera toxin and cAMP was casual, not secondary. In all reported cases, cholera toxin caused the same tissue response, after an initial lag period, as did cAMP (21, 22). Fat cells responded by releasing their stored lipids, and liver cells and blood platelets released their stored glycogen (23).

Final proof that the intestinal response to cholera toxin was mediated by an increase in the secondary messenger, cAMP, came when it was shown that after exposure to cholera toxin mucosal adenyl cyclase activity increased causing heightened levels of cAMP (24, 25, 26, 27, 28, 29, 30).

The next question was whether cholera toxin caused an increase in the synthesis of adenyl cyclase or if it just activated the adenyl cyclase already present in the cells. Protein synthesis seemed implicated because cycloheximide, a potent protein synthesis inhibitor, given ninety minutes before exposure to the toxin, inhibited secretion, and because of the lag time between exposure to the toxin and the start of secretion (31).

Enhanced adenyl cyclase activity after cholera toxin exposure was demonstrated by Sharp and Hynie (32) in rabbit intestinal mucosa. They also showed that the phosphodiesterase level in both control and cholera toxin exposed animals was the same. NaF added to the toxin-activated adenyl cyclase did not further enhance the cyclase activity. This was interpreted to mean that no new adenyl cyclase was synthesized, but that the adenyl cyclase already present was activated fully by the cholera toxin. Later studies confirmed this by showing that cAMP levels were still elevated after cholera toxin exposure even if cycloheximide had been given ninety minutes prior to cholera toxin exposure and secretion was inhibited (33).

The mechanism by which cholera toxin activates the adenyl cyclase is not known. Studies have shown that the adenyl cyclase is located in the basal membrane of the intestinal epithelial cells (Figures 1 and 2) with very little activity being found in the brush borders (34, 35). Since cholera toxin first comes in contact with the brush borders (36), which are on the opposite side of the cell from the lateral membrane, this could be a contributory factor in the latent period between toxin exposure and fluid production.

When colchicine, a drug that disrupts the microtubules in cells, was given any time up to forty minutes after the administration of cholera toxin, secretion was inhibited.

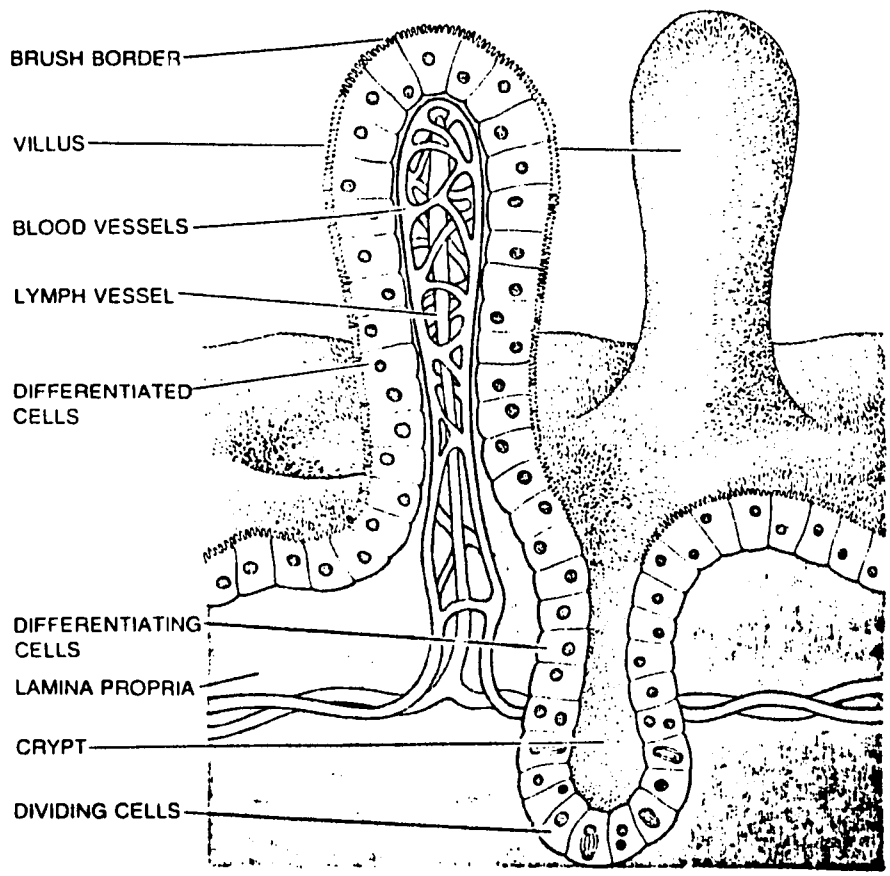
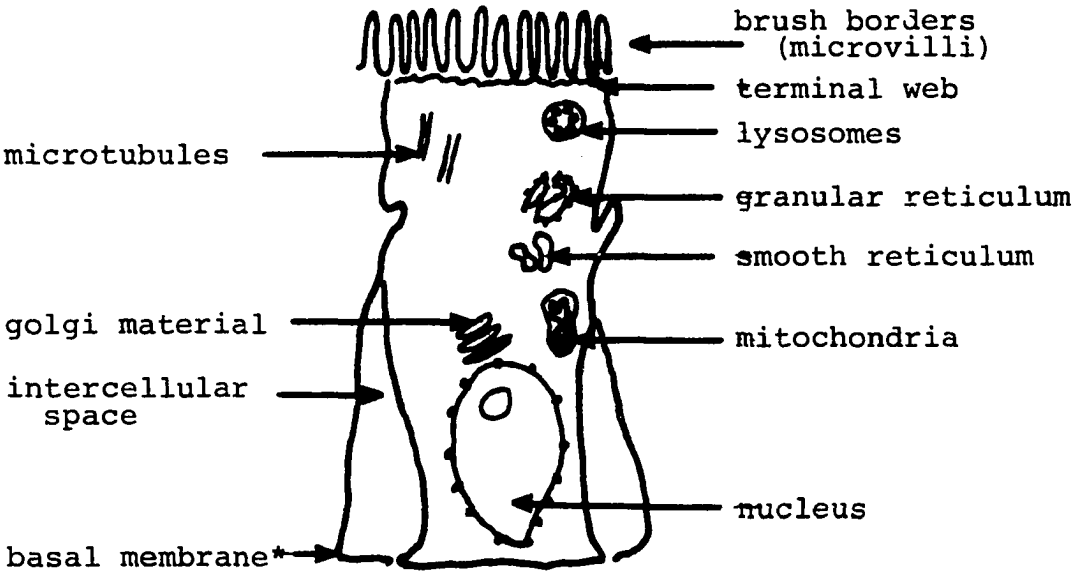


Figure 1 - Cross-sectional view of the villi of the small intestine. The mucous membrane has a connective-tissue layer (lamina propria), which contains blood and lymph capillaries, and an inner surface of epithelial cells. The cells multiply and differentiate in the crypts and migrate to the villi, where they are shed into the lumen from the villi tip. Each day 74 per cent of the cells lining the intestine are lost. [From "Lactose and Lactase" by Norman Kretchmer. Copyright © 1972 by Scientific American, Inc. All rights reserved.] (37)



*location of adenyl cyclase and Na^+, K^+ - ATPase

Figure 2 - Schematic diagram of an epithelial mucosa cell from a mammalian intestinal villi.

This was seen as evidence for some portion of the cholera toxin molecule being transported to the basal membrane and thus accounting for the lag time (38).

In summary then, all the evidence to date suggests that cholera toxin acts by causing an irreversible modification of the existing cellular adenyl cyclase, causing it to become fully activated and thereby increasing cellular levels of cAMP.

But the mechanism by which cAMP regulates intestinal secretion has not yet been elucidated.

Kuo and Greengard have noted that cAMP dependent protein kinases are distributed widely among various tissues and species and have hypothesized that all physiological effects of cAMP are due to the activation of such a kinase (39, 40). Figure 3 presents this generalized concept of cAMP action in a diagramatic form.

Control by chemical modification, i.e. phosphorylation, has several advantages and characteristics that distinguish it from other methods of regulation. Proteins modified by allosteric effectors or regulatory proteins revert back to their original form when the effector is removed. Chemically modified proteins retain their modified character even after the effector has been removed. Reversion of the chemical modification is not initiated unless an effector for the back reaction is present.

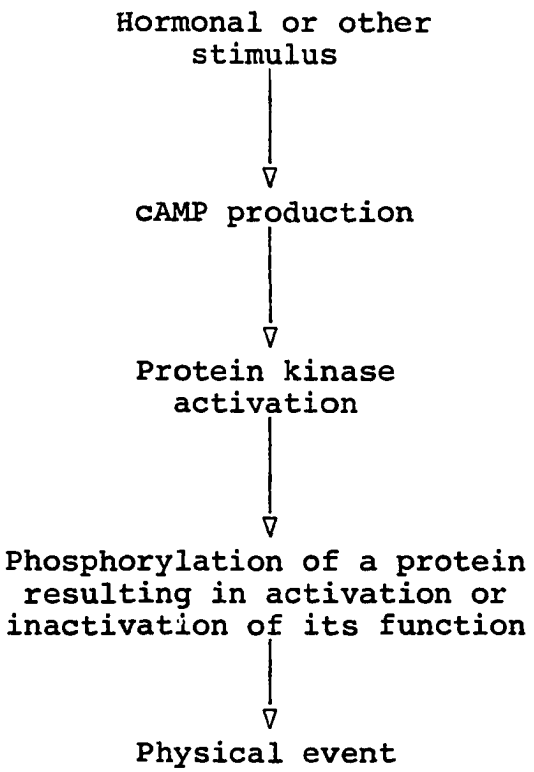
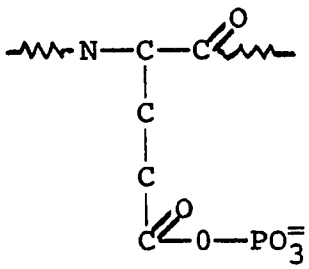


Fig. 3 - Hypothetical scheme for hormonal regulation of cellular functions involving cAMP serving as an activator of protein kinase.

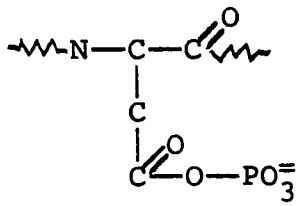
Time and energy conservation are two more advantages of chemical modification. In mammalian tissue, adaptation to metabolic requirements by de novo synthesis of the proteins requires several hours before becoming effective. Phosphorylation and dephosphorylation of intraconvertible enzymes can be completed in a matter of minutes. The energy requirements for phosphorylation are several magnitudes lower than that needed for de novo synthesis of an enzyme. Also, inactivation by protein degradation wastes more energy than dephosphorylation (41).

Figure 4 shows the amino acids that have been found to be phosphorylated by kinase action in proteins. The acyl phosphate bond of glutamic and aspartic acid has been found in Na^+, K^+-ATPases (42). Histidine has been found to be the phosphorylated species in the phosphate carrier protein of bacterial sugar transport systems (43). These two types of phosphorylation are mediated by kinases that are not activated by cAMP. The cAMP dependent kinases described to date phosphorylate the protein on serine or threonine (44, 45, 46).

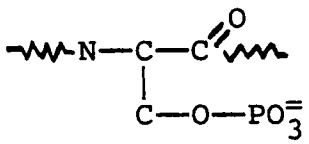
There have been an increasing number of examples reported in the literature that support this hypothesis of kinase activation. Modification by phosphorylation and dephosphorylation has been demonstrated for the following enzymes from mammalian tissues: glycogen phosphorylase (47, 48), phosphorylase b kinase (49, 50, 51), glycogen



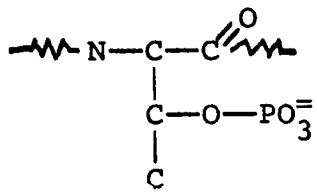
phosphorylated
glutamic acid



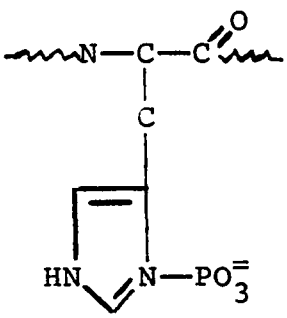
phosphorylated
aspartic acid



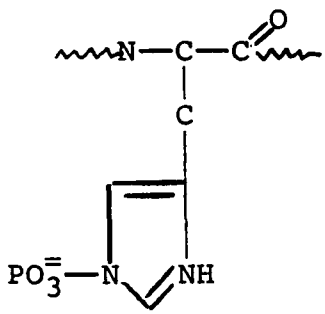
phosphoserine



phosphothreonine



N-1-phosphohistidine



N-3-phosphohistidine

Figure 4 - Known phosphorylated protein structures.

synthetase (52), fructose diphosphatase (53), and the pyruvate dehydrogenase complex (54, 55), phosphorylase a phosphatase (56, 57, 58), glycogen synthetase phosphatase (59, 60), lipase (61), and palmityl-CoA synthetase (62, 63, 64). In vivo histone phosphorylation has been detected and the suggestion has been made that phosphorylation and dephosphorylation in histones are involved in selective gene expression (65, 66). RNA polymerase is activated by the transfer of phosphate from ATP to the sigma factor by a cAMP-activated kinase (67).

Examples of cAMP stimulated phosphorylation have been reported (68, 69) and this type of phosphorylation may be a common mechanism for regulating membrane permeability or secretion. Kuo and Greengard have shown that there is a kinase and substrate in the brain plasma membrane (45). They postulated that its function is to change by phosphorylation and dephosphorylation the permeability of the brain membrane and that this could be the mechanism for short term memory.

Isolated neurotubule subunits are phosphorylated in the presence of a cAMP stimulated intrinsic protein kinase (70). A mediation in this way of the cAMP effect on neurotransmitter release and of secretory processes in general is a possibility.

It should be noted that the final protein phosphorylated could be an enzyme, a nuclear protein, a membrane

protein, a ribosomal protein, or some other protein involved in a critical control point (71).

Therefore, I decided to determine whether the cholera toxin elevated levels of cAMP in intestinal mucosa activated a mucosal kinase which phosphorylated protein involved in intestinal secretion (Figure 5).

According to the scant literature on intestinal secretion, the brush borders are not involved in the secretion process. For the following reasons the major portion of intestinal secretion has been attributed to the crypts of Lieberkühn (Figure 1). Biopsies of intestinal tissue that had been exposed to picrocine, a drug that induces secretion, revealed both merocrine and aprocrine secretion occuring in the crypts of Lieberkühn (see Figure 6 for term definitions). Such processes were not seen in the more differentiated cells of the villi (72).

