ENZYMES IN HUMAN DIARRHOEAL AND CHOLERA STOOLS

FLORENCE K. BHATTACHARYYA AND A. NARAYANASWAMI

Indian Institute of Experimental Medicine, 4, Raja S. C. Mullick Road, Calcutta-32, India

FROM their observations on the effects of cholera stool and vibrio cell extracts on the short-circuited frog-skin system of Ussing and Zerahn (1951), Fuhrman and Fuhrman (1960) and Huber and Phillips (1960) proposed that the cause of diarrhoea in cholera is inhibition of the "sodium pump" in the intestine. However, this hypothesis is still the subject of much controversy and the evidence in favour of it has been recently reviewed by Phillips (1968).

It is now well established that the sodium ion is actively transported. The process in most tissues, including the intestinal mucosa, is mediated by a membrane-bound adenosine triphosphatase (ATPase) first described by Skou (1957) in crab nerve cells. It was therefore thought pertinent to examine cholera stools for an inhibitor of the ouabain-sensitive membrane ATPase, by using the guinea-pig brain microsomal preparation of Schwartz, Bachelard and McIlwain (1962). During these experiments we made some unexpected observations.

Cholera stools were found to contain a potent adenosine triphosphatase with unusual properties. The stool enzyme was found to be insensitive to ouabain and fluoride and to be non-specifically activated by 150mM concentrations of alkali metal cations. This enzyme was not found in whole-cell or lysate preparations of *Vibrio cholerae*, but was demonstrable in rabbit intestinal mucosa. These findings prompted the systematic assay of tissue enzymes found in cholera and non-cholera stools reported below.

METHODS

Treatment of samples

Cholera stools were taken by rectal catheter from patients of the Cholera Wards of Satyabala Devi Hospital, Howrah, and the Infectious Diseases Hospital, Calcutta. The stool was filtered free from gross particles through glass wool, and centrifuged above 10,000*g* for 30 min.; the supernatants were taken for assay.

Other stool samples were collected in sterile paraffin-lined tins, emulsified in a small volume of normal saline where necessary and centrifuged at 10,000*g* for 30 min. The supernatants were filtered through glass wool with suction and recentrifuged.

From the time of collection to testing, samples were maintained at 4°C.

Dry-weight determinations were made on representative samples for the estimation of stool water.

Bacteriological and microscopic examination

Clinical cholera cases were confirmed, after isolation of vibrios from rectal swabs in peptone water at pH 8.0, by agglutination with diagnostic sera and phage-typing.

Other diarrhoeal samples were examined microscopically and cultured in enrichment and selective media for enterobacteria.

Received 29 Sept. 1969; accepted 10 Dec. 1969.

Enzyme assays

Alkaline phosphatase (APase) was estimated in bicarbonate buffer pH 9.3 with 50mM phenyl phosphate as substrate in a reaction volume of 3 ml with incubation at 37°C for 15 min. The reaction was stopped by addition of perchloric acid to 0.2M concentration. Inorganic phosphate liberated was determined by the method of Fiske and Subbarow (1925).

Adenosine triphosphatase (ATPase) was estimated by the liberation of inorganic phosphate from 2.5mM adenosine triphosphate in the presence of 60mM-NaF, in 30mM tris buffer pH 9.0, in a reaction volume of 2 ml at 37°C for 10 min. The reaction was stopped by the addition of perchloric acid to a concentration of 0.4M and the liberated phosphate estimated as before.

Lactic dehydrogenase (LDH) was assayed spectrophotometrically by the oxidation of reduced nicotinamide adenine dinucleotide with sodium pyruvate as substrate (Bergmeyer, Bernt and Hess, 1963). The determinations were carried out without delay as the stool enzyme was rapidly inactivated.

RESULTS

Table I shows the activities of the enzymes studied in seven groups of patients classified bacteriologically.

"Normal" samples were taken from healthy laboratory staff. The non-specific gastro-enteritis samples were characterised by high counts of *Escherichia coli* and *Streptococcus faecalis*, and could not be associated with any enteric or parasitic infection. *Entamoeba histolytica* was identified in the amoeba group along with other parasites in one sample. It was also found in two of the diarrhoeas of the salmonella-dysentery group. In the latter the increased enzyme levels were considered to be due primarily to the bacterial infection, as enzyme activities in the amoeba group were in a lower range. These diarrhoea samples were liquid (88–90 per cent. water) except two that were watery (95 per cent. water). Six of the nine stools associated with enteric bacilli showed microscopic evidence of tissue destruction.

