ENZYMES IN VIBRIO CHOLERAE—A REVIEW.

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Cellular metabolism represents a summation of multiple enzyme reactions in which complex substances are synthesized or broken with great precision under very mild conditions. Considering the large number of links in the chain, one would ordinarily imagine the number of enzyme systems operating in the cells to be innumerable. There has, however, been "a growing suspicion that highly specific macro-molecules—proteins, DNA and presumably RNA—do not attain their complexity through a summation of simple enzyme controlled reactions, each adding one building block at a time, but rather are formed through some quite different mechanism involving a multi-head template" (Davis, 1956). This concept would imply that the number of enzymes in a micro-organism is after all limited. Whatever the number of enzymes, it is obvious that they would play an important role in determining the antigenic behaviour and pathogenicity of a micro-organism and hence it is necessary to obtain an intimate knowledge of the constituent enzymes for a rational approach to the chemotherapy of any infection.

The main emphasis in the work on *Vibrio cholerae* has been on the study of the gross antigenic structure for classification of vibrios into the choleragenic and the non-choleragenic types and not much attention has been given to their enzymic make-up. Only stray investigations on enzymes are reported in the earlier literature, and these have been referred to in the comprehensive "Cholera Studies" Part III by Pollitzer (1955). The present review is concerned with the more recent studies in enzymes of *V. cholerae.*

The work of Burnet and Stone (1947) and Burnet (1948,49) stimulated a great interest in enzymes in *V. cholerae.* They showed that filtrates from *V. cholerae* cultures were capable of producing desquamation of the intestinal epithelium *in vitro;* the principal agent being presumably a mucinase. Singh and Ahuja (1953) demonstrated the presence of the intestinal-epithelium-destroying enzyme not only in smooth cholera strains but also in most El Tor and cholera-like strains. The enzymes did not appear to have any relationship with the antigenic behaviour of *V. cholerae.* Similar conclusions were arrived at by Narayanan and Menon (1952) and Narayanan *et al.* (1953) in their studies on the collagenase and elastinase activities of *V. cholerae.* Felsen-

feld's investigation (1944) showed lecithinase activity in four true cholera strains as well as in one El Tor strain.

Since 1952 a series of papers have been published by workers at the Central Drug Research Institute, Lucknow, embodying their studies on the enzyme systems of *V. cholerae*. This work has been carried out either with resting cells or with cell free extracts from vibrios, on the assumption that the results so obtained would represent the correct picture of the enzymes in the cell. In order that the results may be comparable, the enzyme studies have been carried out with the Inaba sub-type obtained by treating Ogawa with its homologous monospecific antiserum (Shrivastava and Bruce White, 1947). Similarly, the rough variant was obtained by treating the Inaba with its homologous antiserum. Some of the important enzyme systems involved in the protein and carbohydrate metabolism of vibrios have been studied and the observations are discussed in the present review.

Deaminases :

Dudani *et al.*, (1952) studied the deamination of aspartic acid, serine, arginine, glycine, glutamic acid, lysine and threonine by *V. cholerae*. The rate of deamination was found to differ from one amino acid to another and from strain to strain. In general the Ogawa sub-type showed a higher deaminase activity than the Inaba. Deamination took place under strictly aerobic conditions and was optimum at a pH range of 7.0 to 8.0.

Since aspartic acid was most readily deaminated, it was studied in greater detail by Iyer *et al.*, (1953). The deamination was found to be aerobic in nature and of the oxidative type. Resting cells of *V. cholerae* deaminated aspartic acid over a pH range 6.5 to 9 with an optimum at 8.0. Activation inhibition studies indicated that the activity of the enzyme was associated with essential metal radicals and sulphydryl, amino and carbonyl groups. The exact nature of the coenzyme was, however, not clear.

Iyer *et al.*, (1954) found that sodium chloride played an important part in maintaining the stability of aspartic deaminase of *V. cholerae* ; the degree of stability being greater at higher concentrations of the salt. With intact cells suspended in 1.7 and 3.4 per cent sodium chloride, the enzyme activity was found to be low just at the time of preparation but it increased on incubation due perhaps to the altered permeability of the cell membrane in the presence of high salt concentration. Increase of the salt concentration in the growth medium brought down the aspartic deaminase activity of the organism.