When cycloheximide was administered it was found that most mitotic activity of the crypt cells stopped in the G2 phase. No such mitotic arrest is seen in the villi cells (73). When cholera toxin is given ninety minutes after cycloheximide, secretion caused by the toxin is markedly reduced, thereby suggesting that the crypt cells are the major site of fluid production (74).

Further evidence for the crypt involvement in secretion was obtained by selectively damaging the villi but not the crypt cells by injecting hypotonic doses of sodium

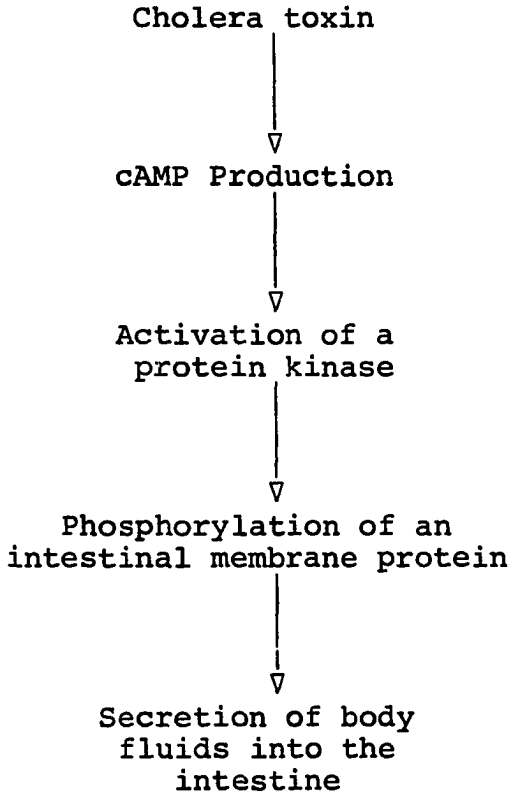
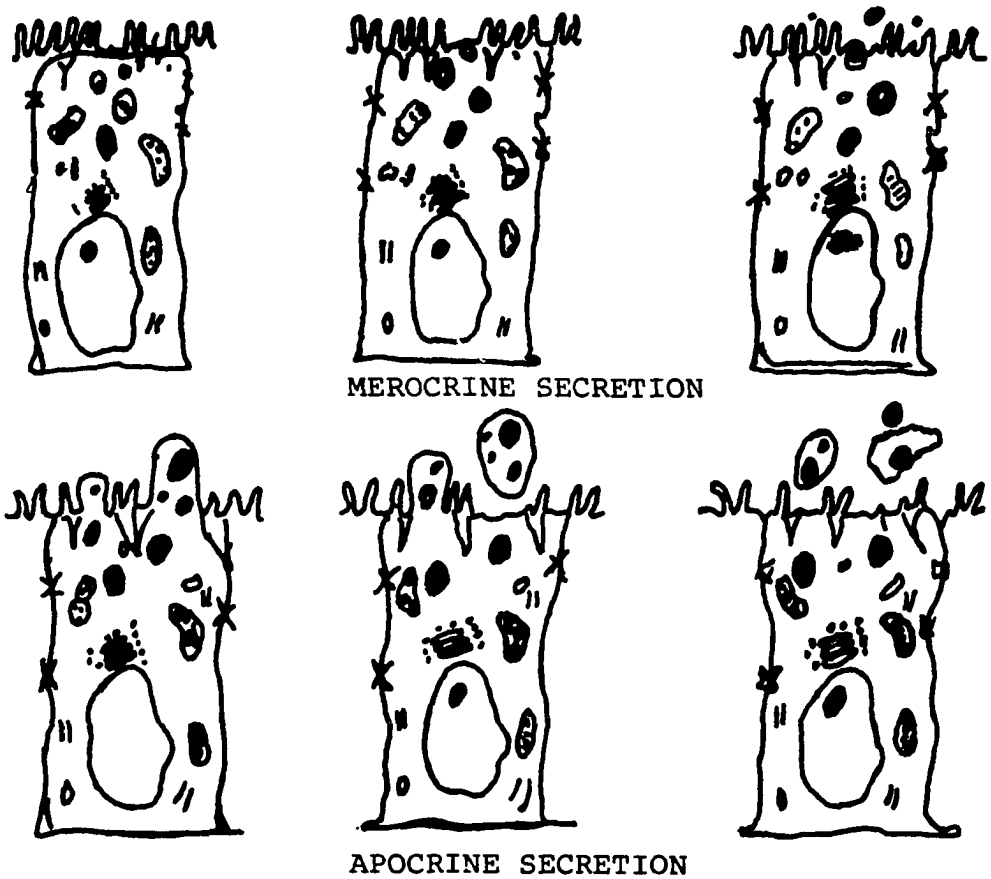


Fig. 5 - Diagrammatic scheme of the proposed mechanism of action of cAMP to explain intestinal secretion.



MEROCRINE SECRETION - the secretory granules migrate through the undifferentiated cell cytoplasm to the apical surface of the cell, the fine membranes surrounding the granules then fuse with and become part of the apical cell membrane as the contents of the secretory granules enter the crypt lumen.

APOCRINE SECRETION - portions of the cytoplasm in addition to preformed secretory substances such as secretory granules are shed by the cell.

Figure 6 - Schematic illustration and definitions of merocrine and apocrine secretion by undifferentiated crypt cells (72).

sulfate into the intestine. Although the villi were indeed damaged, as evidenced by the lack of glucose absorption, typical secretion occured after exposure to cholera toxin (75).

This evidence for the crypt cells playing the major role in intestinal secretion is largely circumstantial. It does not necessarily follow that these cells play a major role in cholera induced secretion.

Cycloheximide is not selective in arresting just crypt cells in the G2 phase. After administration, mitotic arrest can be seen in any tissue that has rapidly dividing cells (75). Since the cells that are proliferating the fastest in intestinal mucosa are the crypt cells (76) they would be the ones that would be expected to be found in mitotic arrest after cycloheximide treatment. Cycloheximide is not specific for the protein synthesis involved in mitosis; it inhibits any protein synthesis. Therefore, the inhibitory effect that cycloheximide has on intestinal secretion after cholera toxin exposure could result from inhibited formation of a protein necessary to the enhanced secretion process. This would not result in a change visible with microscopy.

Cycloheximide appears to inhibit the synthesis of a protein necessary to choleric secretion apart from inhibiting the proteins necessary for the crypt cell to leave the G2 phase. Evidence for this view is as follows: when

cycloheximide is given to animals with a well established choleric secretion, the decrease in secretion does not occur on the same time scale as the changes in mitotic activity. This decrease in choleric secretion after cycloheximide treatment supports the concept of a protein with a fairly short half life being synthesized that is important in the secretion process (77).

The villi exposed to hypotonic sulfate solutions have been assumed to be non-functional because glucose absorption was stopped. But if the villi were responsible for both absorption and secretion then it seems reasonable that absorption could be stopped without secretion being damaged. Absorption and secretion are assumed to be entirely separate processes because cholera toxin affects one but not the other. In choleric secretion, the absorption remains normal (78).

Furthermore, cholera toxin is known to become attached to the brush borders (79) and it is known that cholera toxin affects the cells with which it comes in contact with by raising the cAMP level. The higher levels of cAMP in the villi cells would undoubtedly have some effect on cellular processes.

It does not seem reasonable to say that it has unequivocally been shown that the crypt cells are primarily responsible for secretion since the evidence supporting this concept is largely circumstantial. It would appear that a

role for brush borders in secretion is a possibility.

Elucidation of the mechanism by which cAMP affects intestinal secretion would have several benefits. A better understanding of intestinal function would be obtained. It would provide the pharmacologist with a rational approach to finding a drug treatment for cholera. A drug that would inhibit intestinal secretion would be a much preferable treatment in terms of time, cost, availability and patient discomfort than the fluid replacement treatment now in use. Also, a better understanding of diarrhea in general would be obtained, because whatever this mechanism turns out to be, it is not unique to cholera. Diarrhea from other causes seems to be mediated in the same way, i.e., an increase in cAMP levels in intestinal mucosa.

It has been shown that toxin derived from the pathogenic E. coli can also stimulate adenyl cyclase in the small intestine (80). However, the mechanism by which the E. coli toxin affects the adenyl cyclase appears to be different, because after the toxin is washed off, the adenyl cyclase returns to its normal level of activity (81) unlike that of cholera toxin.

Similarly an impure preparation of phospholipase C from Clostridum welchii caused fluid production and increased adenyl cyclase activity in rabbit intestinal mucosa (27). Medullary carcinomas of the thyroid with metastases are commonly accompanied by watery diarrhea. These tumors are

associated with increased production of prostaglandins (82).

Thus the understanding of the action of cholera toxin and the development of an antidote would have far reaching effects for the treatment of cholera and many other serious diarrheas.

CHAPTER II

MATERIALS AND METHODS

In Vivo Phosphorylation of Intestinal Mucosa by ^{32}P

White female rats of the Stanley-Gumbreck strain were injected intraperitoneally with 0.5 mCi of ^{32}P (as Na_3PO_4) and fasted twelve hours before use. They were then anesthetized with ether and an abdominal incision made. The intestine was tied into two segments of 10 cm each which were separated by a 4 cm segment. The first loop started about 1 cm distal to the pylorus. Cholera toxin (1 ml of 1 µg cholera toxin per ml of 0.145 NaCl solution) was injected into one loop and normal saline into the other. Since it has been shown that the elevation of cAMP levels due to the action of cholera toxin varies in the various parts of the intestine, upper and lower loops were alternatively used for controls. The incision was then closed. After four hours, the rats were again anesthetized and the loops removed. The original injected fluid was rapidly absorbed by the intestine, but at the end of four hours, the loops injected with cholera toxin had refilled in response to the toxin. The removed loops were immediately rinsed with cold saline

and the epithelial cells isolated.

Preparation of Epithelial Cells (83)

One end of the intestinal loop was clipped off. The contents were then drained and the loop was washed with cold saline. It was filled with a pH 7.2 solution containing 96 mM NaCl, 1 mM KH_2PO_4, 50 mM sodium citrate, 5.6 mM Na_2HPO_4, 1.5 mM KCl, 2.5 mg/ml serum albumin, and 1.5 mg/ml hyaluronidase. The citrate and hylauronidase dissociated the epithelial cells while the BSA has been reported to stabilize the epithelial cells. The loop was then retied and placed in a beaker containing 30 ml aerated saline and incubated 15 minutes at 33° with agitation, The solution was removed and the intestine flushed with cold saline. It was then partially filled with an oxygenated medium containing 137 mM NaCl, 11.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.2 mM KCl and 5 mg/ml BSA, pH 7.2. The segment was then squeezed with the fingers on the serosal side. The contents of the lumen were drained. The manipulation of the tissue was repeated twice and the contents collected each time. The collected luminal contents were observed microscopically and then centrifuged at 200 g in the cold. The pellet was washed in 20 volumes of collection fluid without the BSA and then re-centrifuged. Part of the epithelial cells were used to prepare brush borders and part of them were precipitated with 5 per cent TCA. This precipitated protein was then washed and analyzed for protein and ^{32}P.

Brush Border Preparation

Initial brush border preparation was done by the method of Miller and Crane (84). Subsequent preparations were done by the method of Forstner (85).

Miller and Crane Method

The prepared epithelial cells were put into 5 mM EDTA, pH 8.0. They were then homogenized for 30 seconds on the Polytron homogenizer at a speed setting of 2.8. The homogenate was then passed through bolting silk and centrifuged at 450 g for 10 minutes at 4°. The pellet was washed twice with the 5 mM EDTA buffer. The brush borders were examined microscopically and then added to 5 per cent TCA. This precipitated protein was then washed and analyzed for protein and ^{32}P.

Forstner Method

The intestine was washed with normal saline and then everted. The mucosa was scraped off with a glass slide. The scrapings were placed in 20 ml cold 2 mM EDTA adjusted to pH 7.4 with NaOH and homogenized in a Dounce homogenizer for 15 seconds. This was called the homogenate in the various experiments. The homogenate was then centrifuged for 5 minutes at 450 g. The supernatant solution from this centrifugation was designated the "supernate." The sediment was washed in 20 ml of 5 mM EDTA buffer and recentrifuged. It was then suspended in two volumes of 90 mM NaCl-0.8 mM EDTA

buffer, mixed thoroughly and kept in ice until a well-defined sediment developed, about 20 minutes. The supernate and the sediment were then poured through a pad of glass wool to remove aggregated particles. After the sediment on the glass wool had been washed with another 20 ml of 5 mM EDTA buffer, brush borders in the total washings were sedimented by centrifugation at a 450 g for 10 minutes. After washing once with 5 mM EDTA buffer and recentrifugation the pellet of brush borders was suspended in a convenient volume of 2.5 mM EDTA buffer. This method of brush border preparation is outlined in Figure 7.

Washing of In Vivo Phosphorylated Protein

Each TCA precipitate was washed consecutively with the following solutions: 2 x 5 ml of H_2O, 5 ml of H_2O: ethanol (1:4), and 3 ml of ethyl ether: ethanol (1:3). Then 3 ml of 5 per cent TCA was added and the mixture was heated at 96^{o} for 10 minutes. After cooling to room temperature, the precipitate was rewashed with 5 ml of 5 per cent TCA (86). The precipitate was then dissolved in 0.5 ml of 0.1 M NaOH and 100 µl aliquots were taken for protein determination and for counting.

