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EVIDENCE FOR THE INVOLVEMENT OF A GENETIC DETERMINANT CONTROLLING FUNCTIONAL SPECIFICITY OF GROUP VI B ELEMENTS IN THE METABOLISM OF N_2 AND NO_3^- IN THE BLUE-GREEN ALGA NOSTOC MUSCORUM.

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SUMMARY- The parent N. muscorum is Mo-requiring for growth on N_2 or NO_3^- as nitrogen source. W and Cr both are observed to competitively inhibit the function of Mo in growth on N_2 and NO_3^- media in parent strain. Spontaneous mutants growing in the presence of W or Cr were isolated and when examined, found to be W- or Cr-requiring for growth with both N_2 and NO_3^- as nitrogen source. The results of the characterization of the three strains with respect to mutation frequency, interaction among Mo, W and Cr in growth on N_2 as nitrogen source, and requirement of W or Cr for NO_3^- inhibition of heterocyst formation in W- or Cr-requiring strain growing in NO_3^- medium, all suggest the operation of a single genetic determinant in specifying whether Mo, W or Cr (group VI B elements) is required for growth with both N_2 and NO_3^- as nitrogen sources. They also further suggest that this single genetic determinant is common to nitrogenase and nitrate reductase.

INTRODUCTION - Chromium (Cr), molybdenum (Mo) and tungsten (W) belong to group VI B of the periodic table, forming a series of chemical analogues. Mo is a definite requirement for the growth of organisms on N_2 or NO_3^-, since the N_2-metabolizing enzyme nitrogenase and NO_3^--metabolizing enzyme nitrate reductase, both are molybdo-proteins. W is found to be a competitive inhibitor of Mo function in vivo in bacteria (1, 2, 3,) in green alga Chlorella (4) : and in

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higher plants (spinach) (5). The replacement of Mo by W in molybdo-enzymes is shown to render them biologically functionless (3, 6). Similar studies with Cr are virtually non-existent.

Nason et al (7) reported that a Mo-containing sub-unit of nitrate reductase of Neurospora crassa can be replaced functionally by a Mo-containing sub-unit of nitrogenase of Soybean nodule bacterioids. This and subsequent studies have led to the concept that there is an ammonium-repressible, Mo-containing low molecular weight co-factor of polypeptidic nature which is common to different molybdo-enzymes including nitrogenase and nitrate reductase (8, 9). Whether this ammonium-repressible common co-factor which complexes with Mo, is the molecule determining the functional specificity of group VI B elements in the biological activity of nitrogenase and nitrate reductase, is not known. We have isolated mutants of the nitrogen-fixing blue-green alga Nostoc muscorum, which require W or Cr but not Mo for growth in N_2 or NO_3^- medium. The results reported here clearly show the involvement of a single genetic determinant in the control of biological specificity of group VI B elements in N_2 or NO_3^- metabolism and further suggest that this genetic determinant is also common to both nitrogenase and nitrate reductase.

MATERIALS AND METHODS - The axenic clonal culture of parent alga Nostoc muscorum grows well in N_2 or NO_3^- chu no. 10 medium (10) containing 0.0177 µg/ml of sodium molybdate. While N_2 growing such cultures convert 5-6% of their vegetative cells into heterocysts, NO_3^- growing similar cultures remain non-heterocystous. The molybdate free cultures show very little growth in N_2 or NO_3^- medium and molybdate-free NO_3^- cultures produce heterocysts with a frequency almost similar to those in molybdate-free N_2 cultures (8-9%). The molybdate-free NO_2^- or NH_4^+ cultures grow as well as molybdate-containing NO_3^- cultures.

The parent alga was grown in chu no. 10 medium with or without combined nitrogen (5mM KNO_3) at a temperature of 28 ± 2°C and a continuous light intensity of 2500 lux as described previously (11). The inocula for experimental cultures, intended to examine the growth effect of W or Cr were prepared in molybdate-free chu no. 10 medium. W and Cr like Mo were used as sodium tungstate and sodium chromate (Sigma grade), respectively. The

biological effect on growth of increasing concentrations of tungstate or chromate separately or together with that of molybdate was measured as optical density at 663 nm in a spectronic-20 spectrocolorimeter. 10 μg/ml of tungstate and 50 μg/ml of chromate caused complete inhibition of algal growth in liquid or on plate cultures in N_2 medium. In contrast NO_3^- cultures required nearly 100 μg/ml of tungstate or chromate to show complete inhibition of growth.