Taking advantage of the observation that 3.4 per cent of sodium chloride confers maximum stability to aspartic deaminase, Iyer and Krishna

Murti (1955) prepared the cell-free enzyme and studied its properties. The high sensitivity of the vibrio enzyme to pH changes and the inability of biotin, adenosine and yeast extracts to protect the enzyme indicated that this was different from the aspartic deaminase from *Escherichia coli* (Gale, 1938).

Arora *et al.*, (1956) further studied the effect of sodium chloride on three other enzymes, tryptophanase, adenosine deaminase and serine deaminase known to be involved in the intermediary metabolism of *V. cholerae*. It was found that in contrast to the earlier observations on aspartic deaminase, the production of these three enzymes by *V. cholerae* was stimulated to a considerable degree by increasing concentrations of sodium chloride in the growth medium even upto 3.4 per cent. On the other hand, increasing concentrations of the salt in the suspending medium used for enzymatic studies with the resting cells, have a deleterious effect on the initial activity of the enzymes. 0.85 and 1.7 per cent salt solutions had, however, a partial stabilizing effect on adenosine and serine deaminases but no such action was observed on tryptophanase. The general trend of results would indicate the importance of a certain concentration of sodium chloride in maintaining optimal activity of these enzymes. The mechanism of action of the salt was, however, not clear.

Transaminase :

Saxena *et al.*, (1955) showed that amino acids were metabolized by vibrios both through deamination and transamination. This activity was demonstrated in all the strains and sub-types tried, and the observed differences did not reveal any correlation between antigenicity and the transaminase content. The reaction with 16 donor amino acids and 3 Keto acid acceptors was found to be most active in the α (-Ketoglutarate system, maximum activity being observed with L-leucine; D-isomer of the amino acid being completely inactive, indicating an optical specificity for the enzyme in contrast to that in *Bacillus subtilis* (Thorne *et al.*, 1955). Pyridoxal phosphate had only a very partial stimulatory action on the enzyme extract subjected to prolonged dialysis.

Dehydrogenases:

Dudani *et al.*, (1953) reported dehydrogenase activity of an Ogawa strain and the Inaba and rough variants derived from it. The results indicated that dehydrogenase activity was highest in the Rough followed by Inaba and Ogawa strains. This was in contrast to the pattern in deamination wherein the activity was in the order Ogawa - Inaba - Rough as reported above. Almost all amino acids and aliphatic acids employed in this study acted as hydrogen donors for the respiratory activity of this organism. Dehydrogenases of *V. cholerae* appeared to be linked with the cytochrome system present

in this organism but the possibility of other co-enzyme systems taking part in this process could not be excluded.

Peptidases :

Agarwala *et al.*(1953b) studied the hydrolysis of glutathione by *V. cholarae* cells and found evidence of the presence of γ-peptidase in the cells. This was the first observation of the hydrolysis and metabolism of glutathione by any pathogenic or non-pathogenic bacteria.

Study of the peptidase activity of *V. cholerae* has been continued by Saxena *et al.* (1959). Cell-free extracts of the organism prepared by extraction with 0.85 per cent KCl of cells broken by grinding with abrasives or treating in an ultrasonic oscillator, hydrolysed a variety of di- and tri-peptides. The pH optimum was between 7 and 8 and the crude enzyme (s) could be precipitated with acetone without any appreciable loss of activity. In the case of almost all the peptides Co^{++} had an activating influence.

Tryptophanase :

Culture filtrates of *V. cholerae* give a positive reaction for indole presumably due to the breakdown of tryptophane. A detailed investigation on this enzyme in resting cells and in a cell-free state, the action of various inhibitors and co-enzymes, was, therefore, carried out by Arora *et al.* (1959a). The enzyme was found to have a lower optimum pH in the intact cells than in the cell-free state as in the case of tryptophanase of *E. coli* and certain other bacterial enzymes (Dawes *et al.* 1947 and Stephenson, 1949). Sodium and potassium chlorides in the growth medium stimulated the enzyme activity.