Of the 29 clinically typical cholera diarrhoeas examined for LDH, *V. cholerae* was isolated from 18. Without serological information, the remaining 11 cholera-like diarrhoeas cannot be regarded as certainly not choleraic.

The distribution of enzyme activities covers a wide range and is not statistically "normal". The skewness could not be corrected by the usual transformations, and the data were consequently compared non-parametrically by rank analysis (Snedecor, 1956). The probabilities are recorded in table II.

Enzyme activity per ml of stool liquid was statistically higher for LDH and ATPase in cholera as compared with that of normal stool. APase activity, on the other hand, was similar to if not lower than that of normal stool.

The LDH data showed a division of the diarrhoeas into two groups (*a*) bacterial infections—cholera and salmonellosis, (*b*) non-bacterial—amoebiasis and non-specific gastro-enteritis; values in bacillary dysentery stool were intermediate. The activities are clearly zoned, decreasing in the order

Cholera > salmonellosis > cholera-like > dysentery >
amoebiasis > non-specific > normal.

The difference between cholera and all groups except salmonellosis is significant. The distribution of LDH in the salmonella group is essentially the same as in cholera.

TABLE II

Comparison by rank analysis of enzyme activities per ml of stool liquid in normal, non-cholera and cholera diarrhoeas

Groups compared	LDH			ATPase			APase		
	T*	n ₁ /n ₂ †	Probability	T*	n ₁ /n ₂ †	Probability	T*	n ₁ /n ₂ †	Probability
Cholera and normal	78	16/12	0.01	28	15/5	0.05	43	18/3	>0.1
Cholera and cholera-like diarrhoea	112	16/11	0.05	...	...	...	...	...	...
Cholera and non-specific diarrhoea	6	16/3	0.01	3	10/2	0.05	...	...	...
Cholera and amoebiasis	10	16/4	0.01	10	15/2	>0.1	...	...	...
Cholera and bacillary dysentery	14	16/4	0.01	57	15/4	>0.1	24	18/4	0.07
Cholera and salmonellosis	40.5	16/5	>0.1	48	15/5	>0.1	42	18/4	>0.1
Stools containing leucocytes, and cholera	39.5	6/18	0.05‡	49	6/16	0.08	35	6/19	0.01

* Sum of ranks.

† Size of samples.

‡ LDH in the non-cholera group is lower.

TABLE III

Relation of enzyme activity to tissue damage in non-cholera diarrhoeas

Sample no.	Associated infective agents	Microscopic evaluation			Enzyme activity per ml of stool liquid		
		RBC	Leucocytes	Mucus	LDH	ATPase	APase
1	Salmonella bacilli . .	+	+++	+	1.28	2.7	12.2
2	Dysentery bacilli . .	—	+++	+	0.021	0.69	8.2
3	Dysentery bacilli . .	—	++	+	0.11	0.81	6.7
4	Salmonella bacilli . .	—	+	+	0.11	2.73	5.8
5	Salmonella bacilli . .	—	+	+	0.38	0.57	3.2
6	Dysentery bacilli . .	+	+	—	0.031	0	0.28
7	Salmonella bacilli . .	—	—	+	0.52	0.18	0.24
8	Dysentery bacilli and <i>Entamoeba histolytica</i>	—	—	—	0.28	0.11	0.57
9	Salmonella bacilli . .	—	—	—	0.16	0	0.21
10	<i>Entamoeba histolytica</i> . .	—	—	—	0.07	0.12	0.96
11	None	—	—	—	0	0.11	2.2
Rank correlation coefficient					0.423	0.798	0.809
Probability of difference from normal					>0.05	0.01	0.01

The distribution of APase and ATPase in cholera and non-cholera diarrhoeas was different: activities tended to be higher in non-cholera diarrhoeas, particularly the enteric group. Table III shows the positive correlation between microscopic tissue damage and the phosphatase activities in non-cholera diarrhoeas. Comparison of cholera activities with the samples containing pus cells (table II) shows that in the latter group, APase ($P = 0.01$) and possibly ATPase ($P = 0.08$) are higher, whereas LDH is lower ($P = 0.05$).

The histograms in the figure (p. 459) show the association of enzyme activity to the time of collection of stool after onset of cholera symptoms. In each case, activity tends to be lower in the early stage of diarrhoea rising to a peak after about 10 hr. The high content of LDH in cholera stool appears to be maintained thereafter. Data for ATPase and APase beyond this time are insufficient, but suggestive of a drop in activity.

