REFERENCES

- Akeley, D. F. & Gosting, L. J. (1953). *J. Amer. chem. Soc.* **75**, 5685.
- Anson, M. L. & Stanley, W. M. (1941). *J. gen. Physiol.* **24**, 679.
- Cammack, K. A. (1962). *Nature, Lond.*, **194**, 745.
- Cann, J. R. (1953). *J. Amer. chem. Soc.* **75**, 4218.
- Caspary, E. A. & Kekwick, R. A. (1957). *Biochem. J.* **67**, 41.
- Cecil, R. & Snow, N. S. (1962). *Biochem. J.* **82**, 255.
- Charlwood, P. A. (1959). *Biochem. J.* **73**, 126.
- Creeth, J. M. (1958). *J. phys. Chem.* **62**, 58.
- Doty, P. & Edsall, J. T. (1951). *Advanc. Protein Chem.* **6**, 70.
- Fevold, H. L. (1951). *Advanc. Protein Chem.* **6**, 187.
- Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. 1, 174.
- Fleischman, J. B., Porter, R. R. & Press, E. M. (1963). *Biochem. J.* **88**, 220.
- Gosting, L. J. & Morris, M. S. (1949). *J. Amer. chem. Soc.* **71**, 1998.
- Green, N. M. (1963). *Biochem. J.* (in the Press).
- Halwer, M., Nutting, G. C. & Brice, B. A. (1951). *J. Amer. chem. Soc.* **73**, 2786.
- Harrington, W. F., Johnson, P. & Ottewill, R. H. (1956). *Biochem. J.* **62**, 569.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956a). *J. biol. Chem.* **219**, 623.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956b). *J. biol. Chem.* **221**, 385.
- Kekwick, R. A. & Cannan, R. K. (1936). *Biochem. J.* **30**, 226.
- Kraemer, E. O. (1940). In *The Ultracentrifuge*, p. 58, Eqn. (110), by Svedberg, T. & Pedersen, K. O. Oxford University Press.
- Lamm, O. (1929). *Z. phys. Chem.* **A143**, 177.
- Richards, E. G. & Schachman, H. K. (1959). *J. phys. Chem.* **63**, 1578.
- Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 204. New York and London: Academic Press.
- Svedberg, T. (1925). *Zsigmondy-Festschr.: Kolloidzchr.* **36**, (suppl.) 53.
- Trautman, R. & Crampton, C. F. (1959). *J. Amer. chem. Soc.* **81**, 4036.
- Van Holde, K. E. & Baldwin, R. L. (1958). *J. phys. Chem.* **62**, 734.
- Yphantis, D. A. (1959). *J. phys. Chem.* **63**, 1742.
- Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.

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The Role of Molybdenum in Nitrate Assimilation by *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens initiates specifically the crown-gall disease of plants. Relatively little is known about the metabolic characteristics of the bacterium since most of the biochemical researches have been concerned with the tumour tissue itself (Klein & Link, 1955). Vardanis & Hochster (1961a, b) have studied the nucleic acid and glucose metabolism of the bacterium. A study of its nitrogen metabolism has been undertaken in this Laboratory to try to elucidate the mechanism and biochemical aspects of tumour induction.

The requirement of molybdenum in nitrate assimilation is specifically due to its involvement in enzymic nitrate reduction. This element has been shown to be the metal component of nitrate reductase from *Neurospora* (Nicholas & Nason, 1954a, b), soya-bean leaves (Nicholas & Nason, 1955a; Evans & Hall, 1955). *Escherichia coli* (Nicholas & Nason, 1955b; Taniguchi & Itagaki, 1960) and *Pseudomonas aeruginosa* (Fewson & Nicholas, 1961), and to undergo a reversible oxidation-reduction from the 5+ to the 6+ oxidation

state during enzyme action (Nicholas & Stevens, 1956; Fewson & Nicholas, 1961). The present results on growth and nitrate-reductase activity under conditions of tungstate inhibition and molybdenum deficiency indicate that molybdenum is required for the nitrate metabolism of *Agrobacterium tumefaciens* also.

MATERIALS AND METHODS

Cultural conditions. *Agrobacterium tumefaciens* (strain B6) was grown in modified Czapek-Dox medium (Salle, 1954) containing 30 g. of sucrose and 3 g. of NaNO₃ or 4.68 g. of ammonium sulphate/l. of medium. This was distributed in 200 ml. amounts in 1 l. conical flasks, sterilized by autoclaving for 15 min. at 15 lb./in.² pressure, sown with a suspension of cells from potato-agar slopes and incubated in the dark at 30° with occasional shaking.