Tryptophanase activity was found in all the choleragenic and non-choleragenic vibrios but there were quantitative differences among the strains. The indole formed by an Inaba strain was significantly higher than that formed by its parent Ogawa or the Rough, whereas the non-pathogenic W. Khal had the maximum activity.

Cell-free preparations of the enzyme were made by extracting the viable cells of the vibrios with KCl solution to avoid the possibility of injuring the tryptophanase complex. Pyridoxal phosphate alone appeared to be the co-enzyme for the cell-free tryptophanase of *V. cholerae* as in the case of *E. coli* (Dawes and Happold, 1949).

Metabolism of purine and pyrimidine compounds :

Agarwala *et al.* (1954) studied the deamination and oxidation of 18 purine and pyrimidine compounds and the factors influencing the deamination of adenosine by resting cells of *V. cholerae*. Deamination appeared to be

the only active process involved in the utilization of purine nitrogen. In this respect the organism behaved differently from *Mycobacterium tuberculosis* (Di Finzo, 1952) and aerobic soil bacilli which have considerable oxidative action on these bases. Phosphorylated derivatives of purine and pyrimidine ribosides were readily hydrolysed by the cells of *V. cholerae* to give inorganic phosphate which would suggest the presence of ribose phosphorylases. This is an interesting finding and a detailed study of these phosphorylases would help in the elucidation of the synthesis of nucleic acids by the vibrio cells. The comparative importance of the deamination of purine and pyrimidine ribosides in the metabolism of vibrios, as indicated by these studies would suggest that analogues of these substances may function as structural antagonists and thereby possess potential inhibitory activity against vibrios.

Nucleotidases :

The consistent failure to detect any phosphatase activity in the vibrio cells led Krishna Murti and Shrivastava (1955a) to study the factors influencing the dephosphorylation of nucleotides, reported above, both by intact vibrios and by enzyme extracts obtained from the cells. A high reactivity towards the phosphate attached to the 5 carbon atom of ribose in nucleotides was detected. The phosphoric esters of glycerol, phenol, phenolphthalein and hexoses and inorganic meta-and pyrophosphates were not affected at all. These findings are in contrast to the action of 5-nucleotidases reported in mammalian tissues by Reiss (1938) and Gomori (1949) and in microbial cultures by Gros and Macheboeuf (1948) and Auger and Macheboeuf (1951).

The principal finding of this study on the high specificity of nucleotidase is of significance in the biosynthesis of nucleic acids by vibrios. This, together with the resistance of the enzyme to common inhibitors and activators, reported by the authors, might again point out a fruitful line of attack on the metabolism of the organism by suitably designed metabolic antagonists.

Ribonuclease .

In view of the nucleotidase activity of vibrio cells reported above, Sagar *et al.* (1958) made an attempt to find out whether vibrio cells and cell-free preparations from them catalyse the degradation of yeast RNA. The results showed that the *V. cholerae* had an efficient mechanism to depolymerize the macro RNA molecules and this manifested itself only after the rupture of the cells as in the case of *E. coli* reported by Manson (1953).

Gelatinase :

Agarwala and Shrivastava (1953) reported on the gelatinase activity of a few strains of *V. cholerae*, El Tor, Rough and Water vibrios and the effect of certain inhibitors and activators on it. The pH optimum was 8.0. Water vibrios

showed the maximum activity and Inaba had in general slightly higher activity than the parent Ogawa. Further, activation studies showed that glutathione, ascorbic acid and the antibiotics activated the gelatinase of Ogawa and Inaba but did not affect the activity of the Water vibrio. This might be due to the enzyme in Water vibrio being present in a fully activated form, while in the two sub-types it may not be so.

Penicillinase :

The low susceptibility of *V. cholerae* to penicillin led Iyer *et al.*, (1954a) to a screening of several strains of this organism and other vibrios for the presence of penicillinase. Of the 56 strains examined only 2 water and 2 non-agglutinable vibrios were observed to produce penicillinase.

