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Influence of Bacterial Growth on the Forms of Cadmium in Defined Culture Media

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Bacteria play an important role in the transformation, activation and detoxification of heavy metals in the environment (SAXENA and HOWARD 1977). Since the form or speciation of heavy metals may determine how they are mobilised and their potential toxicity, the action of bacteria is an important factor to be considered in the control of heavy metal pollution. The ability of many bacteria to remove metals from solution by active uptake and adsorption is important both in terms of resistance to toxicity (BITTON and FREIHOFER 1978) and in detoxifying the environment. However, other species of bacteria may be equally important in causing remobilisation of heavy metals (RAMAMOORTHY et al. 1975) or converting them into forms with enhanced toxicity (SAXENA and HOWARD 1977).

The forms in which heavy metals exist may affect their toxicity and availability for uptake by bacteria. Chelating agents, such as EDTA, NTA and cysteine may reduce the toxicity of metals to bacteria (JEFFRIES and BUTLER 1975, LOVELESS and PAINTER 1968) and their presence in culture media should be taken into account in studies of metal toxicity. The formation of insoluble salts may also reduce the toxicity of heavy metals (JONES 1967). The ability of some bacteria to change the forms of heavy metals by adsorption, transformation or production of chelating agents is influenced by nutrient source (DUGAN and PICKRUM 1972), growth phase of the organisms (FRIEDMAN and DUGAN 1968) and the presence of other species (STUTZENBERGER and BENNET 1965).

This study has been undertaken to investigate the uptake of cadmium in three different culture media by a bacterium isolated from activated sludge and to evaluate the use of gel filtration chromatography for the identification of changes in the form of the metal resulting from bacterial growth.

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MATERIALS AND METHODS

Isolation of Bacteria. Samples of mixed liquor from a laboratory scale activated sludge simulation treating a synthetic sewage were serially diluted and plated onto the CGY medium of PIKE and CARRINGTON (1972). Pure cultures were obtained by successively replating on the same medium. One organism was selected for its ability to grow on citrate, acetate or glucose as sole carbon sources. Results from several biochemical tests indicated that this organism was a Pseudomonas sp.

Culture Media. Batch cultures were grown in a basal medium containing (mgl^{-1}) NH_4Cl, 1000; K_2HPO_4, 200; KH_2PO_4, 150; $MgSO_4.7H_2O$, 100; $CaCl_2.6H_2O$, 10; $MnSO_4.4H_2O$, 5; and $FeSO_4.7H_2O$,5. This basal medium was supplemented with sodium acetate (558 mgl^{-1}), tri-sodium citrate (2000 mgl^{-1}) or D-glucose (1225 mgl^{-1}) to complete the medium. Citrate was sterilised separately by membrane filtration and all other components were autoclaved. The phosphate salts were autoclaved separately and added to the other components after cooling. Cadmium was added to the media as cadmium nitrate after sterilisation to avoid precipitation.

Batch Cultures. All batch cultures were carried out in 2 l conical flasks containing 200 ml of medium. The flasks were incubated at 25° ± 1° on a lateral shaker operating at 70 oscillations min^{-1}. Inocula were taken from 48 h cultures on CGY slopes. These were grown in 125 ml of culture medium and subcultured twice in the same medium prior to transferring 5 ml of the dense culture obtained to the final 200 ml of medium used for batch culture studies.

Cell Counts. Routine determinations of bacterial growth were made by turbidity measurements using a nephelometer (Evans Electroselenium Ltd., U.K.). Turbidity was converted to cells ml^{-1} by using a calibration curve of turbidity against viable counts performed on CGY medium (PIKE and CARRINGTON 1972) with 0.1 M phosphate buffer (pH 7) as a diluent.

Treatment of Samples. Samples withdrawn from the batch cultures of Pseudomonas sp. were filtered through 0.22 µm pore size cellulose acetate membrane filters (Oxoid Ltd., U.K.). The filters were leached in 1% v/v HNO_3 for 4 h followed by extensive rinsing in distilled water prior to use. After mounting in an all-pyrex filter holder the filters were washed with 10 ml of distilled water and 10 ml of the sample prior to collection of 10 ml of sample filtrate. Part of the filtrate was acidified to 1% v/v HNO_3 prior to analysis of total "soluble" cadmium. The remainder was retained for gel filtration chromatography.

Gel Filtration Chromatography. The molecular size distribution of cadmium in culture filtrates was determined by fractionation on columns (90×1.5 cm internal diameter) of Sephadex G-15 (Pharmacia Ltd., U.K.). The eluant used was 0.02 M NaCl,

adjusted to pH 6.80 with 0.02 M NaOH. The columns were run at constant flow rates of 35 ml h^{-1} with a loading volume (Vl) of 2.0 ml and a gel volume (Vt) of 150 ml. Fractions of 5.0 ml were collected, acidified to 1% v/v HNO_3 and sealed prior to analysis of cadmium.