W- or Cr-requiring mutants of the parent alga were sought by plating a heavy suspension of the culture on N_2 medium containing 100 μg/ml of tungstate or chromate and incubating them for two weeks in the growth chamber. A dozen colonies from tungstate or from chromate plates were examined for their ability to grow in the absence of tungstate or chromate. None of the tungstate or chromate colonies grew without tungstate or chromate, and were thus designated as tungsten (W)-or chromium (Cr)-requiring colonies. One clonal tungstate culture and one clonal chromate culture were then examined quantitatively for growth and heterocyst formation in N_2 and NO_3^- media containing or lacking tungstate, chromate or molybdate. In vivo interaction of molybdate with tungstate or chromate were also examined in terms of quantitative changes in growth and heterocyst frequency.

The W-requiring strain was examined for reversion to Mo-requirement or mutation to Cr-requirement for growth on N_2 by plating a heavy suspension of a W-requiring culture on 100 μg/ml molybdate-supplemented or 100 μg/ml chromate-supplemented N_2 plates. The Mo-requiring and Cr-requiring mutants were also tested for the same requirement for growth with NO_3^- as nitrogen source.

RESULTS AND DISCUSSIONS - The parent strain of N. muscorum grows well with N_2, NO_3^-, NO_2^- or NH_4^+ as nitrogen source but while its growth with N_2 or NO_3^- is Mo-dependent, that with NO_2^- or NH_4^+ is not. Tungstate and chromate both are found toxic for growth in N_2 and NO_3^- media but not in NO_2^- and NH_4^+ media. This obviously suggests that the parent N. muscorum is auxotrophic for Mo for growth in N_2 and NO_3^- media and that W or Cr growth-toxicity is specific to those cultures growing with N_2 or NO_3^- as only nitrogen source. However, W-or Cr-toxicity is less in NO_3^- medium than that in N_2 medium (Table 1). Addition of molybdate to molybdate-free, to tungstate-containing or to chromate-containing media always leads to the quick recovery of the

Table 1. Growth of parent N. muscorum in N_2, 5 mM NO_3^-, 5 mM NO_2^- and 1 mM NH_4^+ media with various concentrations of molybdate, tungstate or chromate.

Concentrations (µg/ml)	Molybdate				Tungstate				Chromate			
	N_2	NO_3^-	NO_2^-	NH_4^+	N_2	NO_3^-	NO_2^-	NH_4^+	N_2	NO_3^-	NO_2^-	NH_4^+
Zero	0.005	0.005	0.450	0.455	0.005	0.005	0.445	0.450	0.005	0.005	0.450	0.455
10	0.370	0.435	0.455	0.455	0.000	0.015	0.450	0.455	0.105	0.105	0.455	0.445
50	0.225	0.350	0.410	0.450	0.000	0.010	0.455	0.445	0.005	0.105	0.455	0.450
100	0.190	0.355	0.445	0.455	0.000	0.000	0.455	0.445	0.000	0.000	0.450	0.455

Growth was measured in terms of the increase in optical density after 8 days of the inoculation of alga in media containing various concentrations of molybdate, tungstate or chromate.

growth of the parent alga. Addition of tungstate or chromate to molybdate-containing cultures causes abrupt decline in algal growth (Fig. 1). This apparently suggests that W and Cr are acting as competitive inhibitors of Mo function for growth with N_2 or NO_3^- as nitrogen source.

The W-requiring strain does not grow without W in N_2 or NO_3^- medium (Fig. 2) but does grow without it in NO_2^- or NH_4^+ medium. Mo or Cr is unable to substitute for W function in the W-requiring strain. On the other hand, they appear to inhibit W function in this mutant strain as W or Cr do in the Mo-requiring parent strain. This seems to suggest that the W-requiring strain is like the Mo-requiring parent strain in all other respects except that Mo function has completely been replaced by W for growth on N_2 or NO_3^-.