Catalase :

Since the catalase activity had been associated with virulent strains of *Pasteurella pestis* (Rockenmacker, 1949), Saxena *et al.*, (1955) carrried out an investigation to see if choleragenic vibrios could be distinguished from the non-choleragenic on the basis of catalase activity. No such difference was, however, observed.

Organic Nitroreductase :

Arora *et al.*, (1959b) examined the distribution of organic nitroreductase in *V. cholerae*. Cysteine was found to have a stimulatory action on the enzyme system of the cells and cell-free extracts. The metal binders had no action. Whereas isonicotinic acid hydrazide inhibited the nitroreductase of *V. cholerae* strongly, chlorotetracycline had no action.

Oxidative Metabolism :

With a view to find out whether antigenic differences in vibrios result in enzymic differences, Agarwala *et al.*, (1953a) carried out a systematic study of the oxidative metabolism of six Ogawa cultures including two recently isolated case strains, four Inaba and one each of a Water vibrio, El Tor and Rough strains with eight carbohydrates, seventeen amino acids, one purine, one tripeptide and seven aliphatic acids. The results showed that *V. cholerae* could metabolize oxidatively a variety of substrates, the rate of oxidation, however, varied from strain to strain and substrate to substrate. No significant difference was observed in the metabolic activity of freshly isolated cultures and those maintained in the laboratory so that it would appear that cultivation in artificial media does not bring about any appreciable changes in the enzymic make up of the organism. Differences were, however, noticed in the metabolic activities of Ogawa and the Inaba derived from it, on a variety of substrates. Whether these would indicate differences in the enzyme contents due to antigenic differences could be clarified only after further and more detailed work.

Some of the substrates that feed directly or indirectly into Krebs' tricarboxylic acid cycle were found to be readily metabolized by vibrios in the oxidative metabolic studies reported above. The oxidation of some of the Krebs' intermediates were studied by Krishna Murti and Shrivastava (1955b) with resting cells of *V. cholerae* using specific metabolic inhibitors. Their observations showed that all the reactions involved in the Krebs' cycle from the stage where α-ketoglutarate participates upto the terminal oxidation of acetate, were demonstrable in the resting cells of *V. cholerae*.

Cystine and cysteine were found to stimulate the uptake of oxygen by resting cells of *V. cholerae* (Agarwala *et al.*, 1953a) and since a major sector of the Krebs' cycle was found to operate in this organism, Krishna Murti and Shrivastava (1956a) made an attempt to find out whether cysteine could participate in this cycle through pyruvic acid, the end product of its metabolism. The results indicated in general that cysteine could participate in the terminal respiration of *V. cholerae* and that the desulphydrase system of this organism acted aerobically in contrast to that associated with *Proteus vulgaris* and *Proteus Morganii* (Kearney and Singer 1953 a & b, and Singer and Kearney, 1953).

Further, hexoses have been reported to undergo both glycolytic and oxidative breakdown in the presence of resting cells of vibrios (Linton *et al.*, 1936 and Agarwala *et al.*, 1953a). Krishna Murti and Shrivastava (1956b) studied the enzymes involved in the utilization of carbohydrates. The results indicated that the following reactions were mediated by cell-free extracts of *V. cholerae*:

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{hexokinase}]{\text{Mg}} \text{G-6-P} + \text{ADP}$$

$$\text{Fructose} + \text{ATP} \xrightarrow[\text{hexokinase}]{\text{Mg}} \text{F-6-P} + \text{ADP}$$

$$\text{Ribose} + \text{ATP} \xrightarrow[\text{pentokinase}]{\text{Mg}} \text{R-5-P} + \text{ADP}$$

$$\text{G-1-P} \underset{\text{phosphoglucomutase}}{\overset{\text{Mg}}{\rightleftharpoons}} \text{G-6-P}$$

$$\text{G-6-P} \underset{\text{Isomerase}}{\rightleftharpoons} \text{F-6-P}$$

$$\text{F-6-P} + \text{ATP} \xrightarrow[\text{fructose phosphokinase}]{\text{Mg}} \text{HDP} + \text{ADP}$$

