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Nutritional Studies on *Vibrio cholerae*

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SUMMARY: One hundred and fifty-eight strains of vibrio, predominantly *Vibrio cholerae*, were tested for their nutritional requirements. Half of the strains were able to grow on a simple inorganic medium with ammonium ions as the sole source of nitrogen; the other strains required purines in addition. The simplest purine which produced adequate growth was hypoxanthine. The purine-requiring strains were able to grow on inorganic media containing human, rabbit or goat serum, but not on the serum from mouse, rat, guinea pig or horse.

Little information is available about the nutritional requirements of *Vibrio cholerae*. Some strains are non-exacting, as they are capable of growth in minimal media containing only ammonium salts and glucose as nitrogen, carbon and energy sources (Anderson, 1946; Saxena, Bhaskaran, Agarwala & Shrivastava, 1953); other strains do not grow in such simple media, indicating that they need additional factors for growth. These factors are obviously available in ordinary protein digest media in which all strains grow profusely. This investigation is concerned with elucidating the precise constituents of these complex media which are essential for the growth of *V. cholerae*.

METHODS

Organisms tested. *Vibrio* strains were collected for this investigation from various laboratories in India and from the National Collection of Type Cultures, Colindale, London N.W. 9, England. One hundred and fifty-eight strains were true *Vibrio cholerae* O Group I (Gardner & Venkatraman, 1935) including the specific antigenic types Inaba, Ogawa and Rough forms. Ten strains were non-agglutinable by the specific cholera anti-sera and were not cholera vibrios, presumably isolated from water sources in India.

Auxanographic technique. For the differentiation between exacting and non-exacting strains, thoroughly washed suspensions of each strain from overnight growth on nutrient agar, were streaked on a solid minimal medium. The medium contained ammonium sulphate (0.1 %), glucose (0.1 %), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02 %), NaCl (0.5 %) and K_2HPO_4 (0.1 %) in distilled water; the pH value was adjusted to 8.0, and the medium solidified with 2 % Bacto Agar (Saxena *et al.* 1953). Colonies of non-exacting strains appeared after overnight incubation at 37°; no growth could be seen with the exacting strains.

The nutritional requirements of the exacting strains were examined by the technique of Pontecorvo (1949). Possible growth-promoting materials such as

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Difco Casamino acids, yeast nucleic acid, *p*-amino benzoic acid, pantothenic acid, glutathione, biotin, vitamin B₁₂, nicotinamide, and thiamine, were tested by adding them dropwise, individually or in groups, on to the surface of the basal medium seeded with a washed suspension of the test strain, to see whether growth of the organism could be obtained. Test substances were made up to a concentration of 1 mg./ml. with the exception of biotin and vitamin B₁₂ which were employed in strengths of 1 and 50 µg./ml., respectively.

RESULTS

Exacting and non-exacting strains. Strains were equally distributed between the two groups, irrespective of their antigenic character (Table 1). Study of the 79 exacting strains, by the auxanographic technique, showed that with the exception of one strain (Ogawa M74) which required methionine for growth, all grew in the presence of yeast nucleic acid.

Table 1. *Distribution of vibrios in two nutritional groups*

Nutrition	strains of vibrios				Total
	Inaba	Ogawa	Rough no. of strains	N.A.G.	
Non-exacting	39	36	2	2	79
Exacting	38	31	2	8	79

N.A.G., not agglutinated by specific cholera anti-sera.

Simpler components of the complex polynucleotides of yeast nucleic acid were examined for growth stimulation. In preliminary tests, it appeared possible that the purine or pyrimidine bases themselves might provide the necessary factors for growth of these strains, as complete hydrolysis of yeast nucleic acid by 6N-hydrochloric acid did not abolish the growth-promoting property. Tests carried out with known purines and pyrimidines confirmed this supposition and showed that the growth of exacting strains of *Vibrio cholerae* responded to the addition of purines either in the form of bases (hypoxanthine, adenine or guanine), nucleosides (adenosine or guanosine) or nucleotides (adenylic acid or guanylic acid). The pyrimidines cytosine or uracil were without effect. The most marked growth stimulation was by guanosine in the strains tested (Table 2). One of the supposed precursors of purines is 5-imidazol carboxamide (Greenberg, 1953) but there was no growth stimulation by this compound in the case of *V. cholerae*.

In spite of this general uniformity as regards growth promotion by some component of yeast nucleic acid, certain strain to strain differences were evident. While most of the strains grew well in minimal media supplemented with 0.5% (w/v) yeast nucleic acid certain strains, belonging to the antigenic type Inaba, grew poorly by comparison. It seemed possible that these poorly growing Inaba strains might have a secondary or partial requirement for some other growth factor. The addition of methionine or cystine to the

Table 2. *Growth stimulation of Vibrio cholerae strains by purines*

The strains tested showed no significant growth on the minimal medium which contained only ammonia and glucose (as nitrogen, carbon and energy sources) mineral salts and agar.

Strain	Compounds added to minimal medium									
	Purines					Pyrimidines				
	Hypo-xanthine	Xanthine	Inosine	Adenine	Adenosine	Guanine	Guanosine	Cytosine	Uracil	
					Growth responses					
Inaba 74	+	+	+	+	+	+	+	-	-	-
Inaba 243	+	+	+	+	+	+	+	-	-	-
Inaba 117/53	+	+	+	+	+	+	+	-	-	-
Inaba 113/53	+	+	+	+	+	+	+	-	-	-
Ogawa 842	+	+	+	+	+	+	+	-	-	-
Ogawa 871	+	+	+	+	+	+	+	-	-	-
Ogawa 887	+	+	+	+	+	+	+	-	-	-
Ogawa 51439	+	+	+	+	+	+	+	-	-	-
Rough 243	+	+	+	+	+	+	+	-	-	-
Rough 49515	+	+	+	+	+	+	+	-	-	-

-, no growth; +, growth stimulation; ++, growth stimulation (marked).

medium enabled these strains to grow as well as the others. Such strains, which had a partial requirement for growth factors other than constituents of yeast nucleic acid, formed a third of the collection of exacting Inaba strains of *Vibrio cholerae*. One such strain had a partial requirement for glutathione.

