EFFECT OF METAVANADATE ION ON THE GROWTH *IN VITRO* OF *MYCOBACTERIUM TUBERCULOSIS*[1,2,3]

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Received for publication December 18, 1958

Little information is available on the effects of vanadium ion on microbiological systems. At least three investigators have reported that salts of vanadium may be stimulatory to the growth *in vitro* of species of mycobacteria when these organisms are grown in the absence of iron. Frouin (1912) found that growth of the bovine tubercle bacillus was aided by addition of the sodium salt of vanadium to his synthetic medium which contained no added iron. Using a modification of Long's synthetic medium, Boissevain (1926) reported that vanadium chloride effectively replaced ferric ion in growth of the human "Test" strain. Turian (1951) noted that $NaVO_3$ increased growth of *Mycobacterium phlei* by 25 per cent.

Previous investigations have indicated a role for this metal in: (a) replacing molybdenum in microbial nitrogen fixation pathways (Bortels, 1933; Burk, 1934); (b) as a molybdenum-independent, essential growth factor for green alga (Arnon and Wessel, 1953); and (c) in effecting alterations in mammalian lipid oxidation (Bernheim and Bernheim, 1939) and synthesis (Curran, 1954; Curran and Costello, 1956; Mountain *et al.*, 1956; Azarnoff and Curran, 1957).

It seemed that the complex functions of vanadium might best be studied in a species of bacteria which produced enzymes both for the reduction of inorganic nitrogen and for the synthesis of relatively large amounts of lipid. Because of the reported stimulation of species of mycobacteria by vanadium salts, these organisms were initially investigated.

The direct stimulation of growth of the H37Rv strain of *Mycobacterium tuberculosis* by vanadium (as the metavanadate ion) could not be confirmed. Contrariwise, it was found that dispersed growth of the bacilli from small inocula was inhibited by microgram quantities of vanadium. This inhibition was further studied in the hope of obtaining information on the mechanism of inhibition by the metal.

METHODS AND MATERIALS

The H37Rv strain of *M. tuberculosis* utilized in this study was obtained from the Standard Culture Depot of the National Tuberculosis Association in 1949 and maintained on glycerol egg medium. The saprophytic strains used were laboratory stock strains maintained on Lowensteins' agar. All organisms were grown one or more times in liquid Kirchner medium (modified by substitution of NH_4SO_4, 0.3 per cent; for asparagine, 0.5 per cent) containing Tween 80[4] before being transferred to the test system. The modified Kirchner medium used throughout this investigation consisted of sodium phosphate, dibasic, 3 g; potassium phosphate, monobasic, 4 g; ammonium sulfate, 3 g; sodium citrate, 2.5 g; magnesium sulfate, 0.6 g; ferric ammonium citrate, 0.05 g; glycerol, 20 g; water, *q.s.* 1000 ml. Triton WR1339[5] or Tween 80 was used in a final concentration of 0.05 per cent. Standard solutions of vanadium pentoxide were prepared by dissolving the amount required to give 200 μg V^{+5} (as VO_3^-) per ml in a solution containing sodium citrate, 0.25 per cent; sodium phosphate, dibasic, 0.3 per cent; and potassium phosphate, monobasic,

[1] This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service E-914.

[2] A preliminary report of this work was presented at the meeting of the Society of American Bacteriologists at Chicago, Illinois, 1958.

[3] Part of the material presented here was taken from a thesis submitted to the Graduate School, University of Kansas by the senior author.

[4] Polyoxyethylene sorbitol monooleate obtained from Hilltop Laboratories, Inc.

[5] Oxyethylated tertiary octyl phenol formaldehyde polymer obtained from Winthrop Laboratories, Inc.

0.4 per cent. The addition of this buffered citrate solution, without vanadium, to the growth medium was determined to be without effect upon the growth of any of the organisms tested. In some experiments vanadium was also added as the readily soluble NH_4VO_3 with essentially the same results.

All compounds used in the investigation were prepared in sterile solution by filtration through a sintered glass filter and appropriate amounts added to 1 ml of sterile, 2 × medium. The final volume was adjusted to 2 ml with sterile distilled water.