Determination of growth. In the nitrate medium the bacterium completed the lag phase of growth after about 18 hr. and the exponential phase about 48 hr. after inoculation. Thereafter the growth was stationary till about 96 hr. This pattern was unaffected by the presence of molybdate

Table 1. *Effect of tungstate on the growth of Agrobacterium tumefaciens in nitrate medium*

Experimental details are given in the text. The control (tungstate absent) gave 457 μ g. dry wt./ml. of medium.

Expt. no.	Final concn. of tungstate in medium (mM)					
	0	0.05	0.10	0.50	1.00	5.00
	Growth (% of control)					
1	100	53.6	40.9	34.8	33.0	24.8
2	100	53.9	38.7	32.4	28.8	28.9
3	100	51.3	37.8	32.1	32.5	30.4

or tungstate in the medium. With ammonium salt as the source of nitrogen, however, the stationary phase of growth was attained only after 72 hr. This again was unaffected by the presence of tungstate in the medium. Therefore the total growth was determined in the nitrate and ammonium media 48 and 72 hr. respectively after inoculation. The cells were harvested by centrifuging at 5000g and washed twice with glass-distilled water, dried in a steam oven for 3 hr. and the dry weight was recorded.

In molybdenum-deficiency studies the inorganic salts were purified by the 8-hydroxyquinoline co-precipitation method (Hewitt, 1952). Sucrose solution was made metal-free by treatment with Dowex 50 (H^+ form). Ferric chloride at a final concentration of 10 μ M, and manganese sulphate, zinc sulphate and copper sulphate at 0.1 μ M, were added to the purified medium (stock solutions 100-fold concentrated were used). Molybdenum and tungsten were added as sodium molybdate and sodium tungstate respectively. All chemicals used were of analytical grade.

Nitrate-reductase assay. For enzyme assay the washed cells were suspended in glass-distilled water, frozen (at -20°) and thawed twice, and the suspension was then used as an enzyme preparation. The reaction was stopped by the addition of 9 ml. of ethanol to the reaction mixture (3 ml.), the mixture was centrifuged and nitrite was determined in a portion (1 ml. diluted to 3 ml.) by the addition of 1 ml. of 2% (w/v) sulphanilamide in 3N-HCl and 1 ml. of 0.02% *N*-(1-naphthyl)ethylenediamine hydrochloride. The red colour was read in a Klett-Summerson photoelectric colorimeter with a no. 54 filter (λ 520–580 m μ) (Snell & Snell, 1959). The curve was linear in the range 0–90 μ M-moles of nitrite.

Determination of Mo^{5+} ions. To 1 ml. of the uncentrifuged broth were added 1 ml. of 20% (w/v) KSCN and 1 ml. of 10N- H_2SO_4 . After 10 min. the colour was extracted with 5 ml. of pentanol and read in a Klett-Summerson photoelectric colorimeter with a no. 47 filter (λ 445–505 m μ) (Snell & Snell, 1959).

Menadiol. Menadiol was prepared by the method of Fieser (1940). The dry powder was dissolved in 2 drops of ethanol, made up with water and used immediately for the enzyme assay.

RESULTS

Growth inhibition by tungstate. The effect of different concentrations of tungstate on the growth of the organism in nitrate medium is summarized in Table 1. The addition of tungstate inhibited growth by as much as 70%. Even a concentration of 50 μ M was effective in suppressing growth by about 50%. This inhibitory effect of tungstate was

Table 2. *Effect of tungstate on the growth of Agrobacterium tumefaciens in ammonium medium*

Experimental details are given in the text. The control (tungstate absent) gave 212 μ g. dry wt./ml. of medium.

Expt. no.	Final concn. of tungstate in medium (mM)			
	0	0.10	0.50	1.00
	Growth (% of control)			
1	100	102	106	99
2	100	96	100	91
3	100	98	102	97

not manifest when ammonium was substituted for nitrate as the nitrogen source (Table 2). With ammonium as the source of nitrogen the total growth was only about 40% of that in the nitrate medium.

Reversal of tungstate inhibition by molybdate. The results presented in Table 3 indicate that the inhibitory effect exerted by tungstate on growth is completely reversed by the simultaneous presence of molybdate in the culture medium. The reversal appeared to be complete at a molybdenum:tungsten ratio of 1:1.

Effect of tungstate on the formation of Mo^{5+} ions. There was an accumulation of Mo^{5+} ions in the medium during the growth of the cells (Table 4). The quantity of Mo^{5+} ions reduced increased with an increase in the concentration of Mo^{5+} ions in the medium. The presence of tungstate did not appear to affect the formation of Mo^{5+} ions.