Cadmium Analysis. Cadmium was determined in acidified samples by flameless atomic absorption spectrophotometry using a Perkin-Elmer model 305 spectrophotometer in conjunction with a HGA-72 graphite furnace (Perkin-Elmer Ltd., U.K.). The analytical conditions used were essentially those of CARRONDO et al. (1979).

RESULTS

Effect of growth of Pseudomonas sp. on cadmium solubility. Samples of cultures of Pseudomonas sp. in acetate, citrate and glucose media containing 1.0 mg l^{-1} cadmium were taken at the end of the lag phase, the end of the exponential phase and after 48 h in stationary phase. The concentration of cadmium remaining in solution was determined on the filtrates obtained after membrane filtration. The changes in solubility of cadmium in the three media during growth of Pseudomonas sp. are shown in Table 1. The decreases observed in cadmium concentrations may be attributed to precipitation as a result of pH changes, uptake by the bacteria or a combination of both.

In acetate and citrate media there was no change in cadmium solubility prior to the onset of exponential growth, but this was followed by a decrease in the soluble cadmium concentration during the exponential phase. Although the pH in both media increased during this time, which may have promoted the formation of insoluble cadmium salts, it is probable that the decrease in soluble cadmium concentration was due to the increase in cell numbers. The higher removal of cadmium in citrate medium (82%) compared to acetate medium (40%) may have been due to the presence of more than twice as many bacterial cells in the former culture. Cadmium solubility decreased by a further 10% in citrate medium during stationary phase, but no such effect was observed during the same phase of growth in acetate medium.

The removal of soluble cadmium in glucose medium was much less than in the other media, despite the development of comparable cell numbers in the cultures. No change in the soluble cadmium concentration was observed during exponential growth, but small quantities were removed during lag phase (9%) and stationary phase (7%). However, the growth of Pseudomonas sp. in glucose medium led to the production of significant quantities of acid, resulting in a final culture pH of 3.35, whereas in both citrate and acetate media bacterial growth resulted in an appreciable increase in pH to values of 7.90 and 7.95 respectively.

TABLE 1

Changes in solubility of cadmium in acetate, citrate and glucose media during growth of Pseudomonas sp.

Medium	Time after Inoculation (h)	Soluble Cd (mgl^{-1})	Cells ml^{-1}	pH
Acetate	0	1.00	-	6.85
	2	1.01	1.4×10^{7}	6.80
	20	0.60	0.7×10^{8}	7.85
	60	0.62	0.7×10^{8}	7.90
Citrate	0	1.00	-	6.80
	2	1.00	1.8×10^{7}	6.80
	12	0.18	2.0×10^{8}	7.95
	60	0.08	2.0×10^{8}	7.95
Glucose	0	0.96	-	6.90
	2	0.87	1.4×10^{7}	6.80
	25	0.87	1.3×10^{8}	4.50
	60	0.80	1.2×10^{8}	3.35

Forms of cadmium in defined culture media. Samples of the culture media were run on columns of Sephadex G-15 in order to determine the molecular size distribution of cadmium prior to inoculation of Pseudomonas sp. The elution profiles of the three media studied are shown in Fig. 1. These profiles are plotted in terms of Ve/Vt (where Ve is the elution volume and Vt is the total gel bed volume) to correct for differences in bed volumes of different columns. The profiles shown in Fig. 1 are typical of those obtained when the experiment was repeated two or more times.

In citrate and glucose media cadmium was eluted as a single peak, whereas in acetate medium there appeared to be two large peaks close together with a much smaller fraction, equivalent to approximately 1.5% of the cadmium present, eluted ahead of these. In acetate and glucose media the main peaks coincided and were in approximately the same position as a peak previously obtained for an aqueous solution of inorganic cadmium, loaded as $Cd(NO_3)_2$, but in citrate medium the cadmium was eluted at a lower elution volume. These observations suggest that in acetate and glucose media the cadmium was in the form of small inorganic species but that it existed entirely as a complex with citrate in the citrate medium.

