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Injury by Heavy Metals in *Escherichia coli*

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Numerous stressing agents, of physical and chemical nature, could have effects on environmental microorganisms and cause modification in some biological syntheses (i.e.proteins, peptidoglycans, DNA and RNA) and membrane functions (i.e. membrane transport),and so, the exposed cells, not necessarily killed, would result physiologically damaged with a consequent lower ability to adapt to an adverse environment than the uninjured cells (Sjogren and Gibson 1981). This last point, apart from its theoretical implications, is very important in some practical applications, such as recovery of sanitary indicator organisms from polluted or chlorinated waters and in the food-processing industry (Bissonette et al.1977; Tobin and Dutka 1977; Zaske et al. 1980a,b). Although injury to bacteria, particularly to E.coli, has been observed as a consequence of salinity, heat treatment, freezing, irradiation, pH, exposure to some toxic compounds, etc. (Przybylski and Witter 1979; Anderson et al. 1979), little attention has been given to injury by heavy metals which, at their lowest concentrations, could act as stressors of water-borne indicator bacteria (Gadd and Griffiths 1978; APHA 1980; Mc Feters et al. 1982). The aim of the present study, a follow up to a previous research, on the effects of sublethal concentrations of metals on the growth and recovery of E.coli (Morozzi et al. 1982), is to throw light on some of the aspects of the viability and recovery of E.coli as well as changes in its cell enzymatic activity when exposed to sub-lethal concentrations of heavy metals. Total dehydrogenase was used since it is a measure of bacterial activity closely related to energetic metabolic systems (Roller et al. 1980; Liu and Thomson 1983) and ß-galactosidase as a typical inducible enzyme in E.coli (Pardee et al.1959). E.coli was chosen as the test organism, both on account of its importance in public health (sanitary indicators) and for the information available (biochemical and genetic properties) on it.

MATERIALS AND METHODS

Escherichia coli (NCTC 9001) was used in all experiments. The test cultures were grown overnight in 50 ml nutrient broth (approximately 10^9 bacteria per ml). Before each experiment the bacteria were resuspended in a medium as described below.

Mercury and cadmium, respectively from Merck (Germany) and BDH (England), were used as chloride salts and zinc and copper, respectively from Riedel De Haen AG (Germany) and Carlo Erba (Italy), as sulphate salts. The metal doses tested were in the following ranges: Hg 1-10 µM; Cd 40-80 µM:Zn 3.12-25 µM and Cu 25-200 µM. The concentrations for stress study and GAL-assay were chosen referring to TDH test results (metal concentrations which produce more than 50% TDH-inhibition).

Viability and degree of injury in cells exposed to heavy metals were tested, as described by Przybylski and Witter (1979) and Zaske et al. (1980a), using Trypticase soy agar supplemented with 0.5% glucose and 0.3% yeast extract (TSYA) as a non-selective medium, and Violet red bile agar (VRBA) as a selective medium. Dilutions were made with 0.1% Peptone water, just before testing. The survival (S) and injury (I) percentages at time 't' were obtained respectively from the colony forming units on indicated media, by the following equations:

$$S_t\ (\%) = (TSYA_t / TSYA_o)\,100 \quad ; \quad I_t\ (\%) = (1 - VRBA_t / TSYA_t)\,100$$

All cultures were grown in duplicate.

The recovery capacity of cells which had previously been exposed for 30 min to heavy metals was tested in Trypticase soy broth (TSB) recovery medium (10^8 cells/ml), with and without recovery inhibitors. The concentration of exposed cells was 10^9 cells/ml in Tris-buffer pH 7. Results were obtained comparing, at different time intervals, colony forming units in TSYA and VRBA media. D-cycloserine (Sigma) at 750 µg/ml was used as in inhibitor to peptidoglycans or cell wall synthesis and 2,4-Dinitrophenol (Sigma) at 100 µg/ml as an uncoupler of the respiratory chain (Przybylski and Witter 1979).