The inoculum in each case consisted of 0.1 ml of a 14- to 21-day culture of *M. tuberculosis* (or of a 3-day culture of *Mycobacterium butyricum*, *M. phlei*, or *Mycobacterium smegmatis*) which had been washed three times, resuspended and diluted in modified Kirchner medium until an optical density of 0.02 was attained. Cultures were incubated at 37 C. Growth was determined by measurement of the turbidity of each culture in a Rouy Photrometer for which a special adaptor was constructed to accommodate test tubes (15 by 125 mm) which contained 2 ml of medium. In the absence of surface active agents, the extent and appearance of growth were determined visually.

RESULTS

The effect of metavanadate ion on the growth *in vitro* of *M. tuberculosis* when grown in the presence or absence of adequate amounts of iron (Fe^{+2}) is given in table 1. The effect was determined on both surface type growth and dispersed growth obtained by use of a wetting agent (Tween 80). Vanadium ion did not markedly stimulate the nondispersed growth of this organism from small inocula in the absence of added iron. The highest concentration of vanadium appeared to be inhibitory. Addition of iron to the medium did not alter the lack of vanadium stimulation although the inhibitory effect was partially eliminated by this metal. The organism grown in the dispersed state was markedly more sensitive to the inhibitory effect of metavanadate ion. Under these conditions, the growth of the organism was partially inhibited by 4 μg V^{+5} per ml (as VO_3^-). The inhibition was increased by addition of ferrous iron. Again no stimulation of growth occurred.

The effect of varied amounts of metavanadate ion on the dispersed growth of *M. tuberculosis* (H37Rv), *M. butyricum*, *M. phlei*, and *M. smegmatis* in modified Kirchner medium (with normal concentrations of ferric iron) is presented in table 2.

The growth of *M. tuberculosis* was partially

TABLE 1

*Effect of metavanadate ion on growth in vitro of Mycobacterium tuberculosis (H37Rv) in defined medium**

V^{+5} Conc	Fe^{+2} Conc (μg/ml)					
	Medium without Tween 80†			Medium with Tween 80‡		
	0	10	60	0	10	60
μg/ml						
0	2+	2+	2+	0.259	0.244	0.222
2	2+	2+	+	0.244	0	0
4	2+	2+	+	0.046	0	0
10	2+	2+	+	0	0	0
40	2+	2+	2+	0	0	0
100	±	+	+	0	0	0

* Kirchner medium with $(NH_4)_2SO_4$ instead of asparagine, and containing no iron.

† 28-day growth interpreted as: 2+ = good growth with formation of pellicle; + = growth without formation of pellicle; and ± = scant or doubtful growth.

‡ 12-day growth in medium containing Tween 80 (0.05 per cent) given as optical density.

TABLE 2

Metavanadate ion inhibition of mycobacteria

V^{+5} Conc	*Mycobacterium tuberculosis* (H37Rv)*	*Mycobacterium butyricum*	*Mycobacterium phlei*	*Mycobacterium smegmatis*
μg/ml				
0	0.177	0.142	0.226	0.286
1.0	0.174	0.122	0.226	0.274
2.5	0.079	0.068	0.226	0.292
5.0	0	0.054	0.221	0.286
10.0	0	0	0.221	0.286
25.0	0	0	0.165	0.380
50.0	0	0	0	0.348
100.0	0	0	0	0.298
200.0	0	0	0	0.096

Medium: modified Kirchner's with Tween 80.

* *M. tuberculosis*, 10-day growth; *M. butyricum* and *M. smegmatis*, 3-day growth; *M. phlei*, 5-day growth. Given as optical density.

inhibited by 2.5 μg V^{+5} per ml (as VO_3^-) and completely inhibited by 5 μg per ml. With as little as 0.25 μg V^{+5} per ml, a granular appearance evidenced an altered type of growth. *M. butyricum* was sensitive to a similar concentration of vanadium. *M. smegmatis* was affected only slightly by relatively high concentrations of the ion. The sensitivity of *M. phlei* was intermediate, inhibition occurring with 50 μg V^{+5} per ml.