Changes in forms of cadmium during bacterial growth. Filtered samples from the cultures taken during the three phases of growth were run on columns of Sephadex G-15. During growth of Pseudomonas sp. in acetate and citrate media there were no significant changes in the positions of the peaks in the elution

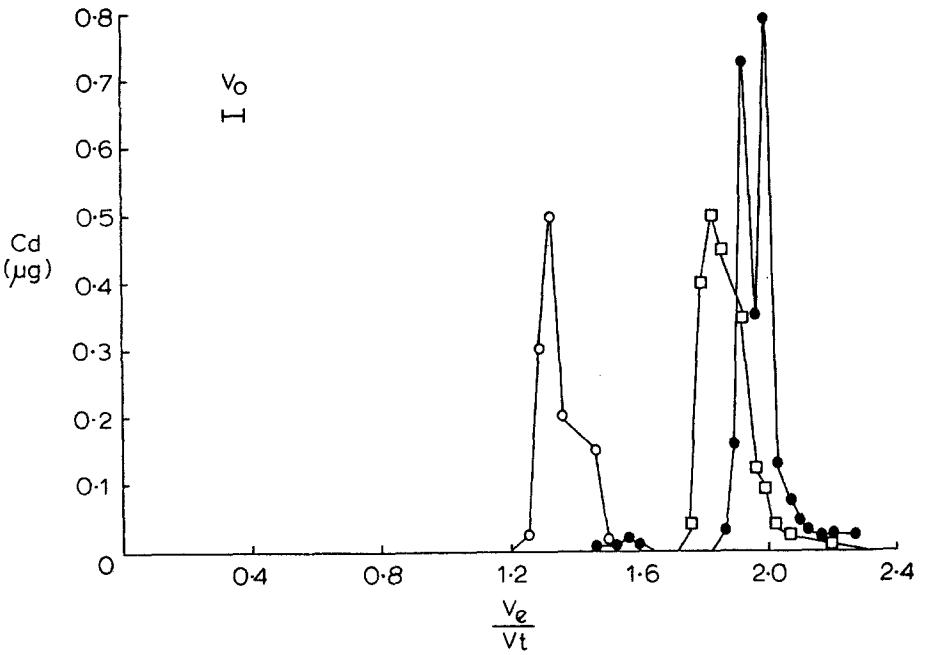

Figure 1. Elution profiles on Sephadex G-15 of cadmium (1.0 mgl^{-1}) in acetate (●), citrate (o) and glucose (□) media

profiles obtained, although the sizes of these peaks diminished as cadmium was removed from solution. However, in glucose medium significant changes were observed; the elution profiles obtained for this medium at different stages of growth are shown in Fig. 2.

Although no significant changes in cadmium form occurred during the lag phase of growth the elution profile corresponding to the end of exponential growth of Pseudomonas sp. in glucose medium (Fig. 2B) shows, in addition of the large inorganic cadmium fraction, two smaller peaks at lower elution volumes. The appearance of these peaks indicates that some cadmium was present as complexes with components not originally present in the medium which were produced during bacterial growth. The elution profile obtained for the same culture after 60 h incubation (Fig. 2C) shows that further quantities of metal-complexing components were generated during stationary phase and that this was concomitant with a reduction in the size of the peak representing inorganic forms of cadmium. The continued generation of these metal complexing compounds after the end of exponential growth may have been the result of some bacterial lysis, although they may reflect products of a change in cell metabolism during stationary phase.