Total dehydrogenase activity (TDH) was determined according to Roller et al. (1980). Overnight cells were washed with 0.06M Phosphate-buffer pH 7 and finally concentrated 10 times in 0.05M Tris-buffer pH 8. Two ml of the whole or sonicated cell suspensions were added to 0.2 ml of TTC-glucose reagent (0.2 Triphenyl tetrazolium chloride and 1.5% Glucose) The suspensions were incubated at 37°C for 15 min in closed tubes, then the reaction was stopped by adding 7.8 ml ethanol and the optical density of the reduction product. Triphenyl formazan measured at 490 nm.

ß-galactosidase activity (GAL) measurement was carried out according to the Pardee et al. (1959) o-nitrophenyl-ß-galactopyranoside (ONPG) method. Induction of enzymatic activity in the cells was obtained by resuspension of overnight sediments in the same volume of 0.5% Lactose broth for 150 min. The cells were then concentrated 5 times in 0.01M Tris-buffer pH 7.4 containing 0.1M $MgSO_4.7H_2O$ and 0.05M NaCl as described by Anderson et al. (1979) and stored at -20°C. Dilutions (1/10 ml) of this suspension with an added drop of toluene, were blended in a Vortex mixer and incubated at 37°C for 30 min. Then 0.2 ml of M/75 ONPG was added and the mixture incubated at 37°C. The reaction was stopped after 20 min, by adding 0.5 ml of 1M Na_2CO_3 and the amount of o-nitrophenol (ONP) related was termined at 420 nm by reference to a calibration curve.

RESULTS AND DISCUSSION

TDH-activity, as a measure for energetic metabolic systems, of E.coli cells in the presence of different heavy metal concentrations specifies the range of dose-response relationship. From the results it is possible to define the order to metal toxicity. The 50 percent inhibition of TDH-activity was: 7.7, 10, 30, 50 µM, respectively for Hg, Zn, Cd and Cu. With respect to previous growth studies (Morozzi et al. 1982) the TDH-inhibition assay shows a marked increase in the effect of Zn when compared to results obtained with other metals tested. However, mercury remains the most active ion (Table 1).

The experiments carried out on the disrupted cells indicated that TDH-inhibition, at the same metal concentrations, was generally higher for whole than disrupted cells. The differences were not significant if Zn and the highest Hg concentration were excluded. TDH-activity of disrupted cells in the presence of Zn was independent of metal concentrations. It was observed that the inhibition of TDH-activity, occurring in the presence of the metal ions tested, was always completely removed by extending incubaction time over 60 minutes (data not shown).

To explain the TDH results, experiments were carried out to define some characteristics consequent to biological damage by heavy metals to the total cell population. Table 2 indicates that the tested concentrations of Hg and Cu markedly inhibit growth in TSYA medium, while the viability of cells treated with Cd and Zn remains constant in the same medium. In VRBA-medium the number of colony forming units from Cd and Zn treated cells decreases slightly, while with Hg and Cu the decrease in cell numbers, after 60 min, can be estimated as more than one logarithm unit. Table 3 shows that the reduction of the total number of viable cells, consequent to Hg exposure for 60 min, can be ascribed both to death (17%) and injury (14%). For Cu a 27% death and 18% injury rate was observed.

Table 1. TDH of whole and disrupted E.coli cells in presence of heavy metals. Percent of control withouth metals.

METAL	Final concn (μM)	% of Control		t
		whole cells	disrupted cells	
Mercury	1	113 (3)[a]	94 (3)	1.86
	5	89 (2)	94 (6)	0.88
	10	7 (3)	20 (3)	3.16*
Cadmium	20	52 (3)	90 (3)	1.96
	40	24 (3)	51 (5)	1.38
	80	10 (3)	18 (3)	0.43
Zinc	3.12	94 (3)	-	-
	6.25	79 (3)	103 (3)	3.88**
	12.5	35 (3)	94 (5)	4.58**
	25	4 (3)	93 (3)	10.03**
Copper	25	55 (1)	54 (1)	-
	50	37 (3)	44 (3)	0.72
	100	21 (3)	18 (3)	0.82
	200	16 (3)	14 (3)	0.49