Tests with animal sera. The purine dependence of exacting strains of *Vibrio cholerae* was also reflected in experiments carried out with various animal sera. While investigating whether these strains would grow in minimal medium containing serum instead of purines it was found that growth depended on the species of animal from which the serum was obtained (Table 3). These experiments were carried out on solid minimal medium to which various sera were added to a concentration of 25 % (v/v). The sera were heated for 30 min. at 55° before their incorporation in the medium. Saline-washed suspensions of purine-dependent as well as non-exacting strains of *Vibrio cholerae* were inoculated in these media and incubated for 24 hr. It will be seen from Table 3 that sera of man, rabbit and goat were effective in supporting growth of exacting strains while the sera of rat, mouse, guinea pig and horse were not. Identical results were obtained in repeated tests with different batches of sera, which indicated that the differences were between animal species and not between different animals of the same species.

Non-exacting strains of *Vibrio cholerae* were able to grow in all the different serum-containing media, so that there was no evidence of inhibitory activity by any species of serum. Furthermore, with those serum media which did not support the growth of purine-requiring strains, a drop of 0.1 % (w/v) hypoxanthine or yeast nucleic acid solution on the plate stimulated the growth of the organisms around it. It seemed probable therefore that these sera could not support the growth of these strains for the simple reason that they were deficient in purines. As the purine content of the various sera was not known, or determined in these tests, this possibility could only be checked by examining similar purine-requiring strains of other bacterial species, to see whether their growth patterns with these sera were similar.

Table 3 also shows the results of experiments in these serum media with adenine-requiring and non-exacting strains of *Salmonella typhimurium*. The adenine dependent strains were made available by the courtesy of Dr M. Demerec, while the non-exacting strains were derived from them by Dr G. Furness, of this Institute, by phage-mediated transduction to prototrophy. It will be seen that the growth patterns of these strains in the serum-media were identical with the corresponding strains of *Vibrio cholerae*. Essentially similar observations were recorded by Wilson (1945) with strains of group A *Streptococcus pyogenes*.

DISCUSSION

The collection of 158 strains of *Vibrio cholerae* examined in this study comprised non-exacting strains capable of growing on ammonia and glucose as the sole sources of nitrogen and carbon, as well as exacting strains, which required purines for growth. The equal prevalence of the two in the present collection suggests that mutation from one to the other may be of high frequency. The

Table 3. *Growth of Vibrio cholerae and Salmonella typhimurium strains in minimal media containing heated animal sera*

Strain	Growth requirement	Sera added							Minimal medium (control)
		Human	Rabbit	Goat	Rat	Mouse	Guinea-pig serum	Horse	
		Growth on minimal medium + 25 % (v/v)							
<i>V. cholerae</i>									
Ogawa 871	Purine	+	+	+	-	-	-	-	-
Ogawa 842	Purine	+	+	+	-	-	-	-	-
Inaba 791	Purine	+	+	+	-	-	-	-	-
Inaba 827	Purine	+	+	+	-	-	-	-	-
Inaba 8024	Non-exacting	+	+	+	+	+	+	+	+
Ogawa 502	Non-exacting	+	+	+	+	+	+	+	+
<i>S. typhimurium</i>									
Ad 2	Purine	+	+	+	-	-	-	-	-
Ad 10	Purine	+	+	+	-	-	-	-	-
Ad 2/p	Non-exacting	+	+	+	+	+	+	+	+
Ad 10/p	Non-exacting	+	+	+	+	+	+	+	+

-, no growth; +, growth.

uniform requirement for purines by the vast majority of exacting strains of *V. cholerae* is not paralleled in any other bacterial species whose nutritional requirements are known. There was no uniform difference in nutritional requirements between the antigenic types Inaba and Ogawa and Rough forms of *V. cholerae*. Both non-exacting and purine-dependent strains occurred in all three groups, and in individual Ogawa strains it was found that Inaba-type mutants had the same nutritional requirement as the parent strain.

It was also found that the sera of man, rabbit and goat were able to support the growth of purine-requiring strains whereas the sera of rat, mouse, guinea-pig and horse could not. It is of interest to note that two of the three sera which supported growth were those of man, to whom the organism is a natural pathogen, and rabbit, in which young animals are susceptible to experimental infection simulating cholera (Dutta & Habbu, 1955). Perhaps the administration of purines, either parenterally or orally, to refractory laboratory animals such as mice and guinea-pigs might increase their concentration in the sera of these animals and so induce susceptibility to *Vibrio cholerae*.

Tests with known purine antagonists (6-mercaptopurine, 8-azaguanine) showed that the former compound inhibited the growth of *Vibrio cholerae*, both purine-dependent and non-exacting, in purine-containing media, when the relative concentration in the medium of purines and antagonist was carefully adjusted. This finding is being further investigated. It is possible that such inhibitory compounds may prove to be of therapeutic value in the treatment of cholera.

The requirement of purines by strains of *Vibrio cholerae* might be applied to laboratory procedures for isolating cholera vibrios by supplementing culture media with purines, or by substituting purines in the place of complex protein digests. By the latter procedure contaminant flora might be restricted, and so permit the omission of suppressive substrates which are sometimes inhibitory even to *V. cholerae* itself.

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