Computation of the data with stooling rate emphasises the magnitude of enzyme loss in cholera as compared with all other diarrhoeas (table I).

DISCUSSION

The enzymes demonstrated in cholera stool could not be found in vibrio cultures *in vitro*. Preliminary work indicates that enzymes of the same specificities do occur in the rabbit intestinal mucosa. It is likely, therefore, that high enzyme activity in cholera stool is a result of damage to epithelial cell permeability.

The non-secretory cellular enzymes of high molecular weight are normally found in extracellular fluids as a result of cell ageing. The intestinal mucosa is considered to contribute largely to the normal enzyme spectrum of serum on account of its high turnover rate (Hess, 1963). Enzyme levels in body fluids increase as a result of (a) gross pathological cell destruction and (b) physiological disturbance of the cell-membrane permeability system; LDH in serum increases after severe exercise owing to the resulting muscle anoxia (Hess); ischaemia and hypokalaemia lead to a rise in LDH in the urine in the absence of morphological kidney damage (Hess; Raab, 1967). The enzymes investigated in these experiments all occur in high activity in the intestinal mucosa. APase is a brush-border, membrane-bound hydrolytic enzyme functioning in the terminal stages of digestion and metabolite transport. Though it is outside the permeability barrier of the epithelial cell, this enzyme is no longer thought to be secreted (Crane, 1966, 1968). The magnesium-independent ATPase demonstrated has been found to be associated with the brush-border membrane. It has been shown by Fernley and Walker (1966) and by Moss and Eaton (1966), and also in this laboratory (Mahajan and Narayanaswami, 1968), to have multiple phosphatase activities, namely, ATPase, APase and pyrophosphatase. The two phosphatases demonstrated in the experiments reported above are considered to represent two separate enzymes, since there was no correlation between activities in paired measurements. The role of brush-border ATPase is not certain and it is thought to be less superficially located than the APase (Crane, 1968). Damage to, or absence of, brush-border enzymes is associated

with malabsorptive diarrhoeas (Shnitka, 1960; Padykula, 1961; Crane, 1966).

LDH is a key cytoplasmic enzyme of the glycolytic pathway, high molecular weight, located within the permeability barrier of the epithelial cell membrane. This enzyme is not thought to play a direct role in the digestion-absorption process.

The question arises what normally happens to the enzymes of the estimated 250 g of mucosa shed daily (Walker and Leblond, 1956); LDH in normal stool is virtually nil. Cell economy would require that the shed enzymes be re-utilised by breakdown and reabsorption. It is reasonable to assume that in the state of violent purging in cholera this system is not functional, so that free enzymes appear in the stools. If this was solely responsible for the LDH activity of cholera stool, it could be expected that at the time of maximal fluid flow, enzyme activity per unit volume of stool should fall owing to dilution. In fact the reverse picture emerges. If enzyme loss is independent of the cholera state, and dependent only on the physiological state of purging, then cholera-like diarrhoea stool should show a similar activity distribution. The figures for LDH in cholera-like stool are significantly lower than those for cholera stool, in spite of the possibility that the former group is likely to include undetected cholera cases.

Data published by Iwert, Leitch and Burrows (1967) and Iwert *et al.* (1968) on serum enzymes in experimental and human cholera follow a pattern similar to the data presented above. They found increased glutamate-oxalate transaminase (GOT), a cytoplasmic enzyme, in both systems. Serum LDH activity in the rabbit ileal loop model was increased; the ten samples obtained from human cholera showed a wide range (180–2000 units), but the mean value was not statistically higher than normal (range 150–580) by the *t* test. This discrepancy between the two models may have been caused by the non-normal distribution of values, and the time of testing the patients' serum after onset of symptoms; LDH is rapidly inactivated in human serum. In experimental cholera, GOT and LDH activities reached a maximum at 10 hr. Preliminary work in this laboratory on a few cholera samples indicates that stool GOT is correlated with LDH in paired measurements. Iwert *et al.* (1967, 1968) also found that serum alkaline phosphatase is not increased in the experimental animal.