Synthesis of [γ^{32}P] Adenosine Triphosphate

This method was essentially that of Glynn and Chappell (87). Into a 12 ml screw cap centrifuge tube the following were added in the order given: 1 ml H_2O, 0.1 ml

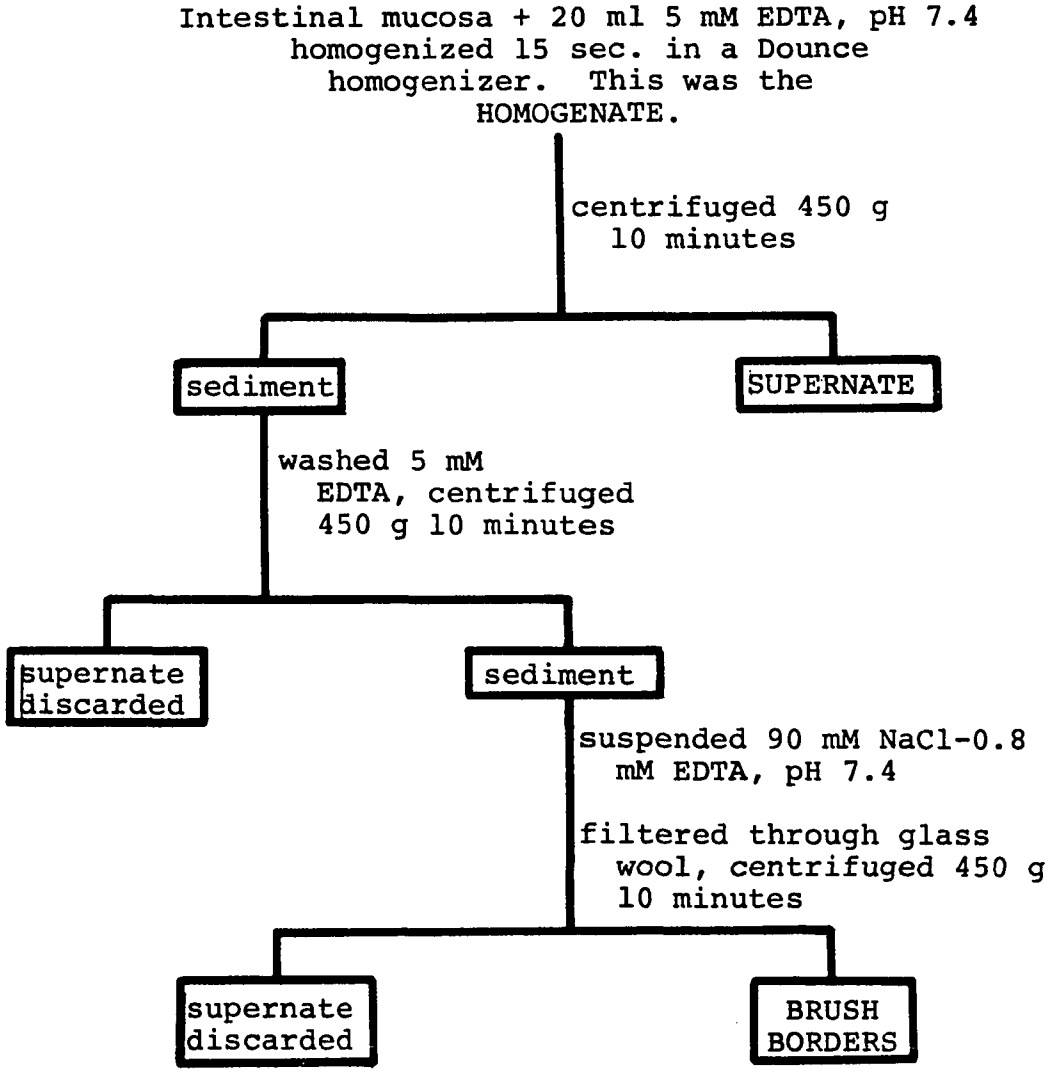


Figure 7 - Flow sheet showing preparation of brush borders by the Forstner method.

0.5 M Tris buffer pH 8.0, 6 µmol $MgCl_2$, 0.1 ml 0.1 M NaOH, 2.0 µmol cysteine HCl, 6.0 µmol disodium adenosine triphosphate, 1.0 µmol phosphoglycerate tricyclohexylammonium salt, 10 µl (14 units) muscle glyceraldehyde 3-phosphate dehydrogenase suspended in 2.5 M ammonium sulfate, 50 µl (160 units) yeast phosphoglycerate kinase in 2.7 M ammonium sulfate, 0.1 µmol NAD and 1 mCi ^{32}P as Na_3PO_4. After complete mixing the tube was capped and incubated for 1 hour. The reaction was stopped by heating at 96° for five minutes. The solution was cooled in ice. After cooling, it was passed through a column prepared by pouring a 1 cm high column of celite, then 300 mg of Norit A on top and then another column of celite, 3 mm high, all in a clean, coarse-porosity sintered-glass Buchner funnel, 2.2 cm in diameter, under a vacuum created by a water aspirator. To wash the loaded column 2 x 10 ml H_2O were used. The [$\gamma^{32}P$] ATP was eluted with 25 ml of 50 per cent w/w ethanol containing 0.25 ml concentrated NH_4OH. This solution was used at room temperature, but the eluant was collected in a flask immersed in ice. The [$\gamma^{32}P$] ATP solution was transferred to a 500 ml round bottom flask and taken to dryness. The dried compound was then taken up in 1 ml of H_2O.

Characterization of the [$\gamma^{32}P$] ATP

One and 5 µl samples were spotted on thin-layer cellulose plates. The plates were run in 125 ml isobutryic

acid, 75 ml 1 M NH_4OH and 2 ml 0.1 M EDTA (88). This solvent separates AMP, ADP and ATP from each other. The nucleotide spots were detected by UV. The spots were scraped off and put in 3 ml of water. After mixing on the vortex mixer and centrifugation in the clinical centrifuge, the aqueous supernate was read at 264 nm to determine the ATP concentration. A 100 µl sample of the aqueous solution was counted. From this data the counts/minute/µmol [$\gamma^{32}P$] ATP were calculated. The specific activity of the [$\gamma^{32}P$] ATP preparations used in the kinase assays were usually about 10^5 counts/minute/µmol [$\gamma^{32}P$] ATP. Each radioactive ATP preparation lasted for about one month; except for sampling it was kept frozen during that period.

Tissue Fractions for Kinase Assay and Endogenous Phosphorylation by [$\gamma^{32}P$] ATP

White female rats, about 100 g in weight, of the Stanley-Gumbreck strain were fasted for 12 hours before use. They were then anesthetized and an abdominal incision made. One loop of 20 cm was made in the intestine starting about 1 cm distal to the pyloric valve. The loop was then injected with 2 ml of cholera toxin (1 µg cholera toxin/ml in 0.145 M NaCl) or for controls with 2 ml normal saline. The incision was sutured. At the end of four hours the intestinal loops were removed and rinsed with cold normal saline and tissue fractions prepared.

Kinase Assay

Kinase activity was measured in a standard assay mixture (45) consisting of 10 µmol sodium acetate buffer pH 6.2, 1.0 mµmol of [γ^{32}P] ATP 2.0 µmol magnesium acetate, 2.0 µmol sodium fluoride, 0.4 µmol theophylline, 0.06 µmol of EDTA, 0.5 mg substrate, usually histone, Sigma Type II, with or without 1 mµmol cAMP and 0.1 ml of the tissue to be assayed in a total volume of 0.2 ml. The reaction was run at 30° C. for 10 minutes. It was stopped by adding 5 ml of 5 per cent TCA. Two-tenths milliliters of 0.63 per cent BSA were then added as a carrier protein. After cooling in ice for 1 hour, the precipitate was collected by centrifugation for 3 minutes in a clinical centrifuge. The precipitate was washed by dissolving in 0.3 ml 0.1 M NaOH and then re-precipitated by adding 5 ml 5 per cent TCA and cooled in ice for an hour. This procedure was repeated twice. Then the precipitate was dissolved in 0.3 ml 0.1 N NaOH, 10 ml counting solution added and the sample counted. All assays were done in triplicate.

Endogenous Phosphorylation

Brush borders from an entire small intestine were suspended in 25 ml 5 mM EDTA and divided equally into 6 parts and centrifuged at 450 g in the clinical centrifuge for 10 minutes. One of the samples was resuspended in 1 ml of H_2O. This sample was used to determine the protein

concentrations of the others. To each brush border sample was added 0.2 ml of the assay mixture (45) consisting of 10 µmol sodium acetate buffer, pH 6.2, 1.0 mµmol [γ^{32}P] ATP, 2.0 µmol magnesium acetate, 2.0 µmol sodium fluoride, 0.4 µmol theophylline, 0.06 µmol of EDTA, with or without 1 mµmol cAMP, and immediately mixed on the vortex mixer. After incubating at 30° the reaction was stopped by the addition of 5 ml of 5 per cent TCA. The protein precipitate was then washed either by the method used when washing *in vivo* phosphorylated protein or by the alkali method described under the kinase assay. Both methods gave comparable results. After washing, the protein was dissolved in 0.5 ml 0.1 N NaOH and counted. All assays were done in triplicate.

Alkaline Phosphatase Assay

Alkaline phosphatase was determined with p-nitrophenyl phosphate (Sigma 104) as the substrate. The assay system contained 25 µmol glycine buffer, pH 9.2, 2.5 µmol of $MgCl_2$, 0.5 µmol of $ZnCl_2$, and 9.0 µmol substrate and 0.1 ml tissue preparation in a total volume of 0.6 ml. Enzyme and substrate were incubated for 10 minutes at 37° and the reaction terminated by the addition of 2.5 ml of 0.02 N NaOH. Free p-nitrophenol was determined spectrophotometrically at 400 nm. One unit of enzyme activity is equal to 1 µmol of substrate hydrolysed/minute (86).

Agarose Column

Agarose (Bio-Gel A-50 m, 100-200 mesh) was equilibrated with 6 M guanidine HCl which contained 0.1 per cent 2-mercaptoethanol. A glass column 100 x 2 cm, thinly coated on the inside with paraffin, was loaded with the agarose. After running 30 ml of the 6 M guanidine HCl, 0.1 per cent 2-mercaptoethanol through the column, 1 ml of dissolved brush border was placed on the top of the agarose. The column elution rate was 0.5 ml/hour. One milliliter fractions were collected. The 280 nm absorbance was measured for each tube. A 100 µl sample of each milliliter was counted for ^{32}P.

Preparation of Brush Borders for Column

Brush borders (from one rat) which had been phosphorylated *in vivo* either with or without cholera toxin in the intestine, were dissolved in 2 ml of 6 M guanidine HCl, 0.1 per cent 2-mercaptoethanol.

SDS Polyacrylamide Gel Electrophoresis

This is essentially the method of Weber, *et al.*(89).

Preparation of the Gels

Three solutions were made. Solution A was produced by dissolving 2.74 g $NaH_2PO_4 \cdot H_2O$, 0.4 g. SDS and 0.2 ml N,N,N,N,-tetramethylethylethylenediamine (TEMED) in 25 ml H_2O. adjusting the pH to 7.20 with NaOH and diluting to 50 ml with H_2O. Solution B was composed of 10 g acrylamide and

0.26 g of methylbisacrylamide dissolved in H_2O and brought to a final volume of 50 ml. These two stock solutions were stored at 4°. Solution C contained 0.76 g of ammonium persulfate in 50 ml H_2O. This solution was made up fresh each time gels were prepared. Gels with increased and decreased cross-linking contained twice and one-half the concentration of methylbisacrylamide, respectively. To prepare the gels, 1 ml of A, 2 ml of B, 4 ml of C and 1 ml H_2O were mixed and immediately placed in clean glass tubes 10 cm long. The tubes were filled to within 2 cm of the top. A few drops of water were placed on the top until the gels formed. After the gels formed, the water was removed and 100 µl of the dissolved brush borders and 1 drop of glycerol were added to the top of the tube.

Running of the Gels

The buffer for the electrophoresis contained 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g of $Na_2HPO_4 \cdot 7H_2O$, and 2 g of SDS per liter. The tubes were run at constant current of 8 ma per tube. The gels were typically run for two hours.

Staining of the Gels

The gels were stained in a solution of 0.145 g Coomassie brilliant blue in 454 ml of 50 per cent methanol and 46 ml glacial acetic acid. Staining was at room temperature for various time periods of 2 to 24 hours. The gels were destained by removing from the staining solution and

washing in distilled water. They were then placed in a destaining solution that consisted of 75 ml acetic acid 50 ml methanol and 875 ml water. The destained gels were scanned on the Gilford spectrophotometer at 570 nm to determine the density of the various bands and then sectioned. The counts of ^{32}P were then measured for each section.

Paper Electrophoresis

The paper chromatogram was run in a pH 1.8 buffer composed of 75 g acetic acid and 25 g of 80 per cent formic acid in 1 liter water, at 50,000 volts for either 1 or 2 hours (90). After the chromatogram was dried it was sprayed with 0.1 per cent ninhydrin in acetone. After development of the spots, the chromatogram was cut into 1 cm strips and the strips were counted.

Hydrolysis of the Protein for Paper Electrophoresis

The TCA precipitated protein from *in vivo* phosphorylation experiments was washed in the standard manner. The washed precipitate was hydrolyzed in a sealed evacuated glass tube at 96° C. for time periods varying from 1 to 6 hours with HCl varying in concentrations from 1 to 6 molar. The hydrolysate was chilled and taken to dryness on the rotary evaporator. The residue was washed once in 0.5 ml H_2O and again taken to dryness. It was dissolved in 0.1 ml H_2O and spotted on the paper for electrophoresis. Phosphoserine and phosphothreonine were hydrolyzed under identical

conditions. Brush borders and phosphoserine were also hydrolyzed together.

Protein Determination

Protein was determined by the method of Lowry, et al., (91). BSA was used as a standard.

^{32}P Determination

The samples were counted in Bray's counting solution (92).

Statistics

The statistical methods used were from Croxton (93).

The data presented in the figures and tables is the arithmetic mean, $\bar{x} = \frac{\Sigma x}{n} \pm \frac{\Sigma [\chi]}{n}$, the average deviation, where χ is the deviation of x from $\bar{x}$. Whether or not the difference between two numbers was significant was obtained by the Student t test.

$$t = \frac{\bar{x} - x_\rho}{\hat{\sigma}_x} \qquad \hat{\sigma} = \sqrt{\frac{\Sigma \chi^2}{n-1}} \qquad \hat{\sigma}_x = \frac{\hat{\sigma}}{\sqrt{n}}$$

Knowing the t value, the value of P, the level of significance of the difference between the numbers compared, was obtained from a Student t test table.

$\bar{x}$: the arithmetic mean of sample one

$\bar{x}_\rho$: the arithmetic mean of sample two

n : the number of items in the sample

χ : deviations

CHAPTER III

RESULTS

The quality of the brush border preparation was ascertained by two methods. One was light microscopy. Under low magnification, brush borders appear as small quarter moons. If they are improperly prepared no discernable structure can be seen under such magnification. The other method was assaying for a marker enzyme, alkaline phosphatase. This enzyme is located in the brush borders (94, 95) and the assay for it is simple.

Since alkaline phosphatase is a membrane enzyme, some assay procedures use sodium deoxycholate to dissolve the membranes to obtain higher and more consistent activities. This enzyme was assayed in several tissue preparations, with and without sodium deoxycholate but otherwise using identical procedures. The same activity for the enzyme was obtained each time. Therefore, sodium deoxycholate was not used.

