DISTRIBUTION OF MOLYBDENUM^{99} IN CELL-FREE PREPARATIONS OF *AZOTOBACTER VINELANDII*[1]

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Azotobacter strains require molybdenum while utilizing N_2 as a source of nitrogen for growth (Bortels, 1930, Horner, *et al.*, 1942, Mulder, 1948, Jensen, 1947, Keeler, 1955). This requirement has prompted us to investigate the distribution of molybdenum in fractions obtained by differential centrifugation of cell-free extracts of *Azotobacter vinelandii*. Assuming molybdenum to be a constituent of the nitrogen-fixing system, it appeared that fractionation might yield a particulate fraction upon which one could center attention in cell-free nitrogen fixation studies. This paper reports the isolation of a molybdenum-rich particulate fraction which contains most of the molybdenum taken up by the growing cells and which has a molybdenum to protein ratio higher than any other fraction.

METHODS

Azotobacter vinelandii strain O[2] was cultured under forced aeration in Burk's (Burk and Lineweaver, 1930) nitrogen-free mineral salt medium with 3 per cent sucrose plus added molybdenum as [$Na_2Mo^{99}O_4$]. Cells grown under these conditions are designated as "normal cells." "Molybdenum-deficient cells" were obtained by successive subculture on "molybdenum-free" media, prepared according to the cupric sulfide co-precipitation method of Nicholas (1952). The cells were harvested by centrifugation, washed twice and resuspended in 0.25 M sucrose containing 0.1 M phosphate (pH 7.2).

Cells were disrupted by one of the following methods: the glass bead technique of Lamanna and Mallette (1954); a modification of this technique substituting washed sea sand for the glass beads; grinding with alumina in a mortar and pestle, or with a 10 KC Raytheon sonic oscillator. Unbroken cells were removed by centrifugation at 10,000 × G for 1 hr. Sedimentable fractions were obtained by successive centrifugations at 25,000 × G for 1 hr, 144,000 × G for ½ hr, 144,000 × G for 1 hr and 144,000 × G for 6 hr and are designated as R_{25}, $R_{144-1/2}$, R_{144-1}, R_{144-6}, respectively, using a Spinco preparative ultracentrifuge, Model L. The corresponding supernatant fractions are designated as S_{25}, $S_{144-1/2}$, S_{144-1}, and S_{144-6}. The disruption and centrifugation steps were accomplished at a temperature range of 0–4 C.

Radioactive assay was performed with a Nuclear 180 scaler with a 1.4 mg/cm^2 end window Geiger-Muller tube. Protein was determined by the optical density values at 260 mμ and 280 mμ according to Warburg and Christian (1941). The ratio of the optical density readings are reported as an indication of the nucleic acid content of the fractions. Specific activity of the fractions is defined as counts/min/mg of protein.

RESULTS

The results of an experiment in which normal cells were supplied 3 ppm Mo^{99} are reported in table 1. The supernatant protein fraction obtained after centrifugation at 144,000 × G for 1 hr, S_{144-1}, contained 86 per cent of the Mo^{99} taken up by the cells. The specific activity of this fraction was approximately 20 times that of any other fraction. When "molybdenum-deficient" cells were supplied a low level (0.0015 ppm) of Mo^{99}, a similar distribution pattern was obtained (table 2).

In another experiment with molybdenum-deficient cells, the time of centrifugation at 144,000 × G was increased to 6 hr. Under these conditions, a particulate fraction, R_{144-6}, was obtained. As shown in table 3, this fraction had the highest specific activity. In addition, this molybdenum-rich particulate fraction had a 260 mμ/280

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mμ ratio considerably higher than any other fraction, indicating that this fraction had a higher proportion of nucleic acids than the others.

Confirmation of these properties of R_{144-6} was obtained from "normal cells" supplied 10 ppm Mo^{99}. A comparison of the data in table 3 with those in table 4, shows that the relative specific activities and the relative 260 mμ/280 mμ ratios are approximately the same in the two experiments even though the cells were supplied Mo^{99} at 100 times greater concentration in the latter experiment.

In one further experiment on normal cells supplied 10 ppm Mo^{99} the R_{144-6} fraction was found to have an even higher relative specific activity (table 5). In addition, in this particular experiment, in contrast to previous experiments, R_{144-6} contained higher percentages of the total activity and of the total protein than any other fraction. These data suggest that the lower percentage of cell rupture resulted in a higher proportion of intact particles.

Dialysis of the fraction R_{144-6} against 0.25 M sucrose-0.1 M phosphate (pH 7.2) and against 10^{-3} M cyanide (pH 7.2) failed to remove any significant fraction of the Mo^{99}. Likewise, the Mo^{99} from S_{144-1} of table 1 was not removed by dialysis against cyanide.

TABLE 1

Distribution of Mo^{99} in fractions from normal cells supplied 3 ppm Mo^{99}†*

Fraction	Total Activity in Fraction, Counts/Min	Optical Density Ratio, 260 mμ/280mμ	Total Mg Protein	Specific Activity, Counts/Min/Mg Protein
R_{25}	11,500	1.15	104	110
R_{144-1}	16,700	1.12	216	77
S_{144-1}	1,400,000	1.63	676	2000

* Disrupted by the method of Lamanna and Mallette (1954) using glass beads.