The results of figure 3 indicate that the Cr-requiring mutant of N. muscorum is like the Mo-requiring parent or the W-requiring mutant in all respect for growth on N_2 or NO_3^- except that Cr has taken over the function of Mo in this mutant. Table 2 shows the heterocyst frequency of the parent, the W-requiring and Cr-requiring mutant strains in relation to different concentrations of molybdate, tungstate or chromate in N_2 and NO_3^- growth media. The Mo-requiring parent strain does not form heterocysts in NO_3^-medium containing Mo but does form heterocysts in the same medium lacking Mo. This suggests a requirement of Mo in the inhibition of heterocyst formation by NO_3^- .

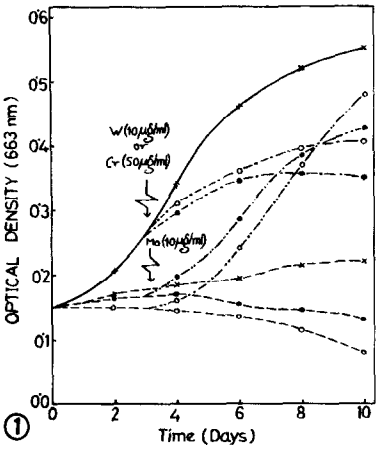
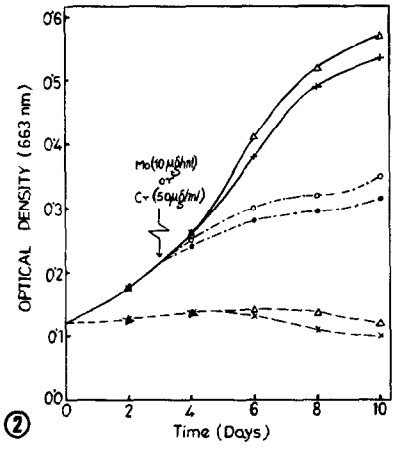


Figure 1. Interaction of Mo, W and Cr on the growth of parent strain in N_2 medium. Interaction was examined by adding Mo, W or Cr in 3 days old cultures. X——X, Mo-containing control culture; X- - -X, Mo-free culture; O- - -O, W-containing culture; ●- - -●, Cr-containing culture; O-·-·-O, W added to Mo-containing culture; ●-·-·-●, Cr added to Mo-containing culture; O-···-O, Mo added to W-containing culture; ●-···-●, Mo added to Cr-containing culture.



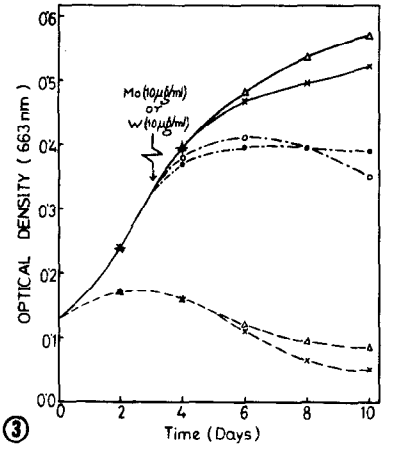


Figure 2. Interaction of W, Mo and Cr on the growth of W-requiring mutant strain in N_2 medium. Interaction was examined by adding Mo, W or Cr in 3 days old cultures. X——X, W-containing control N_2 culture; Δ——Δ, W-containing control NO_3^- culture; X- - -X, W-free N_2 culture; Δ- - -Δ, W-free NO_3^- culture; O-·-·-O, Mo added to W-containing culture; ●-·-·-●, Cr added to W-containing culture.

Figure 3. Interaction of Cr, Mo and W on the growth of Cr-requiring mutant strain in N_2 medium. Interaction was examined by adding Mo, W or Cr in 3 days old cultures. X——X, Cr-containing control N_2 culture; Δ——Δ, Cr-containing control NO_3^- culture; X- - -X, Cr-free N_2 culture; Δ- - -Δ, Cr-free NO_3^- culture; O-·-·-O, W added to Cr-containing culture; ●-·-·-●, Mo added to Cr-containing culture.