Figure 8 shows the distribution of the specific activity of alkaline phosphatase in both control and cholera-toxin-exposed homogenate (the total tissue preparation after

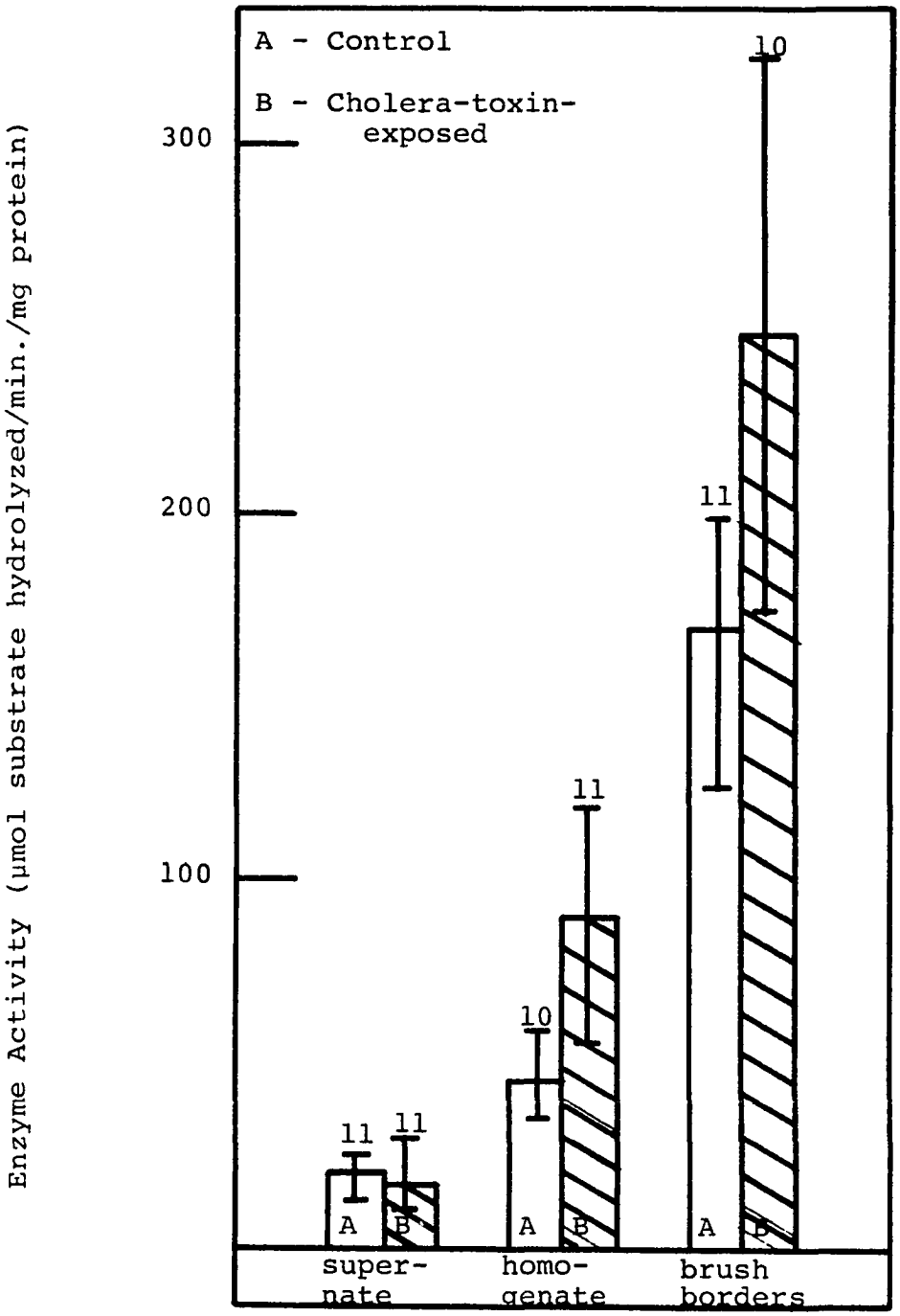


Figure 8 - Alkaline phosphatase distribution in intestinal mucosa fractions. (Each box represents the mean activity ± one standard deviation; number above bar indicates number of animals used.)

being homogenized in 5 mM EDTA), the supernate (supernatant liquid after the first homogenization), and the brush borders. (Figure 7 in materials and methods schematically shows these fractions.) The major activity is clearly in the brush borders. Two reports in the literature compared alkaline phosphatase activity in cholera-toxin-exposed brush borders to that of controls; one reported no difference and the other reported a decrease (96, 34). Figure 8 clearly shows a significant [$P<0.001$] increase.

Table 1 shows a significant increase in phosphorylated protein in epithelial cells exposed to cholera toxin as compared to control cells. An even greater increase was seen in the brush borders of these cells.

In this set of experiments, brush borders were prepared by the method of Miller and Crane (85). There was a definite correlation between the purity of the brush border preparation as seen by light microscopy and the magnitude of the increase of phosphorylated protein. Brush borders prepared by 30 second homogenization at a 2.8 speed setting on the Polytron homogenizer appeared visually at 100 fold magnification to be attached to cytoplasmic debris. These brush borders yielded the lowest ratio of cpm/mg protein (1.30). The highest ratio (2.30) was obtained from brush borders that appeared to be free from cellular material. These were prepared by a 1.5 second homogenization at a setting of 5. These results have been published (97).

TABLE 1

THE EFFECT OF CHOLERA TOXIN ON THE PHOSPHORYLATION OF PROTEIN IN EPITHELIAL CELLS AND THEIR BRUSH BORDERS

Fraction	Ratio of cpm/mg protein of toxin treated fraction to control	Number of animals	T - test of Significance (P)
Epithelial Cells	1.24 ± 0.18	7	0.019
Brush borders	1.68 ± 0.30	8	<0.001

A number of times this method of preparation did not give brush borders but gave a viscous mess instead. However, the method of Forstner for the preparation of brush borders gave consistently good preparations, and was, therefore, used in all subsequent studies. After the switch in brush border preparation, the same experiment detailed in Table 1 was repeated with 3 animals. The ratio of cpm/mg protein of toxin-treated brush border to the controls was 1.75. It was concluded that both methods gave similar results, except that the Forstner method gave more consistent preparations of brush borders.

Since there appeared to be an increase in the protein phosphorylated in cholera-toxin-exposed cells and since this increase appeared to be located in the brush borders of these cells, solubilized brush borders were fractionated using SDS acrylamide gel electrophoresis and column chromatography.

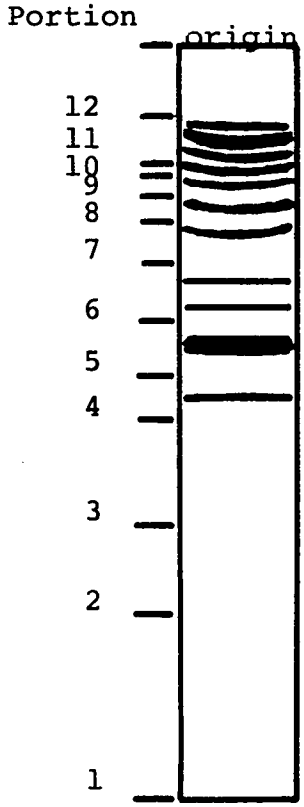
The protein staining patterns for both the cholera-toxin-exposed and control brush borders were the same. The distance from the origin and the intensity at 570 nm was measured for each band in the stained gel. Had different proteins been synthesized the band distance would have been different and if different amounts of protein had been synthesized the band intensities would have been different. If a protein was synthesized, its molecular weight would have to have been great enough to prevent it from

entering the gel.

The radioactivity close to the solvent front that was not associated with any protein staining bands was assumed to be associated with lipids, since lipids might be expected in this area. (Lipids should have been present since they were not removed before the brush borders were solubilized.) The majority of the radioactivity was always found at the origin. With gels that were heavily loaded with material, a whitish layer could be seen at the very top of the gels after they had been run, but not yet stained. Since this behavior is what one would expect from protein of high molecular weight, various changes were made in the gel composition to increase the "pore" size so that the protein could enter. None of these attempts were successful. The radioactivity always stayed at the very top and did not enter the gel (Figure 9).

Figure 10 compares the absorption at 280 nm with the radiometric pattern for both the cholera-toxin-exposed and control brush borders that had been solubilized in 6 M guanidine HCl. (The brush borders were not delipidated before solubilization.) These results are typical of all the agarose columns run. There was only one major radioactive peak, and this peak coincided with the absorbance at 280 nm peak. This material came off the column in the void volume.

When the dissolved brush borders were placed on the column a brown ring formed at the top. When removed and counted it was found to be very radioactive. There was some increase in radioactivity for the last samples from the



Gel Portion	Counts/10 min.
12	2,129
11	391
10	49
9	35
8	38
7	61
6	101
5	46
4	77
3	148
2	217
1	652

Figure 9 - Typical SDS acrylamide gel electrophoresis and radiometric pattern of control or cholera-toxin-exposed brush borders.

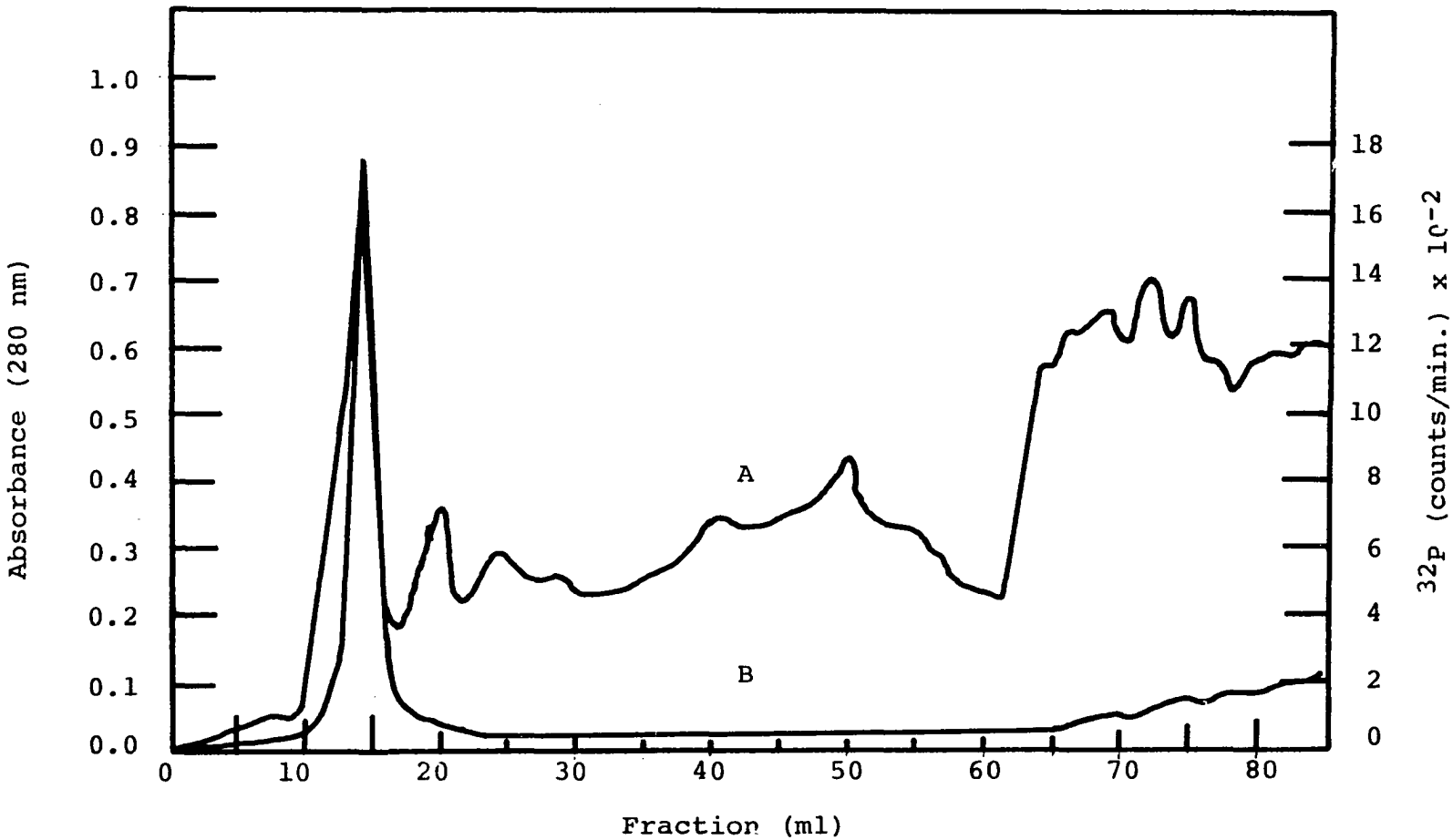


Figure 10 - Typical agarose column spectral (A) and radiometric (B) patterns for control or cholera-toxin-exposed brush borders dissolved in 6M guanidine hydrochloride.

column. This could be from material that had precipitated at the top of the column and was gradually leaking through it.

To determine if the substance in the void volume contained labeled lipids, $CHCl_3$:methanol 2:1 was added to 1 ml of the labeled sample until it was a homogenous phase. The solution was allowed to stand in ice for 10 minutes and then filtered to collect the solids present. The organic solution was taken down to a small volume on the rotary evaporator and counted. The filter paper and solids were also counted. Essentially all of the radioactivity was found to be associated with the precipitate and was not in the solution where the lipids should have been.

A 100 µl aliquot was taken from each of the fractions 10 through 20 and 70 through 80, indicated on Figure 10. To each was added 5 ml of cold 5 per cent TCA and 200 µl of 0.63 per cent BSA. After cooling in ice for 10 minutes, the precipitate was collected by centrifugation, washed in the manner of the in vivo phosphorylated samples discussed in materials and methods, dissolved in 0.1 N NaOH and counted. Of the original radioactivity, 90 per cent was recovered in the precipitate from each of the fractions 10 through 20 and less than 20 per cent was in each of fractions 70 through 80. This behavior would be expected if the material in the void volume was a protein. The column results agree with that of the SDS acrylamide gel electrophoresis, indicating that the

phosphorylated protein has a molecular weight above 1,000,000 (98).

To determine whether cholera toxin itself was a kinase, phosphorylating the brush borders with which it came in contact, or if alkaline phosphatase could act as a kinase under acidic conditions, these two proteins, with and without cAMP, were tested for their kinase ability using the standard assay procedure. Neither caused any incorporation of ^{32}P into the histone substrate. In a similar test, added intestinal juices failed to activate the cholera toxin.

Some kinases have been reported to vary widely in their specificity for various histones when used as substrates (99). Therefore, before the distribution of kinases in the intestinal mucosa was investigated, the ability of brush borders and the supernate to phosphorylate various histone fractions was determined. "Protein kinase 3':5' cAMP dependent from beef heart" (Sigma) was also tested each time an assay was done to verify that the procedure was working properly and to insure that a cAMP effect could be detected. As demonstrated in Figure 11 there was no overwhelming reason to chose one histone over another. Therefore histone type II was chosen because it was the least expensive.