Inhibition of growth by a deficiency of molybdenum. The results presented in Table 5 show that molybdenum is essential for the growth of the organism in nitrate medium. The addition of molybdate to the deficient medium increased growth by about 250%. A concentration of about 0.1 mM-molybdate appeared to be optimum. Higher concentrations tended to exert a depressing influence.

Nitrate-reductase activity under molybdenum deficiency. We have observed that the nitrate-reductase activity in this organism was dependent on menadiol. In this respect it resembled the nitrate reductase reported in *E. coli* by Wainwright (1955), Medina & Heredia (1958) and Heredia & Medina (1960). In the absence of added

Table 3. *Reversal of the inhibition by tungstate of the growth of Agrobacterium tumefaciens by molybdate*

Experimental details are given in the text. The control (both tungstate and molybdate absent) gave 430 µg. dry wt./ml. of medium.

Final concn. of tungstate (mM)	Expt. no.	Final concn. of molybdate in medium (mM)					
		0	0.01	0.05	0.10	0.50	1.00
		Growth (% of control)					
0	1	100.0	100.0	119.2	121.2	118.5	118.3
	2	100.0	100.0	114.3	117.9	110.1	108.7
	3	100.0	100.0	118.0	121.4	113.4	110.7
5.00	1	30.9	45.9	—	51.0	—	64.4
	2	32.2	33.4	—	51.1	—	63.8
	3	41.1	53.4	—	58.5	—	63.1
0.10	1	28.4	—	90.0	104.4	101.7	100.4
	2	33.1	—	93.9	100.7	100.3	100.7
	3	32.9	—	95.2	100.4	99.4	103.0

Table 4. *Effect of tungstate on the formation of Mo⁵⁺ ions during the growth of Agrobacterium tumefaciens*

Experimental details are given in the text.

Final concn. of tungstate (mM)	Expt. no.	Final concn. of molybdate in medium (mM)				
		0	0.05	0.10	0.50	1.00
		Mo ⁵⁺ ion formed (µg.ions/l. of broth)				
0	1	45	40	90	135	235
	2	60	40	95	225	250
	3	45	60	85	220	250
0.10	1	15	40	60	150	215
	2	15	40	60	150	205
	3	15	45	85	205	295

Table 5. *Growth of Agrobacterium tumefaciens in molybdenum-deficient medium*

Experimental details are given in the text. The control (molybdate absent) gave 214 µg. dry wt./ml. of medium.

Expt. no.	Final concn. of molybdate in medium (mM)					
	0	0.01	0.05	0.10	0.50	1.00
	Growth (% of control)					
1	100.0	246.5	244.9	256.6	196.9	179.9
2	100.0	211.9	222.6	229.8	188.7	185.5
3	100.0	208.3	216.8	244.1	188.5	164.4

menadiol no nitrite formation could be detected under the conditions of assay. Electron donors like reduced nicotinamide-adenine nucleotides and flavins had no effect on the system. The enzymic activity assayed with chemically reduced menadione is given in Table 6.

A deficiency of molybdenum caused an appreciable decrease in the specific activity of the enzyme. The total activity was also increased about seven- to eight-fold by the addition of molybdate to the deficient medium. The activity of the preformed enzyme was unaffected by the addition of molybdate or tungstate to the assay system.

DISCUSSION

Higgins, Richert & Westerfeld (1956*a, b*) reported that tungstate was a dietary antagonist of molybdate in animal nutrition, and a competitive inhibitor of molybdate in *Aspergillus niger* grown on nitrate as the sole nitrogen source. The competitive nature of tungstate inhibition of the growth of *Azotobacter vinelandii* has also been reported (Takahashi & Nason, 1957). A report by Hedgecock & Costello (1962) has indicated that tungstate interferes with nitrate utilization by *Mycobacteria*. We have shown the same to be true

Table 6. *Effect of molybdenum deficiency on nitrate-reductase activity of Agrobacterium tumefaciens*

The reaction mixture contained 1 ml. of 0.05M-tris-HCl buffer, pH 8.5, 20 μ moles of KNO_3 , 0.3 μ mole of menadiol and 0.5 mg. of cells in a total volume of 3 ml. Incubation was for 15 min. at 37°. Nitrite was determined as described in the Materials and Methods section. No nitrite was formed in the absence of added menadiol. Specific activity = μ moles of nitrite formed/mg. dry wt./15 min, Total activity = specific activity $\times$ total dry wt. of cells from 200 ml. of medium.