The period of maximal enzyme loss in human cholera coincides with the period of maximum fluid loss, stool vibrio count (Huber, 1965) and enterotoxin activity (Dutt, 1965), suggesting a common cause. The findings of this laboratory and of Iwert *et al.* (1967, 1968) point to the intestinal mucosa as the source of the free enzymes investigated. Unlike what is found in other infective diarrhoeas (Dammin, 1965), the mucosal epithelium is morphologically intact and functional in cholera (Gangarosa *et al.*, 1960; Gordon, 1960). Our results are consistent with this finding, and indicate that free brush-border phosphatase in the stool is a dominant feature of intestinal disorders involving tissue necrosis, but not of cholera. Furthermore, the APase-mediated glucose-sodium ion translocase system in cholera has been shown to be functional

(Love, 1965). Some degree of involvement of the brush-border membrane in cholera is suggested by the two-fold rise of phosphatase activity occurring from 9 to 13 hr, at the time of maximal fluid loss. This may, however, represent a consequential enzyme response to diarrhoea, to increase the absorptive capacity, as happens during starvation. A six-fold increase in APase has been shown in the ruminant small intestinal mucosa, whereas succinic and glutamic dehydrogenases were slightly decreased (Onodera *et al.*, 1967).

A concept of increased cell permeability in cholera, by direct action of vibrio products on the mucosa, is a likely explanation of the presence of soluble cytoplasmic enzymes in stool and serum. In such a situation it is to be expected that, with the development of infection and release of enterotoxin, the integrity of the plasma membrane barrier progressively deteriorates until soluble cell

FIGURE.—The relation of stool enzyme activity to time of sample collection after onset of purging in cholera. Figures in brackets indicate the number of samples tested.

proteins are able to pass through it, the time-threshold and selectivity of protein leak depending on molecular size. From this point onwards, in the absence of inactivation, extracellular enzyme activity will remain at a level dependent on the rate of enzyme biosynthesis until recovery sets in. The figure demonstrates the abrupt five-fold rise of activity in cholera stool LDH after 9 hr; this activity remains high throughout the period of study. More precise information on this hypothesis can best be obtained from parallel clinical studies, not in the scope of this laboratory.

Iwert *et al.* (1967, 1968) attributed their findings to the absorption of vibrio endotoxin leading to peripheral vascular collapse, and consequent release of tissue enzymes due to ischaemia. On intravenous injection of several bacterial endotoxins there occurs, after 6 hr, a dose-dependent increase of serum LDH and GOT (Melby *et al.*, 1959; Vesell, Palmerio and Frank, 1960; Konttinez, Rajasalmi and Paloheimo, 1964), in agreement with the findings of Iwert *et al.* (1967, 1968) with vibrio endotoxin. However, this hypothesis does not satisfactorily explain the stool data available. The patients

admitted to this study were not in a state of endotoxic shock, and ischaemia of the gut is not compatible with the huge fluid movements observed in cholera.

Hypokalaemia is associated with high extracellular LDH activities and also with the cholera syndrome. It is possible that excessive ion loss accounts for the enzyme activities reported. However, the experimental ileal loop reaction in rabbits cannot be associated with potassium depletion, and work in this laboratory has consistently shown high LDH in loop fluid.

The role of cholera toxins in initiating this biochemical lesion, and its relation to cholera pathogenicity, await elucidation.

SUMMARY

Lactic dehydrogenase, alkaline phosphatase and a magnesium-independent adenosine triphosphatase were assayed in normal, diarrhoeal and cholera stools. The diarrhoeas, examined bacteriologically, included bacillary dysentery, salmonellosis, amoebiasis and non-specific gastro-enteritis.

Alkaline phosphatase levels were found to be similar in normal and cholera stools, whereas lactic dehydrogenase was 30-fold higher and adenosine triphosphatase four-fold higher in cholera. The distribution of activity of lactic dehydrogenase was higher, and of the phosphatases lower in cholera than in the stools of bacterial infections showing evidence of tissue necrosis. The levels of activity of all three enzymes in cholera stool were found to reach a maximum between 9 and 17 hr after onset of purging.

It is suggested that these findings indicate an increased permeability of the intestinal epithelium in cholera, leading to a leak of cell enzymes into the intestinal lumen.

The authors are indebted to the authorities of the Satyabala Devi Hospital, Howrah, and the Infectious Diseases Hospital, Beliaghata, for allowing them access to their cholera patients; to Dr B. C. Rudra for help in the collection of specimens; to the WHO for providing them with diarrhoeic specimens; to Miss Anju Ghosh and Mrs Leela Chakravorty for their technical assistance; and to Dr S. Mukerjee, now Director of the Cholera Research Centre, Calcutta, for his advice and help throughout this study.