Figure 12 shows the distribution of kinase activity in intestinal mucosa. As can be seen, there was no detectable difference in kinase activity between the homogenate from the cholera-treated tissue and the control.

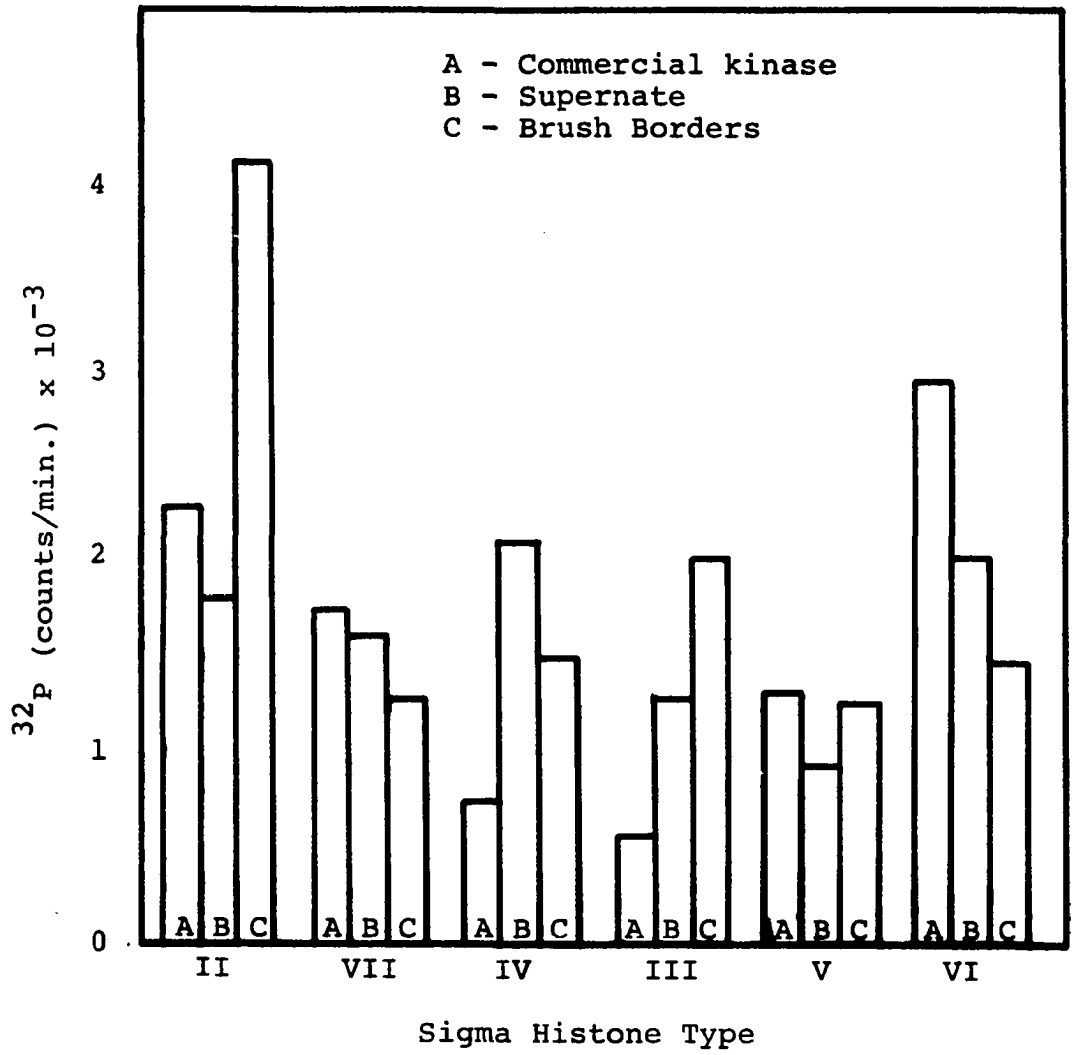


Figure 11 - Kinase assay using various histones as substrate (0.5 mg/ml).

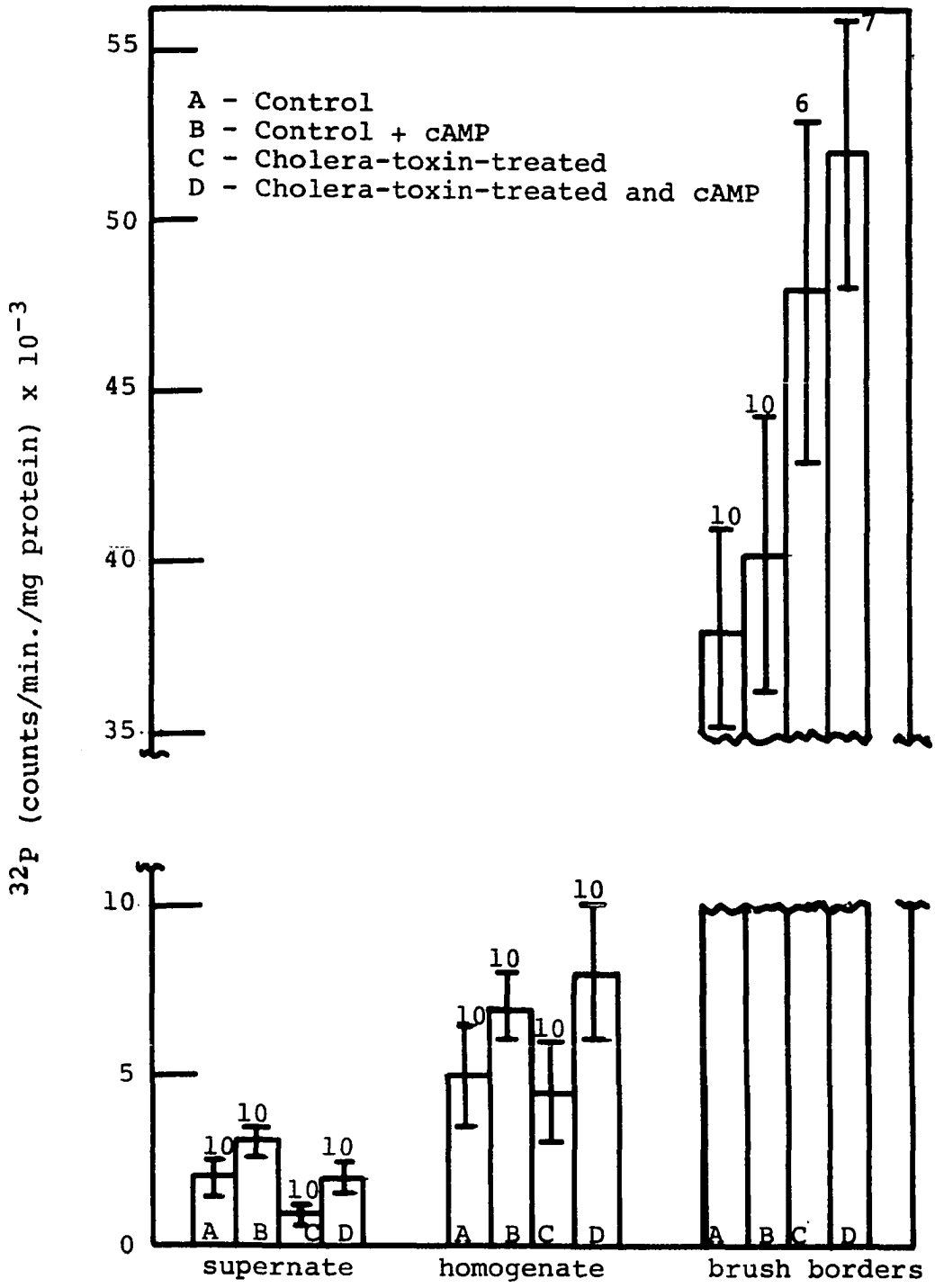


Figure 12 - Distribution of intestinal mucosa kinases and the effect of cholera toxin on their levels of activity. (Histone type II substrate).

The same level of cAMP activation was detected in both samples.

Kinase activity and cAMP-activated kinase activity were also detected in the supernate, although at a lower level than in the homogenate. The cAMP-independent kinase activity was significantly reduced ($P = 0.01$) in the supernate from the cholera-exposed tissue.

The highest kinase specific activity found in intestinal mucosa is in the brush borders. This activity differed from that found in the supernate and the homogenate in that no cAMP activation was found. The difference noted between kinase activity in the cholera-toxin-treated material without cAMP and the same samples with cAMP added is not statistically significant ($P = 0.10$). The difference in the cAMP-independent kinase activity in the controls and the cholera toxin exposed brush borders is significant ($P = 0.005$).

From this set of experiments, the following conclusions may be drawn. In the intestinal mucosa, the highest kinase specific activity is in the brush borders and this activity is not affected by the presence of cAMP. Cholera toxin caused a decrease in the level of specific activity of cAMP-independent kinase in the supernate and an increase in this specific activity in the brush borders.

The lack of cAMP stimulation of this kinase does not fit the original hypothesis of cAMP-directed

phosphorylation. Therefore, various changes were made in the kinase assay in order to determine if cAMP activity could be detected under other conditions.

Activation of liver phosphorylase by cAMP is Mg^{++} dependent (100). At high Mg^{++} concentrations there is no cAMP activation, but activation occurs at lower Mg^{++} concentrations. Figure 13 shows the effect on the brush border kinase activity of varying Mg^{++} concentrations. The kinase in brush borders is Mg^{++} dependent, but at no Mg^{++} concentration was there cAMP activation.

To determine whether cAMP activation of brush border kinase could depend on the substrate, bovine serum albumin, alkaline phosphatase from hog mucosa, cholera toxin, and casein were tried as alternate substrates. The brush borders phosphorylated the casein but the rate of phosphorylation was not increased with added cAMP (Table 2). The other 3 proteins were not phosphorylated.

The kinase activity of the brush borders was not changed by varying the cAMP concentration from 10^{-5} to 10^{-2} M.

The brush borders were suspended in 2 per cent Triton X-100 to dissolve the membranes before being added to the assay mixture. Again there was no cAMP activation. Other workers have reported that the addition of Triton X-100 had no effect on the membrane bound kinase in brain (45).

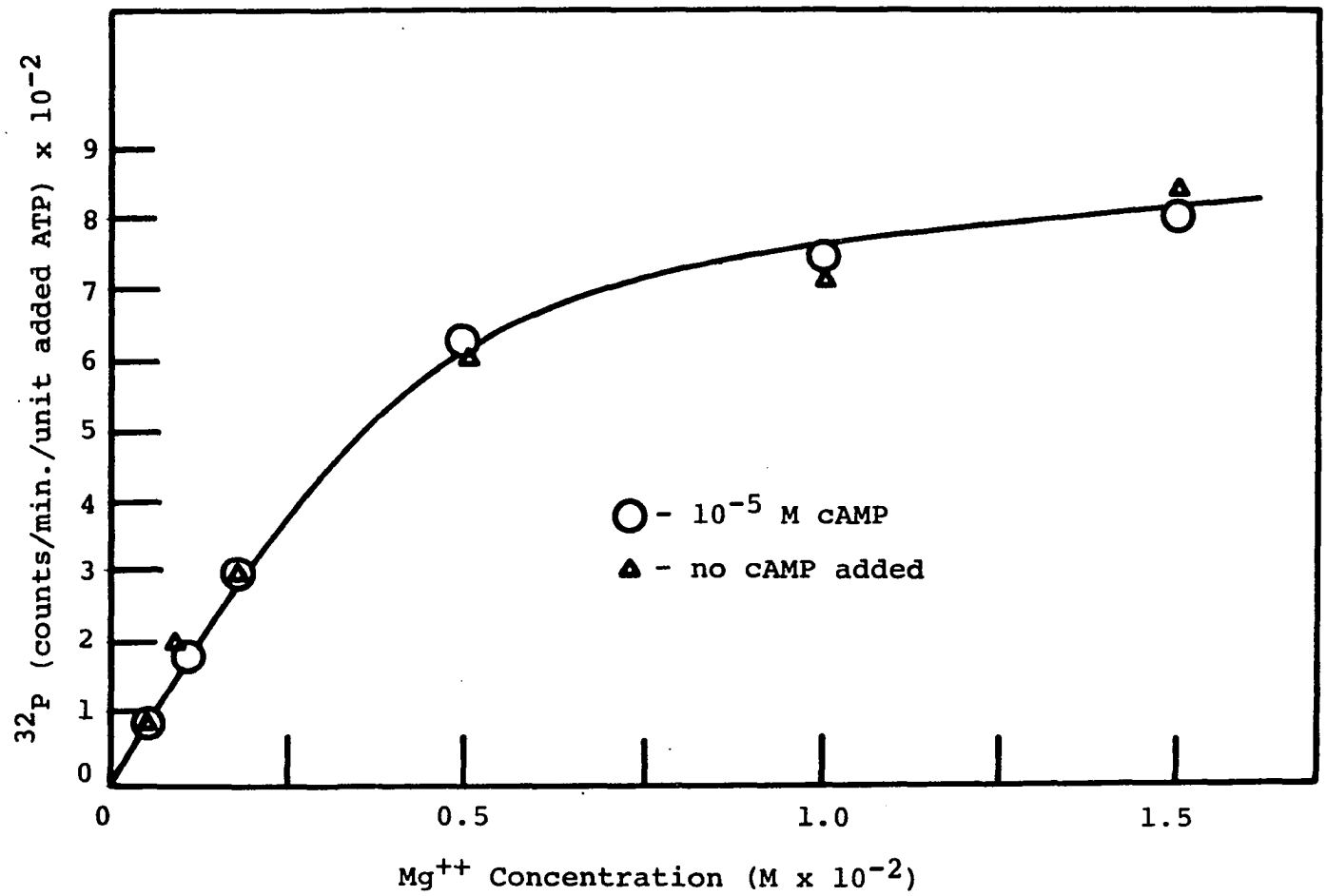


Figure 13 - Phosphorylation of histone Type II substrate with cholera-toxin-exposed brush borders in the presence and absence of cAMP.