Expt. no.	Final concn. of molybdate in medium (mM)			
	0		0.10	
	Specific activity	Total activity	Specific activity	Total activity
1	0.18	10.5	0.60	78.6
2	0.12	6.2	0.60	64.2

of *A. tumefaciens* grown on nitrate as the sole source of nitrogen; with ammonia as the source of nitrogen, however, tungstate exerted no inhibitory effect. The addition of tungstate to the preformed enzyme preparation did not inhibit nitrite formation. Takahashi & Nason (1957), who also observed the same ineffectiveness of tungstate in inhibiting nitrite formation, ascribed the effect when added to the culture medium to the action of tungstate in the replacement of molybdenum in the enzyme protein. Recent reports (Keeler & Verner, 1957; Bulen, 1961), however, seem to suggest that tungstate competitively inhibits the uptake of molybdate but does not inhibit the enzymically functional molybdenum in the preformed enzyme; in other words, tungstate competes with molybdate in the uptake process rather than with the functional molybdenum.

Decreased growth and nitrate-reductase activity under molybdenum deficiency have been reported with *Neurospora* and *A. niger* (Nicholas, Nason & McElroy, 1953), and *P. aeruginosa* (Fewson & Nicholas, 1961). Our results show that the addition of molybdate to the deficient medium resulted in increases of growth to the extent of 250 %. This was followed by a concomitant increase in the nitrate-reductase activity. The addition of molybdate to the deficient cells failed to increase the enzyme activity. This has been a general observation in all molybdenum-deficiency studies and suggests that the element plays a role in the synthesis of enzyme protein.

SUMMARY

1. Tungstate has been found to be inhibitory to the growth of *Agrobacterium tumefaciens* grown on nitrate as the sole nitrogen source.

2. With ammonium as the sole nitrogen source this inhibitory effect was not observed.

3. The effect of tungstate could be completely reversed by the addition of molybdate to the culture medium.

4. Deficiency of molybdenum depressed the growth of the organism in nitrate medium. The nitrate-reductase activity was also depressed by the deficiency.

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REFERENCES

- Bulen, W. A. (1961). *J. Bact.* **82**, 130.
 Evans, H. J. & Hall, N. S. (1955). *Science*, **122**, 922.
 Fewson, C. A. & Nicholas, D. J. D. (1961). *Biochim. biophys. Acta*, **49**, 335.
 Fieser, L. F. (1940). *J. biol. Chem.* **133**, 391.
 Hedgecock, L. W. & Costello, R. L. (1962). *J. Bact.* **84**, 195.
 Heredia, C. F. & Medina, A. (1960). *Biochem. J.* **77**, 24.
 Hewitt, E. J. (1952). *Tech. Commun. Bur. Hort., E. Malling*, no. 22, p. 193.
 Higgins, E. S., Richert, D. A. & Westerfeld, W. W. (1956a). *Fed. Proc.* **15**, 274.
 Higgins, E. S., Richert, D. A. & Westerfeld, W. W. (1956b). *Proc. Soc. exp. Biol., N.Y.*, **92**, 509.
 Keeler, R. F. & Verner, J. E. (1957). *Arch. Biochem. Biophys.* **70**, 585.
 Klein, R. M. & Link, G. K. K. (1955). *Quart. Rev. Biol.* **30**, 207.
 Medina, A. & Heredia, C. F. (1958). *Biochim. biophys. Acta*, **28**, 452.
 Nicholas, D. J. D. & Nason, A. (1954a). *J. biol. Chem.* **207**, 353.
 Nicholas, D. J. D. & Nason, A. (1954b). *J. biol. Chem.* **211**, 183.
 Nicholas, D. J. D. & Nason, A. (1955a). *Plant Physiol.* **30**, 135.
 Nicholas, D. J. D. & Nason, A. (1955b). *J. Bact.* **69**, 580.
 Nicholas, D. J. D., Nason, A. & McElroy, W. D. (1953). *Nature, Lond.*, **172**, 34.
 Nicholas, D. J. D. & Stevens, H. M. (1956). *Symposium on Inorganic Nitrogen Metabolism*, p. 178. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
 Salle, A. J. (1954). *Fundamental Principles of Bacteriology*, p. 135. New York: McGraw-Hill Book Co. Inc.
 Snell, F. D. & Snell, C. T. (1959). *Colorimetric Methods of Analysis*, 3rd ed., vol. 2, pp. 804, 478. New York: D. Van Nostrand Co.
 Takahashi, H. & Nason, A. (1957). *Biochim. biophys. Acta*, **23**, 433.
 Taniguchi, S. & Itagaki, E. (1960). *Biochim. biophys. Acta*, **44**, 263.
 Vardanis, A. & Hochster, R. M. (1961a). *Canad. J. Biochem. Physiol.* **39**, 773.
 Vardanis, A. & Hochster, R. M. (1961b). *Canad. J. Biochem. Physiol.* **39**, 1165.
 Wainwright, S. D. (1955). *Biochim. biophys. Acta*, **18**, 583.