REFERENCES

- BERGMAYER, H. U., BERNT, E., AND HESS, 1963. *In* Methods of enzymatic analysis, ed. by H. U. Bergmeyer, *Weinheim*, p. 736.
- CRANE, K. 1966. *Gastroenterology*, **50**, 254.
- ” 1968. *A. Rev. Med.*, **19**, 57.
- DAMMIN, G. J. 1965. *Fedn Proc.*, **24**, 35.
- DUTT, A. R. 1965. *Ind. J. Med. Res.*, **53**, 605.
- FERNLEY, H. N., AND WALKER, P. G. . 1966. *Biochem. J.*, **99**, P39.
- FISKE, C. H., AND SUBBAROW, Y. . . 1925. *J. Biol. Chem.*, **66**, 375.
- FORDTRAN, M. D., AND DIETSCHY, M. D. . 1966. *Gastroenterology*, **50**, 263.
- FRESH, J. W. 1965. *Proc. Cholera Res. Symp.*, Honolulu, U.S. Dept of Health Education and Welfare, Public Health Service, p. 296.
- FUHRMAN, G. J., AND FUHRMAN, F. A. . 1960. *Nature, Lond.*, **188**, 71.

- GANGAROSA, E. J., SRIBHIBHADH, R., 1960. Proc. SEATO Conf. Cholera, *Dacca*,
SPRINZ, H., BENYAJATI, C., KINDEL, D.,
BEISEL, W. R., AND HALSTEAD, S. p. 76.
- GORDON, R. S., JR. 1960. *Ibid.*, p. 54.
- HESS, B. 1963. Enzymes in blood plasma, *New York*.
- HUBER, G. S. 1965. Proc. Cholera Res. Symp., Honolulu,
U.S. Dept of Health Education and
Welfare, Public Health Service, p. 41.
- HUBER, G. S., AND PHILLIPS, R. A. . . . 1960. Proc. SEATO Conference on Cholera,
Dacca, p. 37.
- IWERT, M. E., DEB, B. C., SRIVASTAVA, 1968. *J. Infect. Dis.*, **118**, 422.
D. L., AND BURROWS, W.
- IWERT, M. E., LEITCH, G. J., AND 1967. *Ibid.*, **117**, 265.
BURROWS, W.
- KONTTINEZ, A., RAJASALMI, M., AND 1964. *Amer. J. Physiol.*, **207**, 385.
PALOHEIMO, J.
- LOVE, A. H. G. 1965. Proc. Cholera Res. Symp., Honolulu,
U.S. Dept of Health Education and
Welfare, Public Health Service, p. 144.
- MAHAJAN, R. G., AND NARAYANASWAMI, 1968. Proc. LV Ind. Sci. Congr., Part III,
A. p. 173 B.8.
- MELBY, J. C., BOSSENMAIER, I. C., 1959. *Lancet.*, **1**, 441.
EGDAHL, R. H., AND SPINK, W. W.
- MOSS, D. W., AND EATON, R. 1966. *Biochem. J.*, **99**, P19.
- ONODERA, R., TSUNEYUKI, T., KAICHI, A., 1967. *Tohoku J. Agric. Res.*, **18**, 177 (quoted in
AND MOTOYOSHI, U. *Biological Abstracts*, 1968, **49**, abstr.
no. 98915).
- PADYKULA, H. A. 1961. *Gastroenterology*, **40**, 735.
- PHILLIPS, R. A. 1968. *A. Rev. Med.*, **19**, 69.
- RAAB, W. 1967. *Experientia*, **23**, 833.
- SCHWARTZ, A., BACHELARD, H. S., AND 1962. *Biochem. J.*, **84**, 626.
MCILWAIN, H.
- SHNITKA, T. K. 1960. *Fedn Proc.*, **19**, 897.
- SKOU, J. C. 1957. *Biochim. biophys. Acta*, **23**, 394.
- SNEDECOR, G. W. 1956. Statistical methods, *Iowa*, pp. 117 and
190.
- USSING, H. H., AND ZERAHN, K. . . . 1951. *Acta physiol. scand.*, **23**, 110.
- VESELL, E. S., PALMERIO, C. F. P., AND 1960. *Proc. Soc. Exp. Biol. Med.*, **104**, 403.
FRANK, E. D.
- WALKER, B. E., AND LEBLOND, C. P. . . 1956. *Physiol. Rev.*, **36**, 255.