TABLE 2

PHOSPHORYLATION OF VARIOUS PROTEINS WITH CHOLERA-TOXIN-EXPOSED AND CONTROL BRUSH BORDERS AS THE KINASE SOURCE, WITH AND WITHOUT cAMP (10^{-5}M)

Substrate	Counts/min./mg brush border protein x 10^{-3}	
	Cholera-toxin-exposed	Control
Histone	4.6	4.0
Histone + cAMP	4.9	4.1
Casein	3.7	3.6
Casein + cAMP	3.8	3.7
Bovine Serum Albumin	none	none
Bovine Serum Albumin + cAMP	none	none
Alkaline Phosphatase from Hog Mucosa	none	none
Alkaline Phosphatase from Hog Mucosa + cAMP	none	none

To rule out the possibility that something in the brush borders was either binding or destroying the cAMP to make it unavailable for kinase activation, a commercial preparation of protein kinase from beef heart was added to the brush border preparation and assayed at various concentrations of cAMP. Figure 14 shows that with and without brush borders the kinase activity increased with increasing cAMP concentration, indicating that the cAMP was available to react with the commercial kinase and brush border kinase as well. But in the second reaction, even though brush borders were also present which could phosphorylate the substrate, the amount of phosphate incorporated into the substrate was lower. This could indicate that a kinase inhibitor is present in the brush border preparation. Another explanation would be that each enzyme in the system (the commercial kinase and the brush border kinase) was phosphorylating the substrate on a different group. When one group is phosphorylated it might be harder for the other enzyme to phosphorylate a different group, thereby giving non-additive phosphorylation. For instance, the commercial preparation could be phosphorylating the serine groups of the histones and the brush border kinase could be phosphorylating the histidine groups. After the serine groups on the histone were phosphorylated, it might be harder for the brush border kinase to phosphorylate the histidine group.

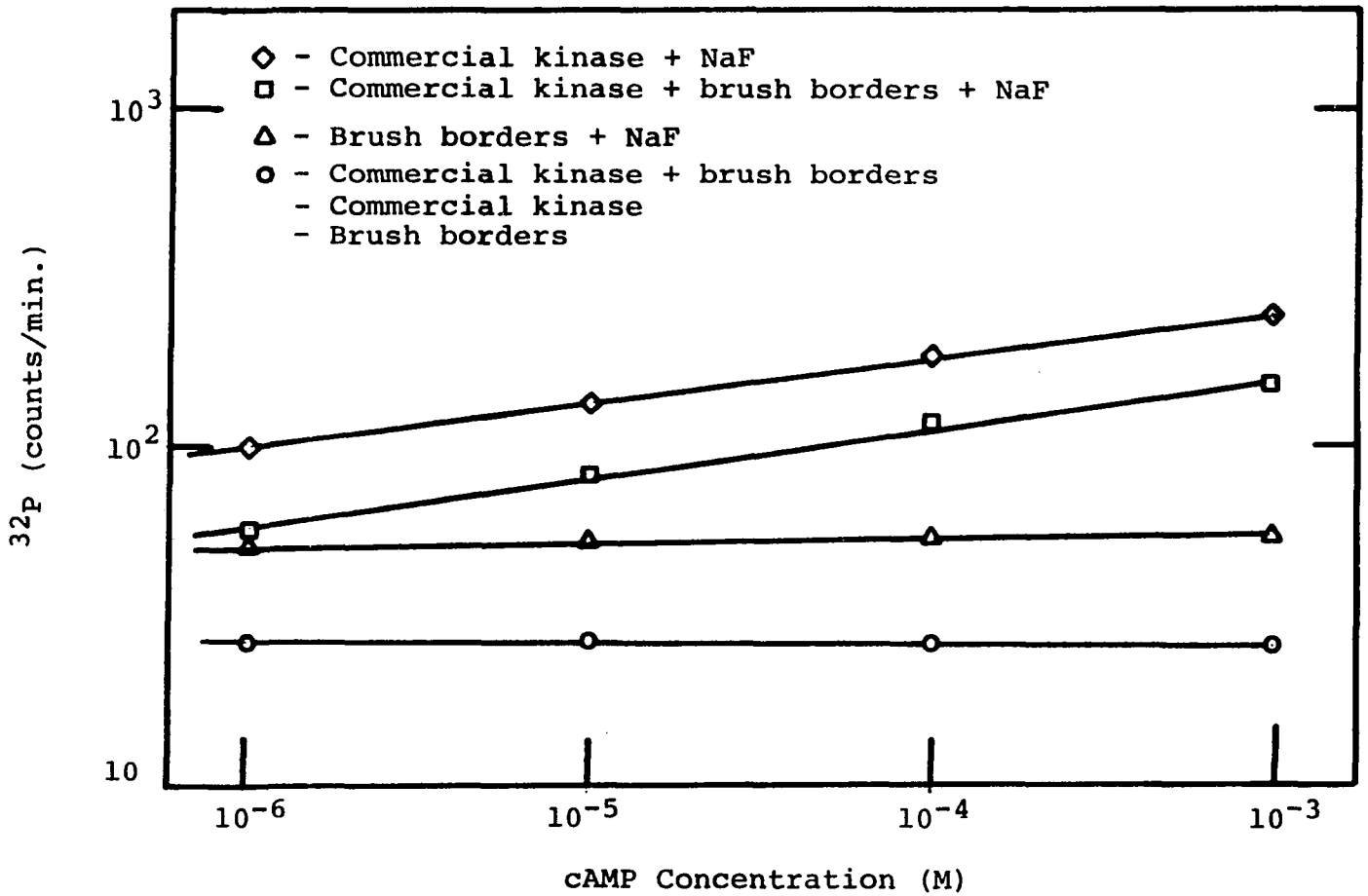


Figure 14 - Phosphorylation of histone Type II substrate by beef heart kinase and brush borders as a function of cAMP in the presence and absence of NaF.

The surprising thing was that the commercial enzyme also exhibited a dependence on NaF for maximal activity. Without NaF being present it was not activated by cAMP at any of the concentrations tried.

Liver phosphorylase is activated by cAMP and NaF but the effects are not additive (101), suggesting that both effectors are competing for the same activation sites on the kinase. The effect of NaF and cAMP on the control and cholera-toxin-exposed brush borders kinase assay was determined as a function of time (Figure 15). When the NaF was left out of the kinase assay, the amount of phosphorylation was decreased by about one-half, but even without the NaF there was no cAMP-induced activity of the kinases.

The fluoride could exert its effect on net phosphorylation by preventing a concurrent dephosphorylation. Liver contains a phosphatase specific for the removal of phosphate from histones that is inhibited by NaF (102). Phosphatases are postulated to always exist with kinases (71). Sometimes the phosphatase is affected by cAMP -- either activated or deactivated (103).

The lowered rate of phosphorylation indicated in Figure 15 could be accounted for by the following reactions:

A) Protein + ATP $\xrightarrow{\text{kinase}}$ Protein-P

B) Protein-P $\xrightarrow{\text{phosphatase}}$ Protein + P_i

When NaF is present, the phosphatase reaction is inhibited and more phosphorylation is observed. Therefore, the brush

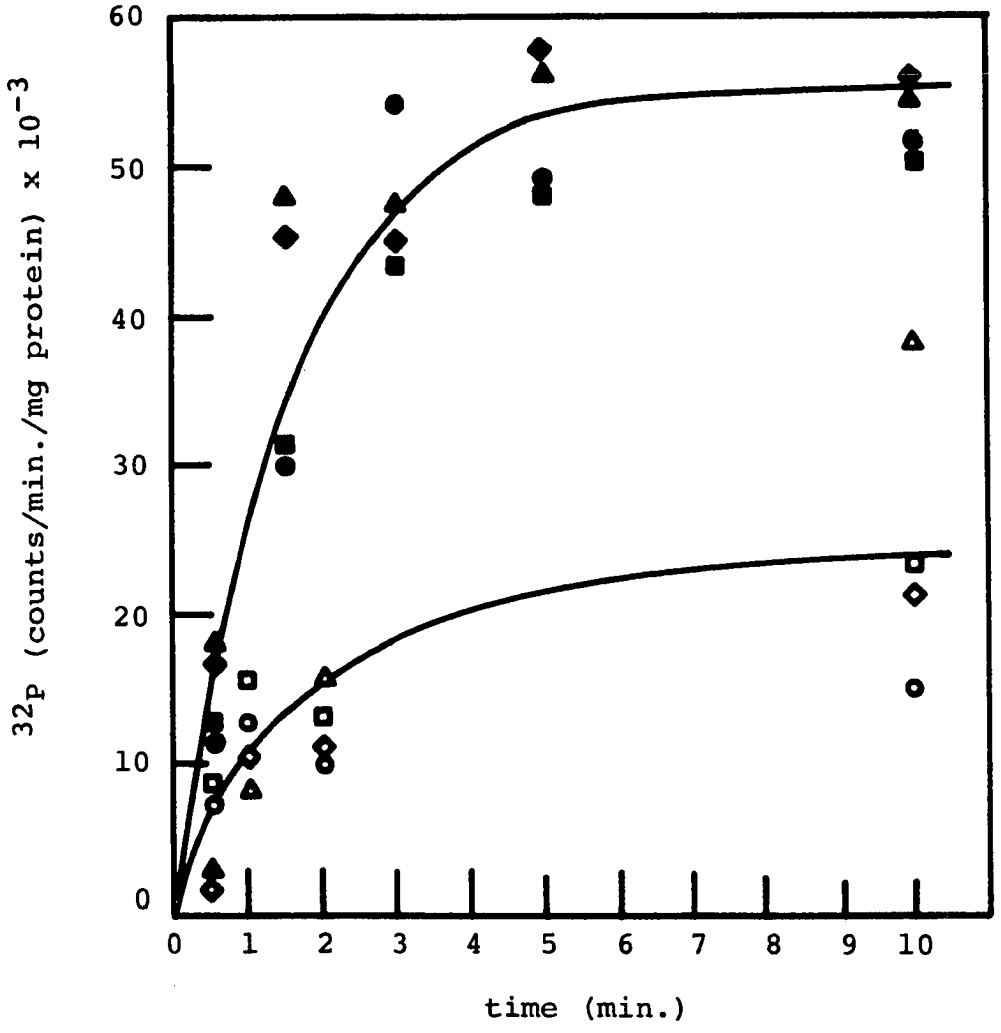


Figure 15 - The affect of cAMP and NaF (closed points) on the rate of phosphorylation of histones by control and cholera-toxin-treated brush borders.

▲- Cholera-toxin-exposed

◆- Cholera-toxin-exposed + cAMP

■- Control

●- Control + cAMP

borders might contain a phosphatase as well as a kinase, but there was no evidence that this phosphatase was affected by cAMP.

The endogenous phosphorylation, i.e., brush borders were used as the substrate and not histones, was investigated to determine the location of the natural substrates for the intestinal mucosa kinases.

Before the distributions of the substrates could be determined, some indication of reaction rates was needed. Figure 16 shows the amount of phosphorylation in control and cholera-toxin-exposed samples of homogenate and supernate as a function of time and cAMP presence. Figure 17 indicates the results of the same experiment with brush borders in the presence and absence of NaF. One minute was chosen for the length of time to run the assay to determine the distribution of endogenous phosphorylation.

Table 3 shows the results of a typical experiment. The pattern of endogenous phosphorylation followed that of the kinase. The lowest specific activity of endogenous phosphorylation was located in the supernate and the most in the brush borders. No cAMP activation was detected.

To determine if the amount of endogenous phosphorylation was significantly different between cholera-toxin-treated brush borders and controls, several samples from different preparations were assayed after a 10 minute reaction time (Table 4). A significant difference ($P = 0.05$)

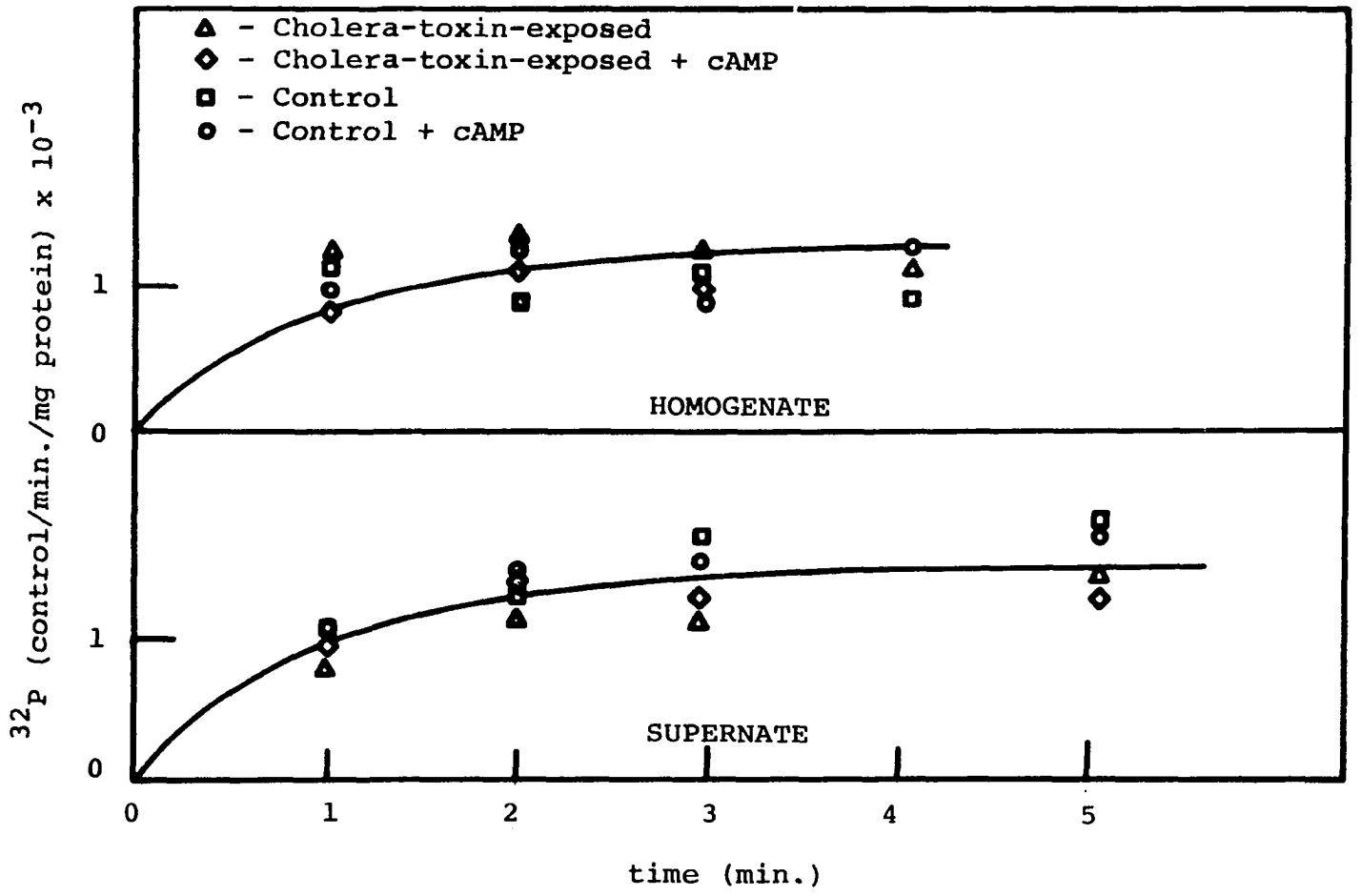


Figure 16 - Rate of endogenous phosphorylation of intestinal homogenate and supernate.