The effect of vanadium on several other strains of *M. tuberculosis* was determined. These included a strain of H37Rv recently obtained from the American Trudeau Society and several strains isolated from patients. These strains varied in their resistance to the antituberculous drugs (PAS, INH, streptomycin). The vanadium sensitivity of all strains were essentially similar to that of the H37Rv strain.

The effect of closely related ions upon growth of *M. tuberculosis* and upon the inhibition of this organism by vanadium is indicated in tables 3 and 4, and in figure 1. Of the series 4 transition metals tested (titanium to nickel), three appeared to be inhibitory. Two others, titanium and ferrous iron, increased the inhibitory effect of vanadium (table 4) although they were not in themselves appreciably inhibitory. Ferric ion was not effective. Manganous and chromate ion inhibited the growth of the organism at levels similar to vanadium but reversed the inhibition caused by the metavanadate ion (figure 1) at a ratio of less than one metal ion to one vanadium ion (1:15 and 1:4, respectively). The manganous and the chromate ion antagonized the metavanadate ion inhibition in a manner which is possibly competitive (figure 1).

Magnesium ion, which is occasionally interchangeable with manganous ion in biological systems, was ineffective in reversing the vanadium inhibition when the concentration in the medium was increased. Nor was any effect noticed when the magnesium ion concentration in the medium was reduced by 1/20. Calcium, copper, and zinc, elements whose addition to medium for the growth of *M. tuberculosis* has been suggested (Dubos and Middlebrook, 1947), were not effective in altering the metavanadate ion inhibition. However, copper was found to be markedly inhibitory. Molybdenum, which appears interchangeable with vanadium in nitrogen fixation pathways, was not inhibitory.

TABLE 3

Effect of transition group metals (series 4) on growth of Mycobacterium tuberculosis

Element and Atomic No.	Added* Ion	Minimal Inhibitory† Conc
		μg/ml
Titanium 22	Ti^{+4}	>100
Vanadium 23	V^{+5}	10
Chromium 24	Cr^{+6}	20
Manganese 25	Mn^{+2}	10
Iron 26	Fe^{+2}	>100
	Fe^{+3}	>100
Copper 29	Cu^{+2}	1

* Metal ions added as $TiCl_4$, V_2O_5, $K_2Cr_2O_7$, $MnSO_4$, $Fe(NH_4)_2(SO_4)_2$, ferric ammonium citrate, and $CuSO_4$. Other metal ions tested that neither inhibited growth at the 100 μg/ml level nor affected the metavanadate ion inhibition: Ca^{+2} ($CaCl_2$), Co^{+2} ($CoCl_2$), Ni^{+2} ($NiCl_2$), Zn^{+2} ($ZnSO_4$), Mg^{+2} ($MgSO_4$), W^{+6} (Na_2WO_4), Mo^{+6} (Na_2MoO_4).

† Minimal concentration of added ion that completely inhibited growth when added to modified Kirchner medium with Tween 80. Effect of iron was determined in the absence of the ferric ammonium citrate normally contained in the medium.

TABLE 4

Effect of transition group metals (series 4) on inhibition of growth by metavanadate ion

Added Ion	Added Ion Conc	V^{+5} Conc (μg/ml)					Relative Effectiveness of V^{+5}†
		0*	5	10	20	50	
	μg/ml						
Ti^{+4}	None	0.677	0.602	0.301	0	0	
	10.0	0.602	0.070	0	0	0	2×
Cr^{+6}	None	0.602	0.523	0	0	0	
	5.0	0.443	0.387	0.568	0.387	0.142	¼×
Mn^{+2}	None	0.456	0.456	0	0	0	
	2.5	0.522	0.494	0.482	0.444	0.420	1/10×
Fe^{+2}	None	0.568	0.602	0.318	0	0	
	10.0	0.552	0	0	0	0	4×

* Optical density of 21-day growth in modified Kirchner medium.

† Ratio of the minimal inhibitory concentrations of metavanadate ion in the presence and absence of the given metal ion.