† 1.5 ppm Mo^{99} taken up by the cells.

TABLE 2

Distribution of Mo^{99} in fractions from deficient cells supplied 0.0015 ppm Mo^{99}†*

Fraction	Total Activity in Fraction, Counts/Min	Optical Density Ratio, 260 mμ/280 mμ	Total mg Protein	Specific Activity, Counts/Min/Mg Protein
R_{25}	11.0	1.15	46	0.24 ± 0.02
R_{144-1}	3.0	1.53	51	0.06 ± 0.02
S_{144-1}	203	1.45	348	0.58 ± 0.06

* Disrupted by grinding with alumina.

† 0.0006 ppm Mo^{99} taken up by the cells.

TABLE 3

Distribution of Mo^{99} in fractions from deficient cells supplied 0.1 ppm Mo^{99}†*

Fraction	Total Activity in Fraction, Counts/Min	Optical Density Ratio, 260mμ/280mμ	Total Mg Protein	Specific Activity Counts/Min/Mg Protein
S_{25}	4,620	1.25	750	6.2
R_{144-6}	460	1.59	55	8.4
S_{144-6}	3,000	1.13	453	6.6

* Disrupted by the method of Lamanna and Mallette (1954) using washed sea sand.

† 0.09 ppm Mo^{99} taken up by the cells.

TABLE 4

Distribution of Mo^{99} in fractions from normal cells supplied 10 ppm Mo^{99}†*

Fraction	Total Activity in Fraction, Counts/Min	Optical Density Ratio, 260mμ/280mμ	Total Mg Protein	Specific Activity, Counts/Min/Mg Protein
S_{25}	24,800	1.60	540	46
$R_{144-1/2}$	180	1.40	50	3.6
R_{144-6}	6,200	1.76	104	60
S_{144-6}	13,900	1.46	306	45

60 per cent cell rupture.

* Disrupted by 10 kc Raytheon sonic oscillator.

† 1.0 ppm Mo^{99} uptake by the cells.

TABLE 5

Distribution of Mo^{99} in fractions from normal cells supplied 10 ppm Mo^{99}†*

Fraction	Total Activity in Fraction, Counts/Min	Optical Density Ratio, 260mμ/280mμ	Total Mg Protein	Specific Activity, Counts/Min/Mg Protein
$R_{144-1/2}$	3,100	1.24	105	30
R_{144-6}	22,600	1.60	143	158
S_{144-6}	7,900	1.59	98	81

33 per cent cell rupture.

* Disrupted by 10 kc Raytheon sonic oscillator.

† 0.85 ppm Mo^{99} uptake by the cells.

DISCUSSION

These experiments clearly establish the existence in cell-free preparations of *Azotobacter vinelandii* of a molybdenum-rich particulate

fraction which is also characterized by a high 260 mμ/280 mμ ratio, and which contains a large proportion of the Mo^{99} taken up by the cells. The conditions shown in table 5 for the preparation of this particulate fraction appear to be nearly optimum. At present we have no information concerning the homogeneity of this fraction.

Magee (1955) has effected a 150-fold purification of a molybdo-protein from *Azotobacter vinelandii* which contained a large percentage of the molybdenum taken up by the growing cells. The stability of our Mo^{99} fraction upon dialysis against cyanide is in contrast to the loss of Mo^{99} from the molybdo-protein purified by Magee.

Alexander and Wilson (1955) have recently discussed the application and values of the differential centrifugation technique in localization of enzymes in *Azotobacter vinelandii*. It would be of interest to know what enzymatic activities are present in this R_{144-6} particulate fraction obtained from *A. vinelandii*. It has been found in this laboratory recently that the activity (per mg of protein) of glutamyl transferase is highest in this fraction. Repaske (1954) has reported 50–75 per cent of the succinic dehydrogenase activity of cell-free extracts of *A. vinelandii* prepared by sonic oscillation to be "soluble" after centrifugation at 144,000 × G for 30 min. Wilson and Wilson (1955) have shown that all of the succinoxidase activity remains in the supernatant from centrifugation of cell-free extracts at 144,000 × G for 5 min, but is 90 per cent sedimented if the centrifugation is continued for 3 hr.

While no enzyme system known to contain molybdenum has been purified from extracts of *A. vinelandii*, a number of molybdenum containing systems are known in other organisms (Mahler and Green, 1954). At present the function of this molybdenum containing fraction of *Azotobacter vinelandii* is not known.

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SUMMARY

A particulate fraction has been obtained from cell-free preparations of *Azotobacter vinelandii* by centrifugation for 6 hr at 144,000 × G. This particulate fraction has a Mo^{99} to protein ratio higher than any other fraction, a high 260 mμ/280 mμ ratio, and contains a large percentage of the molybdenum taken up by the cells.

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