Figure 17 - Rate of endogenous phosphorylation of cholera-toxin-exposed and control brush borders, as a function of NaF (closed points) and cAMP.

▲ Cholera-toxin-exposed brush borders

◇ Cholera-toxin-exposed brush borders + cAMP

□ Control brush borders

o Control brush borders + cAMP

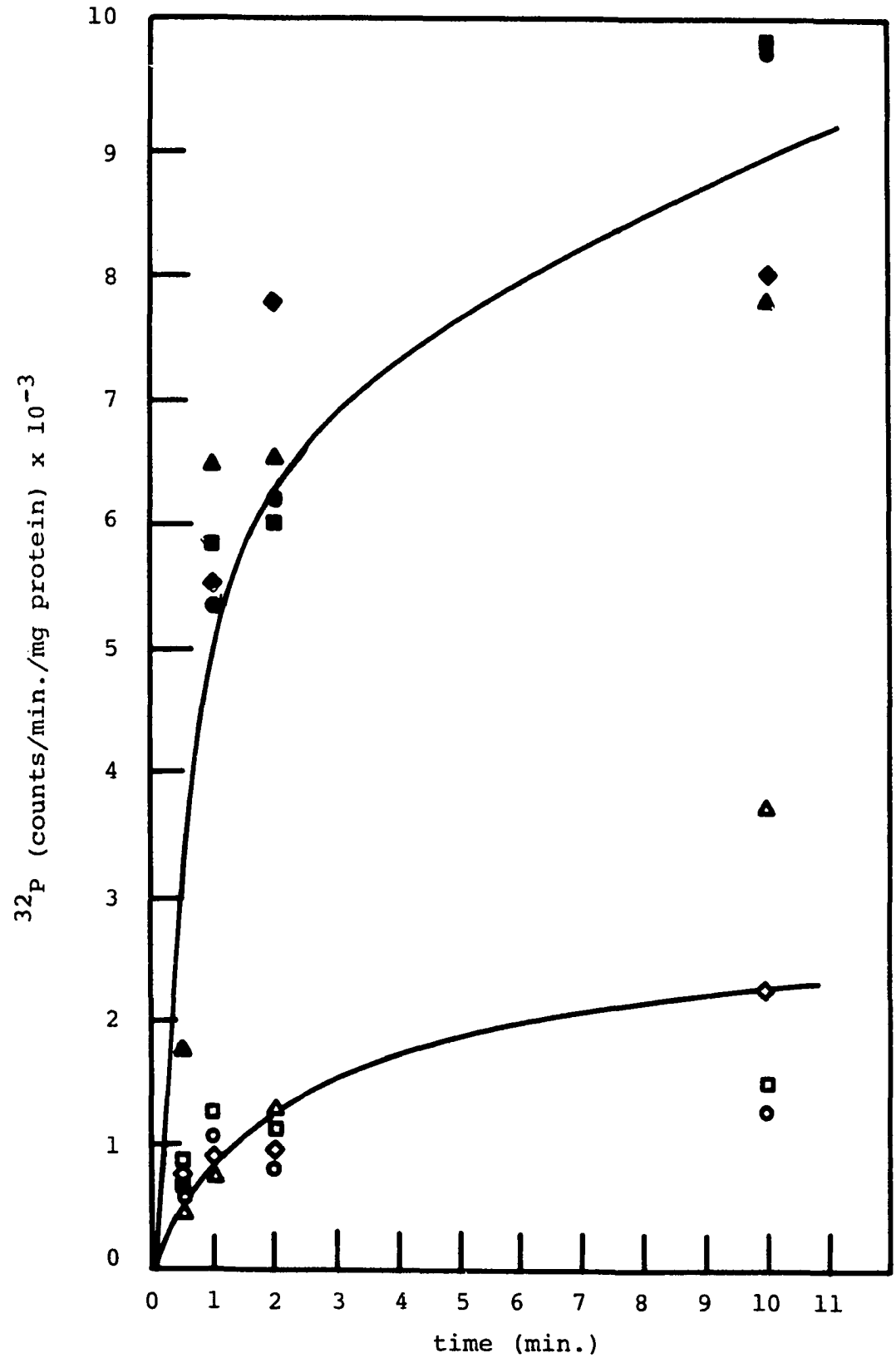

10
9
8
7
6
5
4
3
2
1
0
^{32}P (counts/min./mg protein) x 10^{-3}
0
1
2
3
4
5
6
7
8
9
10
11
time (min.)

TABLE 3

ENDOGENOUS PHOSPHORYLATION OF VARIOUS FRACTIONS OF INTESTINAL MUCOSA AND THE EFFECT OF cAMP (10^{-5}M) ON THIS PHOSPHORYLATION

Fraction	Counts/min./mg protein	
	Cholera-toxin-exposed	Control
Supernate	736	750
Supernate + cAMP	745	876
Homogenate	964	879
Homogenate + cAMP	773	683
Brush borders	6,440	5,845
Brush borders + cAMP	5,560	5,315

TABLE 4

THE EFFECTS OF cAMP (10^{-5}M) ON ENDOGENOUS PHOSPHORYLATION OF CONTROL AND CHOLERA-TOXIN-EXPOSED BRUSH BORDERS

Brush Border Fraction	Counts/min./mg protein	Number of rats
Control	8,144 ± 3,900	5
Control + cAMP	7,538 ± 1,380	4
Cholera toxin	6,827 ± 1,059	5
Cholera toxin + cAMP	9,017 ± 1,829	4

was detected. The endogenous phosphorylation of the control brush borders was not affected by the addition of cAMP ($P = 0.45$). It did increase phosphorylation of cholera-toxin-treated brush borders ($P = 0.02$).

To identify the phosphorylated amino acid residue, samples of *in vivo* phosphorylated protein and endogenously phosphorylated protein were washed in the standard way and hydrolyzed in HCl concentrations varying from 1 to 6 M. None of the conditions produced a radioactive phosphoserine or phosphothreonine spot. A sample of the phosphorylated protein was hydrolyzed along with a known sample of phosphoserine in 2 M HCl for 1 hour. Phosphoserine was partially hydrolyzed as expected, but 65 per cent (104) of it remained intact; still no radioactive phosphoserine was detected. Phosphoserine from the protein should have hydrolyzed at the same rate as free phosphoserine; therefore, no labeled phosphoserine was released. Most of the radioactivity was recovered as inorganic phosphate. Under these mild conditions, some radioactivity was detected in the peptide region of the chromatogram (Figures 18, 19). Also, a radioactive peak that always trailed the inorganic ^{32}P peak in the hydrolyzed samples remains unidentified.

It was concluded that the phosphorylated amino acid residues were not phosphoserine or phosphothreonine.

ATPase activity is associated with the formation of an acyl phosphate intermediate. The following experiments

Figure 18 - Radioactive and ninhydrin staining pattern of brush borders hydrolyzed for one hour in 3 M HCl by themselves and spotted with phosphoserine that had been hydrolyzed under the same conditions. (Electrophoresis was for two hours.)

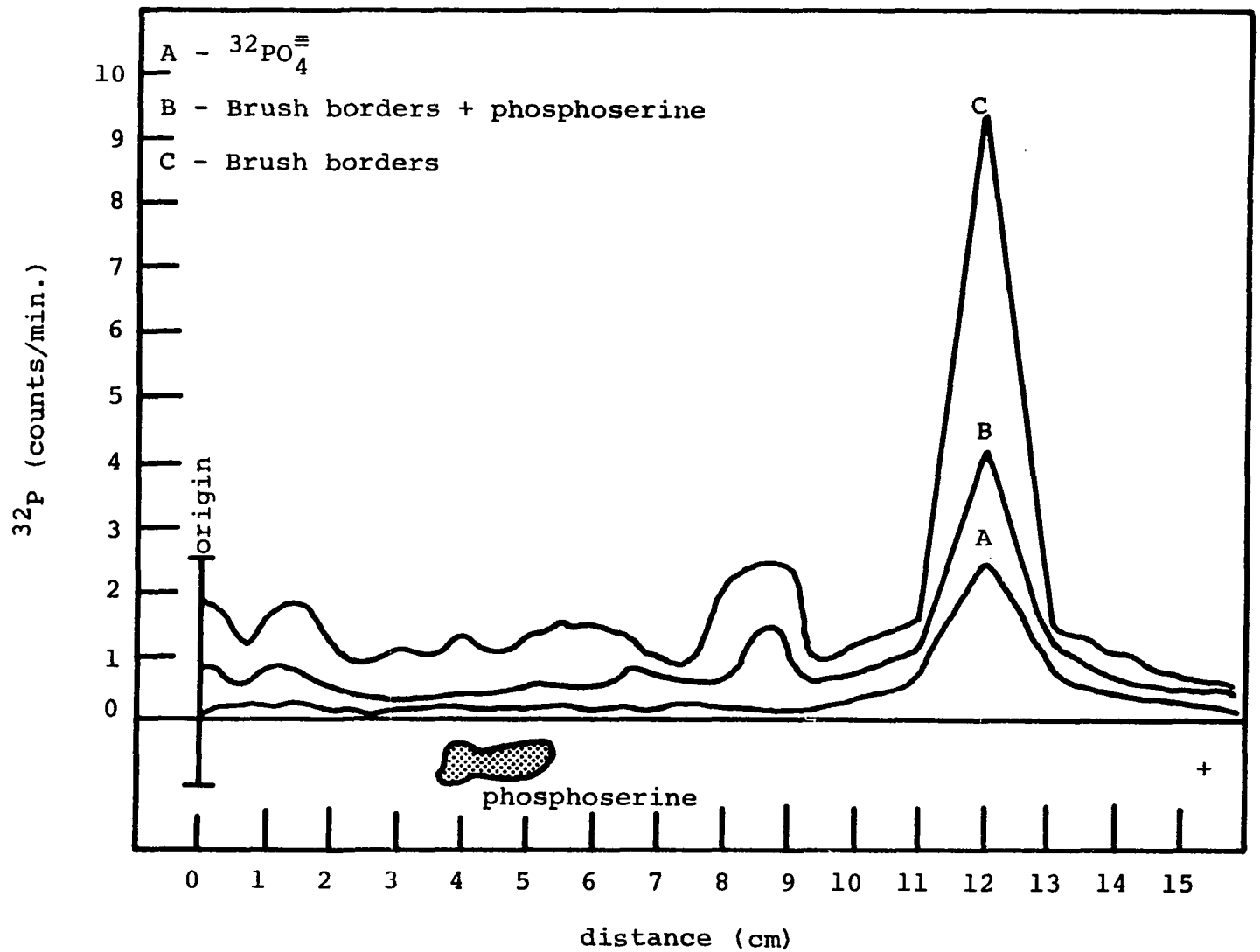
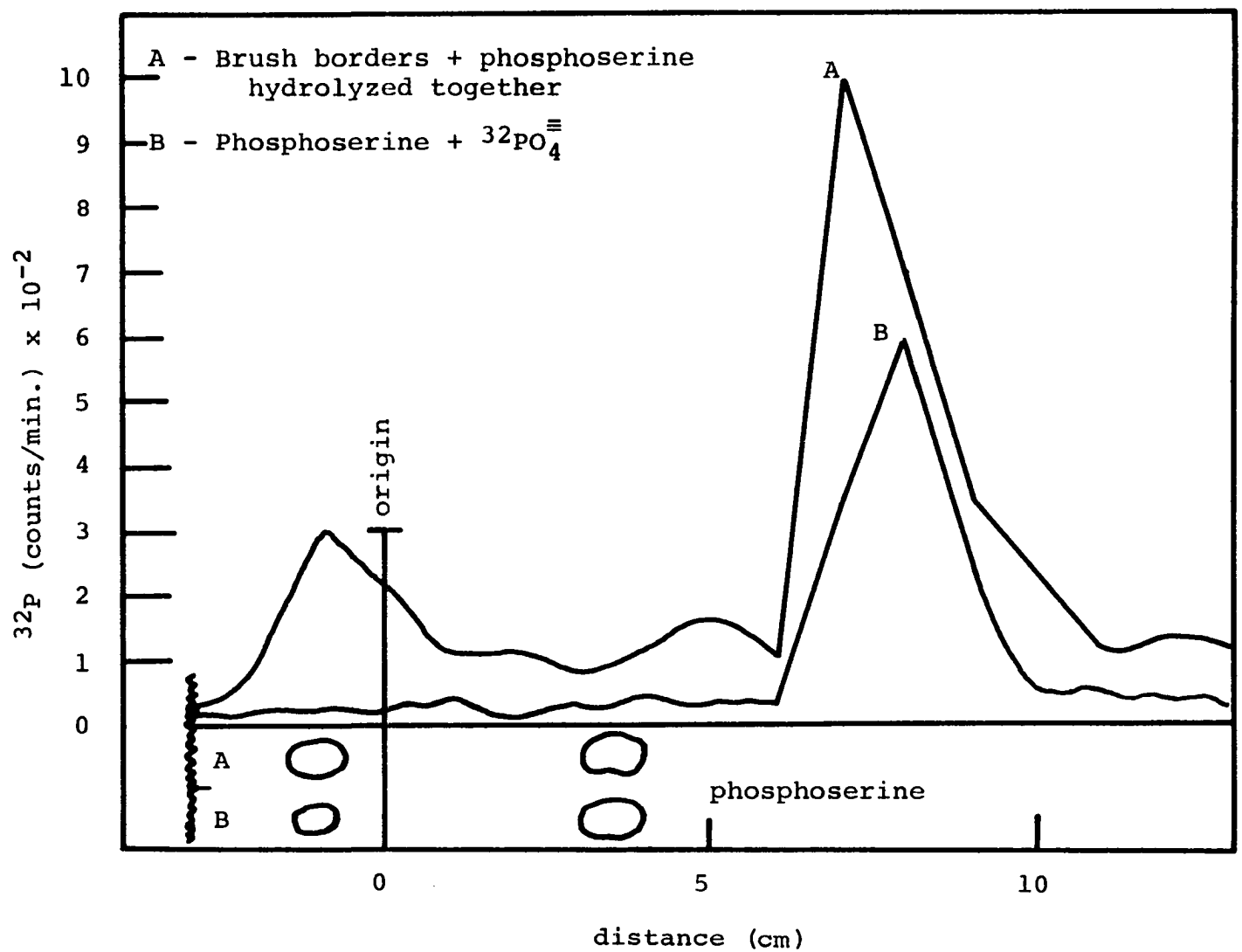

A - $^{32}PO_4^{\equiv}$
B - Brush borders + phosphoserine
C - Brush borders
C
B
A
origin
phosphoserine
+
^{32}P (counts/min.)
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
distance (cm)

Figure 19 - Radioactive and ninhydrin staining pattern of *in vivo* phosphorylated brush borders hydrolyzed by themselves and with phosphoserine in 1 M HCl for one hour. (Electrophoresis was for one hour.)


