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THE ROLE OF TRACE ELEMENTS AND PHOSPHATES IN THE SYNTHESIS OF VASCULAR-PERMEABILITY FACTOR BY *VIBRIO CHOLERAE*

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SUMMARY. Trace element and phosphate requirements for the synthesis of vascular-permeability factor (PF) by *Vibrio cholerae* strains B1307 and VC12 were investigated. While magnesium appeared to be indispensable for strain VC12, small amounts of PF were synthesised by strain B1307 in the presence of iron, zinc and manganese. However, even in the latter strain, maximum synthesis was recorded only in magnesium-containing media. Phosphates in the range 0·75–6·00mM controlled the synthesis of PF by both strains.

INTRODUCTION

The importance of trace elements and phosphates in the synthesis of secondary metabolites in general (Weinberg, 1970, 1974) and microbial toxins in particular (Kato, 1970; Weinberg, 1972) is being increasingly recognised. Although the nutritional and environmental factors influencing the production of cholera toxin have been studied (Callahan, Ryder and Richardson, 1971), the role of trace elements and phosphates has not been investigated. We here report studies of their role in toxin production by two strains of *Vibrio cholerae*. In our studies, toxicity was examined in terms of the effect of culture filtrate on vascular permeability, because Craig (1971) concluded that enterotoxic and permeability activities are two functional manifestations of the same molecule and that they can be described by the simple but appropriate name "cholera toxin". Attempts have also been made to correlate toxin synthesis by vibrios with their metabolic activity as evaluated by the consumption of glycerol and amino acids.

MATERIALS AND METHODS

Vibrios. *Vibrio cholerae* strains B1307 and VC12 were kindly supplied by Dr J. P. Craig, State University of New York, USA. Both these strains are virulent. Strain B1307 is a classic Ogawa biotype used by Craig (1971) and strain VC12 (Ogawa) was used by Evans and Richardson (1968). The cultures were maintained by the procedure of Evans and Richardson

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(1968). The first subculture from the lyophilised culture obtained by us was suspended in 3% (w/v) peptone water containing 15% (w/v) glycerol. These were distributed in 1-ml amounts in small sterile screw-capped bottles and maintained at −20°C. All experiments were done on a single batch of each culture.

Culture media. The TA medium of Callahan, Ryder and Richardson (1971) was modified by omission of the trace-element solution. The final medium had the following ingredients (w/v) in 0·01M Tris (hydroxymethyl) aminomethane (Tris)–HCl buffer, *p*H 7·2: arginine (Centron Research Laboratories, 103 Adhyard Industrial Estate, Bombay 40013, India) 0·25%, serine (Centron Research Laboratories) 0·25%, glutamic acid (Riechel-De Hain Aa Sulzu-Hannovar) 0·25%, asparagine (Merck, Alton, Hants) 0·25%, NaCl (BDH Ltd, Poole, Dorset) 0·25%, KCl (BDH) 0·25%, Na_2HPO_4 (BDH) 0·02%, glycerol (BDH) 0·05%.

Solutions of trace elements were prepared in double glass-distilled water at a concentration of 100 μg of the metal/ml and were sterilised by filtration. The salts used were: magnesium sulphate (BDH), manganese sulphate (BDH), ferrous sulphate (BDH), nickel sulphate (May & Baker, Dagenham, Essex), zinc sulphate (BDH). These salts were added to media individually at different concentrations to determine the optimum level for growth. For further studies on production of permeability factor (PF), these optimum concentrations were used. For experiments with phosphates, a basal medium devoid of Na_2HPO_4 was prepared and various amounts Na_2HPO_4 solution were added to give a final molarity of 0·25–48·0mM. The test media were distributed in 10-ml amounts into 100-ml flasks to give a surface: volume ratio of about 2·5 cm^2: ml.

Methods. From the thawed stock cultures, a loopful was transferred to nutrient-agar slants and incubated at 37°C. A saline suspension of this growth, optical density 0·1 at 660 nm, was used as the seed culture; 0·1 ml of this was inoculated into each flask of test medium which was then incubated at 28°C on a reciprocal shaker adjusted to 230 oscillations/min. Incubation was ended at 14 h for strain VC12 and at 22 h for strain B1307, as recommended by Callahan *et al.* (1971).