$$\text{G-6-P} + \text{DPN} \xrightarrow[\text{dehydrogenase}]{} \text{6-PGl} + \text{DPNH}$$

$$\text{G-6-P} \longrightarrow \text{6-PG} \longrightarrow \text{Pentose}$$

$$\text{HDP} \underset{\text{aldolase}}{\rightleftharpoons} \text{G-3-P} + \text{DHAP}$$

$$\text{G-3-P} + \text{DPN} \xrightarrow{\text{2-PGA}} \text{3-PGA*} + \text{DPNH}$$

$$\text{2-PGA} \rightleftharpoons \text{enol-phosphopyruvate}$$

$$\text{Enol-phosphopyruvate} \rightleftharpoons \text{pyruvate} + \text{ATP}$$

$$\text{Triosephosphate} + \text{acetaldehyde} \underset{\text{aldolase}}{\rightleftharpoons} \text{desoxypentose}$$

*It would thus appear that *V. cholerae* is endowed with the enzymic mechanisms operating the glycolytic as well as the direct oxidative pathways. This is of special significance because of the importance of these two major pathways of carbohydrate metabolism in the synthesis of nucleic acids, proteins and polysaccharides. It is possible that the specific differences in the antigens of *V. cholerae* and other vibrios may be reflected in the mode of utilization of the main constituents of the growth medium by the organism. In this connection an interesting observation has been made by Misra and Shrivastava (1959). The polysaccharide (glycogen) isolated from the uninoculated broth was found to be freely metabolized by vibrios during proliferation, whereas the heteropolysaccharide isolated from them was not utilized. This conforms to the observations in the case of many other bacteria. The importance of enzymes in the anabolism of homo-and hetero-polysaccharides has been discussed in a recent review by Wilkinson (1958), and many of the factors possibly involved in the biosynthesis of polysaccharides pointed out in it may be applicable in the case of vibrios also. This would, however, be clear on further work. The observations so far made on the enzyme

*6-PGl = 6 Phosphogluconate
DHAP = Dihydroxy acetone phosphate
PGA = Phosphoglyceric acid

make-up of vibrios have revealed only quantitative differences between the two sub-types of *V. cholerae* on the one hand and between the cholera and non-cholera vibrios on the other. Investigations regarding the quantitative importance of various enzyme reactions specially that of the glycolytic and the direct oxidative pathways and the role of phosphorylation in the economy of the vibrio cells will help a great deal in the elucidation of the biosynthesis of the specific polysaccharides and other antigens.

LOCALIZATION OF ENZYMES

The work on vibrio enzymes has so far been carried out either with resting cell suspensions or with cell-free extracts prepared after breaking up the cells. One would expect some information on intra-cellular localization of enzymes and the probable sites of certain metabolic activity by testing various fractions obtained by differential centrifugation of the cell-free extracts. Localization of a number of enzyme activities associated with the mitochondria of higher organisms has been reported in a number of bacteria (Alexander, 1956). For example, succinic oxidase is usually associated with mitochondria in higher organisms. By differential centrifugation Sagar *et al.*, (1959a) have showed that almost all the activity of both the components of this system (succinic dehydrogenase and cytochrome oxidase) are present in the particulate fractions sedimented upto 92,000g. The concentration of the enzymes in terms of specific activity decreases in the particles sedimenting at higher speeds particularly in the case of disintegration of cells by ultrasonic treatment. It is thus possible that the smaller particles may have been produced because of the rupture of larger particles or damage of the cell membrane during disruption (Stanier, 1954 and Repaske, 1954).

Further work by Sagar *et al.*, (1959b) at 144,000g has shown that the residue at this speed contains 45 to 50 per cent ribonucleic acid and 15 to 20 per cent deoxyribonucleic acid, whereas almost all the remaining nucleic acids are recovered from the supernatant at this speed. This is suggestive of the particles obtained at 144,000g being analogous to the "microsomal" fraction of higher organisms, considered to be important sites for synthesis of proteins (Brachet 1957). Thus these preliminary studies may well be the starting point in the elucidation of the organisation and functions of enzymes in the vibrio cells.

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