A - Brush borders + phosphoserine hydrolyzed together
B - Phosphoserine + $^{32}PO_4^{\equiv}$
A
B
origin
phosphoserine
A
B
^{32}P (counts/min.) x 10^{-2}
0
1
2
3
4
5
6
7
8
9
10
0
5
10
distance (cm)

demonstrate the observed phosphorylation of brush borders was not via formation of acyl bonds. The *in vivo* phosphorylated brush borders were precipitated with 5 per cent TCA and washed in the usual manner. The washed precipitates were suspended in 4.0 ml of 0.8 M hydroxylamine hydrochloride and 0.10 M sodium acetate, pH 5.3 and incubated for 10 minutes at 30° C. Duplicate controls were treated similarly except for the substitution of 0.8 M sodium chloride for 0.8 M hydroxylamine hydrochloride. After incubation, 1.3 ml of cold 50 per cent TCA were added and the samples were centrifuged at low speed. The protein was then dissolved in 0.5 ml 0.1 N NaOH and counted. Under these conditions hydroxylamine hydrochloride cleaves acyl phosphate bonds but leaves phosphoester bonds intact (41). No acyl bound phosphate was found in the washed precipitate.

CHAPTER IV

DISCUSSION

Cholera-toxin-exposed epithelial cells contained significantly more phosphorylated protein than did the controls. This difference was further enhanced when the brush borders were compared. To determine if this was a direct result of the action of cholera toxin on the cell or whether it was a result of the cholera toxin induced cellular increase in cAMP concentration, the relationship between intestinal mucosa kinases and cAMP was investigated.

When histones were used as a substrate, cAMP dependent kinase activity was found in the supernate and homogenate fractions of intestinal mucosa. Using the same substrate, cAMP independent kinase activity was found in the same fractions as well as in the brush borders.

To show that this kinase phosphorylation of histones by the brush borders was indeed cAMP independent and not just a function of the assay conditions, various changes were made in these conditions. It was found that the brush border kinase was Mg^{++} dependent and that the presence of NaF increased its activity. It was also determined that

this brush border kinase could phosphorylate casein. Under no conditions was cAMP activation of the brush border detected. It was therefore concluded that the phosphorylation of histones by the kinase in the brush borders was cAMP independent.

When the distribution of the kinase specific activity for the phosphorylation of histones was compared in control and cholera-toxin-exposed intestinal mucosa fractions, it was found that both cholera-toxin-exposed and control brush borders had over a five fold increase in kinase specific activity as compared to the homogenate. In addition, cholera toxin caused an increase in this activity as compared to the controls.

Endogenous phosphorylation, i.e., phosphorylation of a natural substrate, was found to parallel that of the specific kinase activity for the phosphorylation of histones. The highest specific activity of endogenous phosphorylation was found in the brush borders. This endogenous phosphorylation was slightly lower in the cholera-toxin-exposed brush borders when no cAMP was added to the assay reaction mixture. When cAMP was added to the reaction, cholera toxin endogenous phosphorylation was increased. The endogenous phosphorylation of control brush borders was not enhanced by cAMP.

The behavior of <u>in vivo</u> and <u>in vitro</u> phosphorylated brush borders in strong acid agrees with that of phospho-

histidine. Phosphohistidine is very susceptible to acid hydrolysis and could not have been detected under the pH conditions used in the protein hydrolysis and electrophoresis. Since phosphoserine and phosphothreonine were not detected, they were not the product of brush border phosphorylation. Nor was the radioactivity of the phosphoprotein an acyl bond as evidenced by the alkali stability of the phosphoprotein bond and by the fact that hydroxylamine hydrochloride did not remove the phosphate at pH 5.4. (This is a rather specific test for acyl bonds [41].) Therefore, the phosphorylated product in brush borders must be phosphohistidine or some other phosphorylated species not previously reported. This is a further suggestion that the cholera toxin induced phosphorylation of brush borders is mediated by a cAMP independent kinase, because all cAMP activated kinases reported to date have phosphorylated the serine or the threonine groups in the proteins.

Since the brush border kinase was found to be cAMP independent, four possible mechanisms whereby cAMP would be directly implicated in brush border choleric secretion are ruled out: 1) cholera toxin induced secretion cannot be caused by cAMP activation of a membrane bound kinase that phosphorylates a membrane protein thereby changing the membrane permeability, 2) cAMP does not activate a cytoplasmic protein kinase that phosphorylates a protein normally present in the cytoplasm, which when phosphorylated would

migrate to the brush border and change the permeability, 3) a cAMP dependent kinase does not migrate to the membrane and phosphorylate a protein present there, or 4) a cytoplasmic protein does not migrate to the brush borders to be phosphorylated by the kinase present in the brush borders.

The first and fourth mechanism are ruled out because the brush border kinase is cAMP independent. The second and third are ruled out because endogenous phosphorylation occurs in the isolated brush borders with all cytoplasmic material removed. In other words, there is a natural substrate for the kinase present in the brush borders.

The increase in kinase specific activity seen in the brush borders when histones were used as a substrate might be explained by cAMP initiated protein synthesis. However, the explanation which seems best supported by the literature and which will best explain all of the experimental data in this work is that the kinase is a lipid dependent protein.

Since adenyl cyclase and Na^+, K^+ -ATPase are known to be membrane bound and lipid dependent, Sharp and coworkers (34) have attributed the effect of cholera toxin on these enzymes to alteration of the membrane lipids by the cholera toxin.

If the brush border kinase were lipid dependent, its activity could be altered by the binding of the cholera toxin

to the brush borders. In other words, the increase in kinase activity could be a result of cholera toxin binding and not related to the increased cellular levels of cAMP. Similar reasoning can be used to explain the decrease in cAMP independent kinase activity in the supernate, providing the kinase whose activity is decreased is bound to the plasma membrane.

This same postulation of Sharp and co-workers can also explain the increase seen in alkaline phosphatase activity in cholera-toxin-exposed mucosa. Certain compounds inhibit alkaline phosphatase in some strains of rats but not in others. That is, the alkaline phosphatases vary in reaction requirements from species to species (105). In the rat strain used in these experiments, the alkaline phosphatase activity may have increased after exposure to cholera toxin because the cholera toxin interacted in some way with the membrane lipids. This would explain the discrepancy between the results in Figure 8 and the results in the literature (34, 100) where rabbits were used and lower or no change in alkaline phosphatase values were obtained after cholera toxin exposure.

Despite the fact that different results for alkaline phosphatase activity were obtained using different animal types, both types responded to cholera toxin in the same manner, i.e., massive secretion. Therefore, it seems unlikely that the alkaline phosphatase activity was directly

involved in the secretory process. Any changes in activity would be because of the relationship of alkaline phosphatase to the particular membrane to which it was bound.

Endogenous phosphorylation of cholera-toxin-exposed brush borders was increased by cAMP. This was not true for controls. There are several possible explanations that would account for this. First, the experimental deviation in the endogenous phosphorylation experiments was quite large. Therefore, the increased phosphorylation that was seen under the influence of cAMP could be due to a deficiency in the method. Second, there could be synthesis of a kinase that is cAMP activated. This protein synthesis could account for the increase in kinase activity in cholera-toxin-exposed brush borders using histones as a substrate. In other words a kinase would be synthesized that would phosphorylate the natural substrate found in the brush borders only when cAMP was present, but would phosphorylate histones without cAMP activation. Third, cholera toxin interaction with brush border lipids could render the brush border kinase cAMP activatable. This activation would be specific for the natural substrate. Assuming the data are real and not merely an artifact of the experimental method, this last explanation seems to be the most likely one.

SDS polyacrylamide gel electrophoresis and column chromatography experiments showed that the phosphorylated protein in the control and cholera-toxin-exposed brush

borders has a molecular weight greater than 1,000,000 daltons. The phosphorylated protein was eluted in the void volume where no separation occured. Thus in the cholera-toxin-exposed brush borders no newly synthesized protein or no increase in a protein already present would have been detected.

Alpers (106) has noted that the larger the molecular weight of a brush border protein, the faster its turnover rate. Turnover rates of 4.5 hours have been measured for some of the larger proteins. However, a protein complex with a molecular weight greater than 1,000,000 daltons has never been found before in brush borders. Such a protein might have a turnover rate similar to the elapsed time observed in cycloheximide inhibition of well-established choleric secretion (78). Thus, it could be the inhibition of synthesis of this protein by cycloheximide that results in the decrease in secretion.

If the brush borders are involved in choleric secretion, the following mechanism can be postulated to incorporate the above data.

Cholera toxin is bound by the brush border. It irreversibly changes the adenyl cyclase in the plasma membrane which leads to an increase in cellular cAMP levels. The cAMP then causes phosphorylation of histones, inducing synthesis of a protein. The protein synthesized could either be brush border substrate or kinase. After synthesis,

the protein would migrate to the brush border where, if a kinase, it would either phosphorylate the substrate present there or be phosphorylated by the kinase in the brush border. If a substrate is synthesized, then the kinase activity is increased by the action of the cholera toxin on the membrane. The phosphorylation and dephosphorylation of the substrate in the membrane is responsible for the control of brush border secretion.

This mechanism does not explain the effect of other adenyl cyclase activators such as prostaglandins. After application to the serosal side, they cause an immediate increase in secretion, although not as profuse as with cholera toxin. Maybe this immediate increase in secretion comes about from stimulating secretion from the crypt cells. It has been suggested that the secretory granules seen in the crypts empty their contents into the lumen under hormonal influence (100). In other words, cholera toxin involves both the crypts and the brush borders.

There is evidence from the literature that choleric secretion involves more than one type of secretion. Goblet cells are known to increase their secretion of mucus after exposure to cholera toxin (107). This mucus accounts for the "rice water" stools of cholera. This secretion from the goblet cells is not inhibited by cycloheximide indicating that the choleric secretion from these cells is not mediated by a protein synthesis step. Their response to heightened

cAMP levels could also be immediate. To summarize, the fluid secretion seen after exposure to cholera toxin would have to be a summation of three different secretion processes. Secretion from the brush borders alone will not account for all the known facts of cholera toxin.

Alternatively, the data does not preclude a hypothesis that the brush borders are involved only in absorption and have no secretory functions. The kinase and substrate located in the brush borders could be involved in the transport of sugars in a manner analogous to that found in bacteria. Kerndig and Roseman (42) have characterized the following system for sugar transport in bacteria.

$$\text{Enzyme 1: PEP + HPr} \xrightarrow{Mg^{++}} \text{phospho-HPr + pyruvate}$$

$$\text{Enzyme 11: phosph-HPr + sugar} \xrightarrow{Mg^{++}} \text{sugar-P + HPr}$$

$$\text{1 + 11: PEP + sugar} \xrightarrow{\text{HPr, } Mg^{++}} \text{sugar-P + pyruvate}$$

HPr = carrier protein; PEP = phosphoenolpyruvate

It has been postulated that the same thing occurs in animal systems, but no direct evidence has been offered to support this contention. The kinase activity found in the brush borders could be that of the kinase (Enzyme 1) that phosphorylates the carrier protein, HPr. Increased phosphorylation levels in the presence of cholera toxin could be explained by enhanced kinase activity. The same reasons

described previously to explain the enhanced kinase activity when histones were the substrate would still be applicable in this instance.

Histidine is phosphorylated in bacterial HPr. None of the data so far accumulated on the brush border kinase is contrary to histidine phosphorylation. Also, the brush border kinase and the sugar transport system in bacteria are Mg^{++} dependent.

Several important differences exist between the bacterial system and the kinase activity found in brush borders. In bacteria the phosphate comes from PEP, not ATP. In addition the kinase, Enzyme 1, and the protein HPr, are not membrane bound but are found in the cytoplasm. HPr is phosphorylated in the cytoplasm before migrating to the membrane. Finally, sodium is not required for the transport of sugars in bacteria as it is for intestinal transport of glucose. A separate mechanism would be required to explain the cAMP dependent endogenous phosphorylation of cholera-toxin-exposed brush borders.

CHAPTER V

SUMMARY

Brush borders that had been exposed to cholera toxin *in vivo* contained more phosphorylated protein than did control brush borders. To determine if this *in vivo* phosphorylation was a direct result of the action of cholera toxin on the cell or was caused by the cholera toxin induced cellular increase in cAMP concentration, the relationship between intestinal mucosa kinases and cAMP was investigated.

Intestinal mucosa that had been exposed *in vivo* to cholera toxin and unexposed intestinal mucosa were scraped and homogenized in 5 mM EDTA, pH 7.4 in a Dounce homogenizer. This was the homogenate fraction. The supernatant liquid from the first centrifugation of the homogenate was called the supernate.

When histones were used as the substrate, cAMP dependent kinases were found in the homogenate and the supernate. Kinase activity that was independent of cAMP was found in these two fractions as well as in the isolated brush borders. The highest specific activity of cAMP

independent phosphorylation of histones was in the brush borders.

Changes in the reaction mixture were made to show that this kinase phosphorylation of histones by the brush borders was indeed cAMP independent and not just a function of the assay conditions. With the histone substrate, added NaF doubled the amount of phosphorylation. Under no conditions was the brush border kinase activated by cAMP. It was therefore concluded that the phosphorylation of histones by the brush borders kinase is cAMP independent. The brush border kinase was Mg^{++} dependent and could phosphorylate casein.

Endogenous phosphorylation was found to occur in the supernate, homogenate and the brush borders of intestinal mucosa. The specific activity of endogenous phosphorylation activity paralleled that of the cAMP independent kinase. The highest specific endogenous phosphorylation was found to occur in the brush borders. With cholera-toxin-exposed brush borders, this in vitro phosphorylation was found to be cAMP activated and to be lower than in the control brush borders.

Agarose column chromatography and SDS gel electrophoresis revealed a phosphorylated protein with a molecular weight above 1,000,000 daltons in both control and cholera toxin treated brush borders. This phosphorylated protein did not contain phosphoserine, phosphothreonine or an acyl

bonded phosphate. The phosphate group in the phosphorylated protein was found to be acid labile and alkali stable.

Alkaline phosphatase activity was elevated in the cholera toxin exposed brush borders.

The results obtained here were incorporated with the information available from the literature to infer several possible mechanisms for the role of brush borders in choleric fluid transport.

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