Growth was measured colorimetrically at 660 nm in a Klett Summerson colorimeter and the readings obtained were recorded as Klett units (KU). After removal of the bulk of bacteria by centrifugation, the supernate was sterilised by passage through a sintered-glass filter G5. The filtrate was maintained at 4°C until assays were completed.

PF was assayed by the technique of Craig (1965). Doubling dilutions of culture filtrate were prepared in borate-buffered saline containing 0·1% (w/v) gelatin; 0·1 ml of each dilution was injected intradermally in the shaved back of a rabbit. After 18–24 h, the amount of PF inducing erythema and induration of 7 mm diameter was taken as one induration dose (ID). Samples of toxin containing more than 1000 ID/ml were also assayed by the limit of blueing (Lb) titration method of Craig (1971). One-ml serial dilutions of toxin were mixed with 1 ml of antitoxin containing 1 antitoxin unit and incubated at 37°C for 1 h; 0·1 ml of each dilution was then injected intradermally into the shaved back of a rabbit. After 24 h, the amount of PF evoking an area of increased permeability 4 mm in diameter was taken as the end-point. Results were expressed as Lb doses/ml.

Glycerol was estimated by the technique of Burton (1957), and amino acids by thin-layer chromatography on silica gel C (Acme Synthetic Chemicals, 308 Veer Savarki Marg, Bombay 400028, India) with chloroform:methanol:17% ammonia (2:2:1 v/v) as solvent (Brenner, Niederweiser and Pataki, 1969).

RESULTS

Tables I and II show the effect of metal ions on growth and PF production of strains B1307 and VC12 respectively. The metal-ion concentrations studied were those found to be optimal for the growth of the particular strain. The presence of magnesium was particularly important. Strain B1307 secreted small quantities of PF when cultured in media containing iron, zinc or manganese. Copper and nickel did not bring about PF production, though

TABLE I

Effect of metal ions on growth and production of permeability factor by Vibrio cholerae strain B1307

Metal ion μg/ml	Density of growth (Klett units*)	Concentration of PF as: ID/ml	Lb/ml
None	38	0	NT
Ni 5	125	0	NT
Zn 15	220	500	NT
Mn 15	230	400	NT
Mg 50	300	100000	2500
Fe 50	190	2000	50
Cu 15	185	0	NT

PF = permeability factor; ID = induration dose (see *Methods*); Lb = limit of blueing dose (see *Methods*); NT = not tested.
* See *Methods*.

TABLE II

Effect of metal ions on growth and production of permeability factor by V. cholerae strain VC12

Metal ion μg/ml	Density of growth (Klett units*)	Concentration of PF as: ID/ml	Lb/ml
None	28	0	NT
Ni	0	0	NT
Zn 1	4	0	NT
Cu 1	0	NT	NT
Mn 1	8	0	NT
Mg 50	400	50000	1250
Fe 50	180	0	NT

Footnotes as in table I.

they supported growth. Comparison of the results obtained with zinc and iron clearly shows that PF production is not a mere function of growth. In iron-containing media, PF 2000 ID/ml was detected despite a low cell density of 190 KU. In zinc-containing media, on the other hand, even at the 220 KU, the PF concentration was only 500 ID/ml. Strain VC12 could synthesise PF only when magnesium was present in the medium, although it grew fairly well in iron-supplemented media. Nickel, copper, zinc and manganese were strongly toxic to this strain.

The utilisation of glycerol did not parallel either growth or toxin production in strain B1307 (table III). Cultures containing copper 15 μg consumed as much glycerol as those containing magnesium 50 μg, though growth in the former was of the order of 185 KU compared to 300 KU in the latter. Furthermore, copper did not support any PF synthesis while high concentrations of PF were produced in magnesium-containing media (table I). Likewise, nickel, manganese and zinc ions brought about more utilisation of glycerol than iron, notwithstanding the greater amount of PF produced in the

presence of the latter. In the case of strain VC12, however, utilisation of glycerol was greatest in cultures containing magnesium ions (table IV).