Table 2. Heterocyst frequency of the Mo-requiring parent, W-requiring mutant and Cr-requiring mutant strains of *Nostoc muscorum* in N_2 and 5 mM NO_3^- media

Medium composition	Mo-requiring parent strain		W-requiring mutant strain		Cr-requiring Mutant strain	
	N_2	NO_3^-	N_2	NO_3^-	N_2	NO_3^-
-Mo -W -Cr	5-9	8-9	5-7	6-10	5-7	5-9
+Mo(10 µg/ml)	5-6	0.0	5-9	6-10	5-7	5-10
+Mo(50 µg/ml)	4-5	0.0	5-10	7-11	5-9	6-11
+W (10 µg/ml)	5-9	6-11	5-6	0.0	5-8	6-9
+W (50 µg/ml)	5-10	6-14	5-6	0.0	5-10	6-12
+Cr(50 µg/ml)	5-8	6-9	5-8	6-8	5-8	0.0
+Cr(100µg/ml)	5-9	6-12	5-9	6-10	5-6	0.0

Heterocyst frequency was measured as number of heterocysts per hundred vegetative cells.

Since active nitrate reductase is a requirement for NO_3^- inhibition of heterocyst formation in N. muscorum (12), it seems reasonable to infer that Mo-requirement for NO_3^- inhibition of heterocyst formation is operating here at the level of Mo-activation of nitrate reductase. The Mo-requiring parent strain forms heterocysts in NO_3^- medium containing W or Cr, which suggests that W or Cr is unable to substitute physiologically for the role of Mo in the activation of nitrate reductase. Mutation from Mo-requirement for growth to W- or Cr-requirement for growth in N_2 and NO_3^- media also results in W-or Cr-requirement for NO_3^- inhibition of heterocyst formation. Since Mo function in the Mo-requiring parental strain is inhibited competitively by W or Cr, W function in the W-requiring strain by Mo and Cr and Cr function in the Cr-requiring strain by Mo and W, it appears that the three s strains are alike with respect to transport system for the three elements. Their genetic difference may, therefore, lie in the system involved in determining the group VI B elements' specificity in the organization of functionally active N_2 or NO_3^- metabolizing enzyme systems, namely nitrogenase and nitrate reductase, respectively.

Both the W-requiring and Cr-requiring mutants, though obtained spontaneously from N_2 medium, show the same requirement even for growth

on NO_3^- as nitrogen source. Apparently the mutation leading to W- or Cr-requirement for growth on both N_2 and NO_3^- media indicate it to be of pleotropic in nature. The W-requiring mutant mutates to Mo-requirement with a frequency of nearly 1×10^{-5}, and to Cr-requirement with a frequency of 1×10^{-7}. This and the observation that the Mo-requiring parent also mutates freely to W or Cr-requirement, strongly suggests that a single genetic determinant controls the specificity of functional dependence of both nitrogenase and nitrate reductase on group VI B elements ; i. e. whether Mo, W or Cr is required for the growth of N. muscorum on N_2 and NO_3^- is specified by this genetic determinant. This common genetic determinent does not seem to operate at the permeation level. Could it be at the level of a Mo-containing co-factor common to nitrogenase and nitrate reductase as suggested by biochemical studies (7, 8, 9) ? The fact that mutants selected for W- or Cr-requirement on N_2, maintain this requirement even with NO_3^- as nitrogen source, clearly implicates the involvement of a common genetic determinant in the organization of active nitrogenase and nitrate reductase in this alga.

It is, therefore, very likely that the postulated single genetic determinant which controls the specificity of Mo, W or Cr for growth on N_2 and NO_3^-, is also the genetic determinant common to both nitrogenase and nitrate reductase, coding for the reported low molecular weight co-factor of polypeptidic nature (13, 14, 15). A class of chlorate-resistant single-site mutant of N. muscorum has been shown to lack both nitrogenase and nitrate reductase activity, an evidence taken to suggest the possibility of a common genetic determinant for nitrogenase and nitrate reductase both (16). The present results further, strongly support the concept of a common genetic determinant of nitrogenase and nitrate reductase in Nostoc muscorum.

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