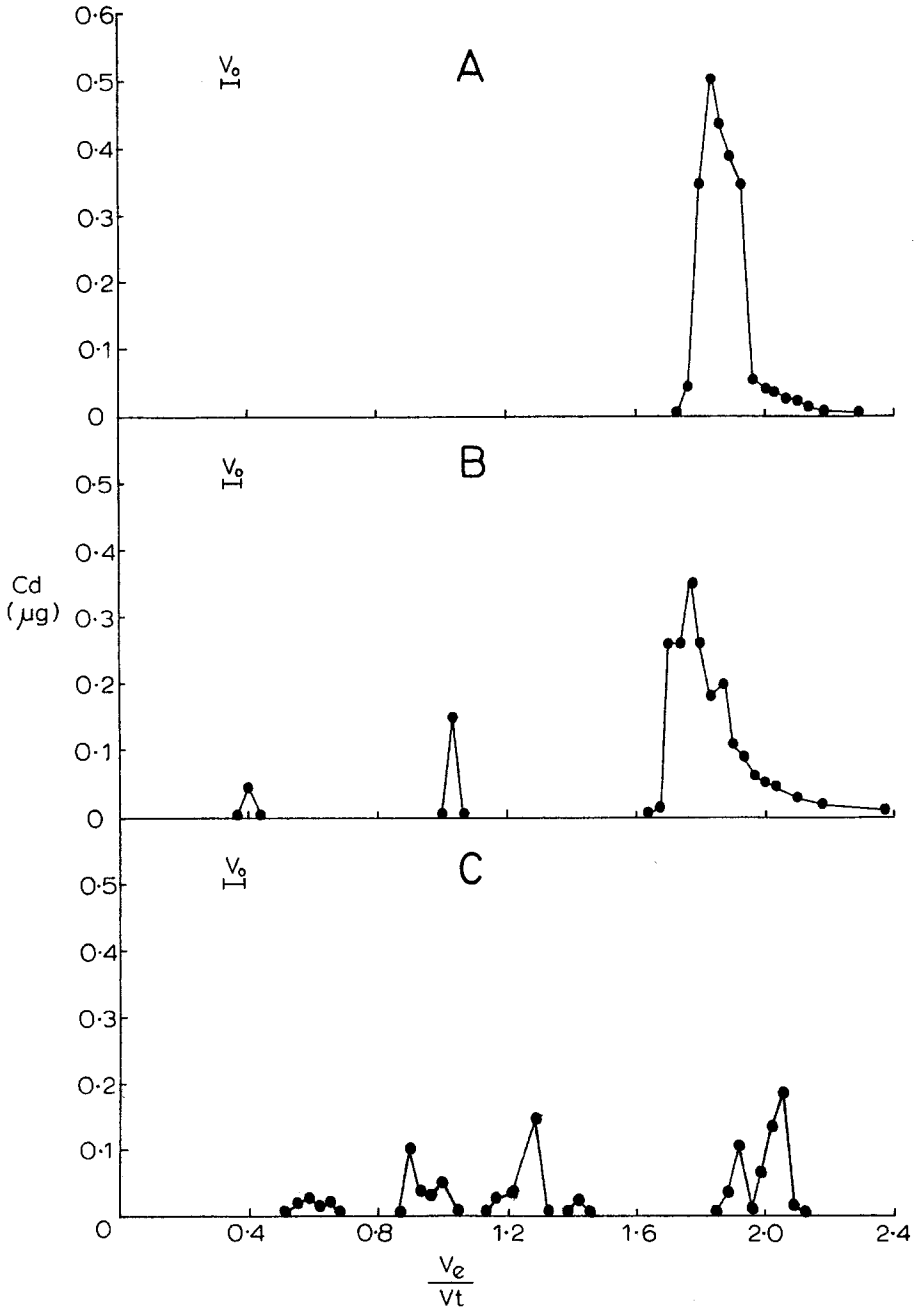

Figure 2. Elution profiles of cadmium in glucose medium during three phases of growth. A : lag phase (2h); B : exponential phase(25h); C : phase (60h)

DISCUSSION

The extent of removal of cadmium from solution in the presence of Pseudomonas sp. differed for each of the three media. Although the differences in removal between citrate and acetate media may have been due to the difference in the sizes of the cell populations, it has been shown (DUGAN and PICKRUM 1972) that the affinity of bacteria for metals is influenced by the composition of the growth medium. This effect could be due to components of the culture media retaining the metal in a form unavailable to the bacteria, but since gel chromatographic analysis of acetate and glucose media showed that cadmium existed in similar forms in both media it is probable that the lower removal of cadmium in glucose medium was due to other factors. One important factor controlling the availability and toxicity of cadmium to bacteria is pH (BABICH and STOTZKY 1977) and it is possible that the acidic conditions generated by Pseudomonas sp. in glucose medium may have caused the cadmium to remain in solution.

HECK et al. (1972) showed that the solubility of copper in the absence of bacteria may be increased substantially in their presence, and this effect was attributed to the production of chelating agents by the bacteria. During the exponential and stationary phases of growth of Pseudomonas sp. in glucose medium elution profiles of the medium on Sephadex G-15 revealed that some of the cadmium was present as complexes with products of bacterial metabolism and possibly cell lysis and may have been retained in solution by this mechanism. The production of these metal-binding components appears to depend upon the nutrient utilised by the bacteria, since no similar effect was observed in citrate and acetate media.

In some instances natural and synthetic chelating agents have been shown to protect bacteria and other organisms from the toxic effects of metals, presumably by making them unavailable for uptake. TYNECKA et al. (1975) found that Staphylococcus aureus was protected from penetration of cadmium into the cell by pretreatment with cysteine. LOVELESS and PAINTER (1968) showed that EDTA abolished the toxic effect of copper to Nitrosomonas. However, in the case of Pseudomonas sp. in citrate medium the presence of cadmium as a complex with citrate did not prevent the bacteria from causing its removal from solution. Since far more cadmium was removed in the citrate medium than in the acetate and glucose media it is possible that the availability of the metal to the bacteria was enhanced ty its association with citrate.

These experiments show that the ability of bacteria to control the distribution of cadmium to some extent, either by removing it from solution or by generating compounds which increase its solubility, is influenced by the growth conditions, pH and nutrients available. Although this work was undertaken

with simple defined media, it is likely that complex interactions of these factors controlling the behaviour of metals in the presence of bacteria in natural systems could be important in terms of the environmental distribution of toxic metals.

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