Breakdown of all the supply of asparagine by strain B1307 was recorded in all media except the metal-free medium (table III). On the other hand, complete consumption of all the available serine occurred only in magnesium-containing medium. Results with strain VC12 were similar (table IV). Iron appeared to be a poor promoter of glycerol utilisation by strain VC12 in contrast to the findings with strain B1307. Furthermore, this ion did not bring about depletion of all the supply of serine.

The effects of serine on PF secretion was further examined with a serine-free medium containing all the other constituents at levels optimum for PF synthesis. Strain B1307 was grown in this medium supplemented with a range of serine concentrations. The results (table V) showed that strain B1307 could not synthesise PF in the absence of serine. Small amounts of PF were detected in the presence of serine 0·5 mg/ml, but maximal production of PF was attained only at 2·5 mg/ml, at which concentration growth was also maximal. Growth and toxin production remained unaltered even when the serine level was raised to 80 mg/ml. Similar results were obtained with strain VC12.

TABLE III

Effect of metal ions on utilisation of glycerol and amino acids by V. cholerae strain B1307

Metal ion μm/ml	Amount of glycerol utilised (μg/ml)	Amino acids remaining in the medium			
		Arginine	Asparagine	Glutamic acid	Serine
None	2000±5	+	+	+	+
Ni 5	3585±6	+	−	+	+
Zn 15	3500±5	+	−	+	+
Mn 15	3620±6	+	−	+	+
Mg 50	3756±4	+	−	+	−
Fe 50	3300±4	+	−	+	+
Cu 15	3750±4	+	−	+	+

+ = Present; − = absent.

TABLE IV

Effect of metal ions on utilisation of glycerol and amino acids by V. cholerae strain VC12

Metal ion μg/ml	Amount of glycerol utilised (μg/ml)	Amino acids remaining in the medium			
		Arginine	Asparagine	Glutamic acid	Serine
None	1150±4	+	+	+	+
Mg 50	4020±5	+	−	+	−
Fe 50	1350±6	+	−	+	+

+ = Present, − = absent.

Tables VI and VII illustrate the effect of phosphates on growth and PF production. The medium used contained optimum concentrations of all the constituents found necessary for PF production except phosphate. Both the strains demanded a minimum of 0·25mM phosphate to grow. However, PF synthesis did not occur at this concentration. Strain VC12 secreted small quantities of toxin at 0·5mM phosphate and maximum quantities were attained at 0·75mM. Strain B1307, on the other hand, did not produce any PF until the phosphate concentration reached 0·75mM but maximum concentrations were attained at 1·25mM. The amount of PF produced declined to one tenth when the phosphate concentration was raised to 6mM, and PF synthesis was totally inhibited by 24mM phosphate (table VI). The pattern of amino-acid utilisation by strain B1307 in the presence of different concentrations of phosphate was also examined (table VI). Serine and asparagine utilisation depended on the presence of phosphate.

TABLE V

Effect of serine on growth and production of permeability factor by V. cholerae strain B1307

Concentration of serine (mg/ml)	Density of growth (Klett units)	Concentration of PF (ID/ml)
None	260	Nil
0·5	270	500
1·0	270	5000
1·5	280	10000
2·0	280	25000
2·5	300	100000
3·0	300	100000
5·0–80·0	300	100000

Footnotes as in table I.

TABLE VI

Effect of phosphates on growth and production of permeability factor by V. cholerae strain B1307

mMolarity of Na_2HPO_4	Density of growth (Klett units)	Concentration of PF (ID/ml)	Amino acids remaining in the medium			
			Arginine	Asparagine	Glutamic acid	Serine
None	10	0	+	+	+	+
0·25	210	0	+	+	+	+
0·5	280	0	+	±	+	+
0·75	290	2500	+	−	+	±
1·0	300	25000	+	−	+	−
1·25	300	100000	+	−	+	−
1·5	300	100000	+	−	+	−
2·0	300	100000	+	−	+	−
3·0	300	100000	+	−	+	−
6·0	300	10000	+	−	+	−
12·0	300	5000	+	−	+	−
24·0	300	0	+	−	+	−
48·0	300	0	+	−	+	−

+ = Present; − = absent. Other footnotes as in table I.