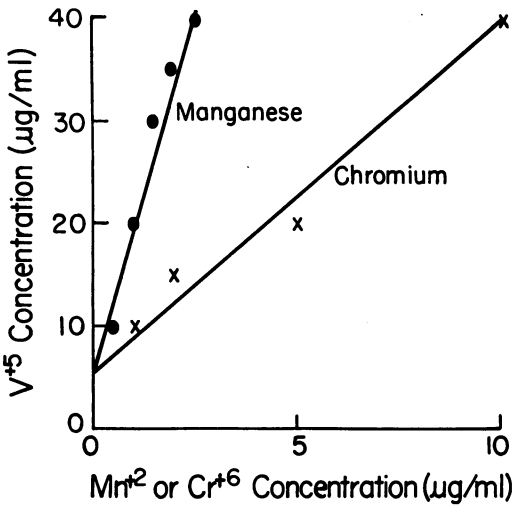

Figure 1. Competitive relation of Mn^{+2} or Cr^{+6} to the inhibitory effect of metavanadate ion. Expressed as the concentration of V^{+5} (μg/ml) required to inhibit the growth *in vitro* of *Mycobacterium tuberculosis* in the presence of the given amount of Mn^{+2} or Cr^{+6}.

TABLE 5

Effect of dispersing agents on metavanadate ion inhibition of Mycobacterium tuberculosis

V^{+5} Conc	Additions to Basal Medium*						
	No addition	Triton WR1339	Tween 80 (mg/ml)				Tween 80 + bovine albumin
			0.25	0.5†	1.0	2.5	
µg/ml							
0	+	0.960	0.470	0.480	0.470	0.220	0.602
1	+	0.900	0.480	0.440	0.440	0.100	0.550
2	+	0.820	0.380	0.420	0.420	0.022	0.570
5	+	0.850	0.270	0.090	0.070	0	0.590
10	+	0.790	0	0	0	0	0.590
50	−	0.870	0	0	0	0	0.550
100	−	0.940	0	0	0	0	0.550

* Modified Kirchner medium containing (a) no dispersing agent, (b) Triton WR1339 0.5 mg/ml, (c) various amounts of Tween 80, (d) Tween 80 0.5 mg/ml + bovine albumin (Armour fraction V) 0.5 mg/ml. Given as optical density of 21-day growth.

† Concentration of Tween 80 normally utilized.

When Tween 80 was absent from the medium, inhibition occurred only at significantly higher concentrations of vanadium (table 5). When Triton WR1339 was substituted for Tween 80 no inhibition by the metavanadate ion occurred.

The quantitative relationship between Tween 80 and the concentration of vanadium required for inhibition is also indicated. The initial change in the inhibitory concentration of the ion due to the surfactant was not greatly changed by increasing the surfactant concentration until the concentration of the Tween 80 became sufficiently high to be inhibitory in itself.

The inhibition of growth in the absence of Tween 80 by larger amounts of metavanadate ion was also reversed by manganous ion.

The addition of bovine albumin obliterated the metavanadate ion inhibition of *M. tuberculosis* either in the presence or absence of Tween 80.

Albumin rendered "lipide-free" by acetone treatment as described by Oyama *et al.* (1953) did not alter the effectiveness of albumin in reversing the metavanadate ion inhibition. It is known that protein will bind the transition group metals. The ability of the protein to remove the metavanadate ion from a simple system was demonstrated by adding an excess of the ion to a solution of fraction V bovine serum albumin, immediately coagulating the protein by heat, and determining the amount of vanadium (Rockhold and Talvitie, 1956) remaining in the solution. It was found that 1 molecule of albumin complexed approximately 30 molecules of the metavanadate ion with a binding that resisted even heat denaturation. A similar removal of vanadium was indicated when the albumin was removed by means of ethanol (approximately 75 per cent, v/v) or $(NH_4)_2SO_4$ (approximately 80 per cent saturated) precipitation.

DISCUSSION

The results described in this paper would seem to contradict the stimulatory role reported for vanadium in the growth of certain species of mycobacteria. This contradiction arises, no doubt, from different experimental conditions utilized. In the previous investigations the bacillus was grown in a medium devoid of iron and devoid of surface active agents. The results presented here would indicate that in the absence of Tween 80 the quantity of vanadium utilized in previous studies may have been insufficient to inhibit growth of the bacillus. Small amounts of iron present in the vanadium salts would readily explain the stimulatory effect found. Turian (1951) has indicated that as little as 0.05 μg Fe^{+2} per

ml would cause stimulation of growth of *M. phlei*. Our results would indicate that in the presence of adequate amounts of iron no stimulation of growth occurs due to added vanadium.