TABLE VII

Effect of phosphates on growth and production of permeability factor by V. cholerae strain VC12

mMolarity of Na_2HPO_4	Density of growth (Klett units)	Concentration of PF (ID/ml)
None	0	0
0·25	325	0
0·5	350	25000
0·75	380	100000
1·0	400	100000
1·25	400	100000
1·5	400	100000
3·0	400	100000
6·0	390	50000
12·0	350	5000
24·0	340	5000
48·0	320	0

Footnotes as in table I.

DISCUSSION

The results demonstrate that magnesium is essential for good toxin production by *V. cholerae* strains B1307 and VC12. However, these strains differed in their response to other metal ions. While magnesium was the only element that could bring about toxin production by strain VC12, strain B1307 could synthesise small quantities of toxin in the presence of other metal ions, e.g., zinc, manganese and iron. Unlike strain VC12, strain B1307 could grow well in manganese- and zinc-containing media. Nevertheless, it cannot be inferred that the traces of toxin observed in these cultures are the result of a direct influence of the increased growth of vibrios. Thus, in iron-containing media, although the cell density attained by both the strains was almost the same, PF was not detected in strain VC12 whereas considerable amounts of PF accumulated in the case of strain B1307. Moreover, zinc and manganese were more stimulatory to the growth of strain B1307 than iron, but gave rise to a lower PF concentration. Comparison of the effects of copper and iron on strain B1307 showed that despite their equal growth promotion, copper failed to elicit synthesis of toxin. These observations suggest that metal ions have a specific part to play in toxin synthesis and that this phenomenon is independent of the influence they may have on the synthesis of cell material. The synthesis or activity of synthetases of cholera toxin, or both, is probably magnesium dependent. Weinberg (1970), reviewing the trace-element requirement for secondary metabolism by a wide variety of microbial systems, concluded that manganese, iron and zinc are the most important. Because cholera toxin has all the characters of a secondary metabolite, the results of the present study emphasise the need to include magnesium in this list. Accumulation of small quantities of toxin by strain B1307 in the presence of iron, zinc and manganese indicate that the process of toxin synthesis is not brought to a complete halt in the absence of magnesium. This is in conformity with the hypothesis of

Weinberg (1970) that secondary metabolism is merely distorted rather than destroyed in an unfavourable trace-metal balance.

A significant point that emerges from this study is that toxin synthesis does not run parallel to glycerol utilisation by the vibrios. This is particularly evident when the results with magnesium and copper are compared. Toxin synthesis appeared to be related, however, to serine metabolism in that complete consumption of this amino acid occurred in cultures yielding maximum toxin. Similar observations were recorded with strain 569B (Sagar, Nagesha and Bhat, 1979). The results shown in table V suggest that, though serine is indispensable for toxigenesis, an excess does not inhibit it. Good growth of the organisms occurred in the absence of serine. Total absence of toxin in these cultures indicates that serine has a specific role to play in the toxinogenesis of vibrios. A relation between serine metabolism and toxinogenesis is further suggested by the results in tables VI and VII. Complete consumption of serine did not occur until the phosphate concentration reached the level optimum for toxin synthesis. However, complete utilisation of serine continued to occur even in cultures in which phosphates had reached concentrations inhibitory for toxinogenesis. Instances of amino acids inducing the synthesis of secondary metabolites have been reported (Demain and Inamine, 1970; Drew and Demain, 1977). Serine possibly has an inductory role in the synthesis of cholera toxin.

In many microbial systems the synthesis of secondary metabolites has a much narrower tolerance for environmental inorganic phosphates than does the growth of producer cells (Demain, 1968; Weinberg, 1974; Witney, Failla and Weinberg, 1977). The present study makes it clear that *V. cholerae* is no exception. Many diverse suggestions have been made to explain the mechanism of phosphate control of secondary metabolism. Dafni, Ulitzur and Shilo (1972) ruled out the possibility that a low concentration of phosphate favours secondary metabolism merely by limiting vegetative growth, because growth-limiting concentrations of nitrogen or vitamins did not increase the yield of secondary substances. Our results also show that phosphates do not control synthesis of cholera toxin merely by influencing the growth of vibrios. However, at this stage it is not possible to explain the mechanism of phosphate control of PF synthesis.

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