The requirement of Tween 80 for demonstration of maximal activity of an inhibitory agent is not unique for the metavanadate ion. It has been reported by Youmans and Youmans (1948) that the presence of Tween 80 increases the inhibitory power *in vitro* of the majority of the tuberculostatic agents which they tested, although the activity of a few of them remained the same or decreased. These workers also found that decreasing (but not omitting) the concentration of Tween 80 in the medium did not markedly affect the bacteriostatic end point of the agents studied. A similar situation occurs with metavanadate ion.

Our findings would appear compatible with the concepts derived by MacLeod and Snell (1950) to explain the toxicity of zinc towards *Lactobacillus arabinosus* and its reversibility by Mn (or by Mn and Mg, Ca or Sr). Those authors theorized that the Zn-Mn ion antagonism was an extension of competitive analogue-metabolite antagonisms. Utilizing that theory, the metavanadate ion could be interpreted as causing inhibition by competing for the site of an enzyme activating metallic ion (Mn or Cr). A metabolite role could not be proved, since significant stimulation of growth by these ions was not demonstrated. However, it is not unlikely that minute quantities of either, sufficient for growth in the absence of vanadium, were present in the basal medium. Contradictory evidence concerning growth stimulation of *M. tuberculosis* by Mn has been reported (Frouin and Guillaumie, 1928; Sher and Sweany, 1939).

Of the metallic ions tested, a similarity of activity of vanadium, chromium, and manganese upon growth of the mycobacteria was noted. A relationship of titanium, chromium, manganese and iron (ferrous) to the vanadium-induced inhibition was also apparent. The valence state of the metals when added did not appear to be of prime importance (with the exception of iron) since inhibition by the hexavalent chromate ion was equivalent to that of the pentavalent metavanadate ion and of the divalent manganous ion; the hexavalent chromate reversed the vanadium inhibition at a ratio similar to the divalent manganous ion and the tetravalent titanic ion increased the vanadium inhibition similar to the divalent ferrous. The trivalent ferric ion was not effective. It is probable that the metal ions are either active at any oxidation state or easily acquire the active oxidation state.

The activity relationship of these low molecular weight series 4 transition metals in this system invites comparison to a similar relationship of the same metals in mammalian liposynthetic pathways described by Curran (1954). This author reported that V and Fe^{+2} ion (but not Fe^{+3}) would cause a decrease in the synthesis of of cholesterol and fatty acids by surviving rat liver suspensions. Cr and Mn effected an increased synthesis and both ions prevented the decreased synthesis due to vanadium. Ti, Co, Ni, Cu, and Zn had no major effect on the synthesis.

The close similarity of the effects of the series 4 metals in the two systems is indicative of a possible link between mammalian liposynthetic pathways and a metabolic pathway present in mycobacteria.

ACKNOWLEDGMENTS

The authors are indebted to Dr. George L. Curran for his helpful advice and criticism.

SUMMARY

The effect of vanadium ion on the growth *in vitro* of *Mycobacterium tuberculosis* in a defined medium was studied. The stimulation of growth by this metal reported by other investigators was not confirmed. Instead, the metal was found to be inhibitory to the growth of this organism. This inhibition was most marked in the presence of polyoxyethylene sorbitol monooleate (Tween 80). The inhibitory effect of vanadium varied with different saprophytic species of mycobacteria.

A similar inhibitory action was found with manganous ion and chromate ion. The inhibition caused by vanadium was reversed, however, by minute quantities of either. A possible competitive relationship was apparent. Titanium and iron were not appreciably inhibitory to the growth of *M. tuberculosis*. However, both titanium and ferrous (but not ferric) iron appeared to produce an additive inhibitory effect with vanadium.

The inhibitory action of vanadium was diminished in the absence of Tween 80 or in the presence of bovine albumin. There was no quantitative relation between the amount of Tween 80

present and the concentration of vanadium required to inhibit growth. Bovine albumin apparently decreased the inhibitory activity by binding the metal.

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