[a]Number of replicates. Cell concentration was about 10^9/ml. TDH mean values, ± SD of controls, TF(μg)/proteins(mg) were 33.1 ± 10.3 for whole and 24.7 ± 11.1 for disrupted cells.
*, $p < 0.05$; **, $p < 0.01$

Table 2. Effect of heavy metals on E.coli viability. Comparison between growth in complete (TSYA) and selective (VRBA) medium after different exposure times.

METAL	Final concn (μM)	Medium	time (min)		
			0	30	60
Control	-	TSYA	9.41[a]	9.11	9.50
		VRBA	8.59	8.97	8.92
Mercury	10	TSYA	9.34	8.17	7.70
		VRBA	8.97	6.95	6.65
Cadmium	80	TSYA	9.04	9.08	9.00
		VRBA	8.78	8.59	8.36
Zinc	25	TSYA	9.14	9.14	9.48
		VRBA	9.08	8.53	9.08
Copper	200	TSYA	9.17	6.90	6.70
		VRBA	8.84	5.97	5.47

[a]Logarithms of colony forming units/ml are given. All plating with the respective media were done in duplicate.

Table 3. Survival and injury % fraction[a] of E.coli cells exposed for different times to heavy metals. Initial cell concentration was 10^9 /ml.

METAL	Final concn (μM)	Survival (%)		Injury (%)	
		30 min	60 min	30 min	60 min
Control	-	97	100	1	6
Mercury	10	88	83	15	14
Cadmium	80	100	99	5	7
Zinc	25	100	100	7	4
Copper	200	75	73	13	18

[a]See Material and methods

Regards Cd and Zn the cells maintain their viability and the slight percentage of injury (Cd 7%, Zn 4%) does not vary much from the control test. This indicated that treatment with Cd and Zn unlike that observed for Hg and Cu, did not cause metabolic injury to E.coli.

In order to define the type of injury caused by the studied metals, tests were carried out in the presence of selected metabolic inhibitors. Figure 1 shows that recovery from damage caused by the

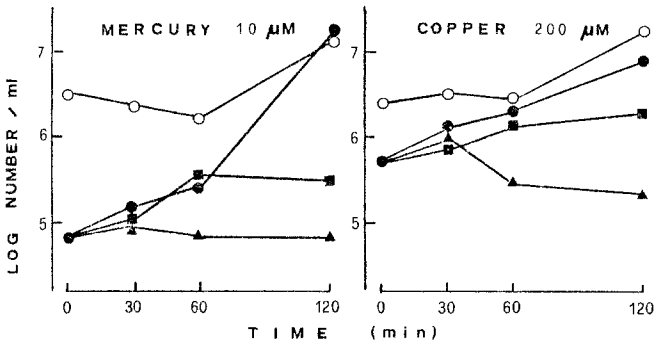

Figure 1. Recovery of metal-injured E.coli in TSB. Inoculum on TSYA (-○-), on VRBA (-●-), on VRBA plus D-CY (-▲-) and on VRBA plus DNP (-■-). See text.

cations Hg and Cu (Cd and Zn do not injure) occurs, as early as 30 min after incubation in TSB medium especially when inhibitors are not present. The figure clearly shows that the recovery from Hg is higher than from Cu after 120 min. In the presence of 2,4-Dinitrophenol recovery diminishes. Recovery was completely blocked, in all cases, by D-cycloserine.

Since the toxic effects of Hg and Cu treatment can in part be de-

fined as metabolic injury, and therefore a recoverable damage, their effect is mainly on inducible enzymes, such as ß-galactosidases, rather than on constitutive enzymes of E.coli. According to this hypothesis Zn and Cd, metals which don't produce injury, could not interact with inducible enzymes. In this connection both the induction and preformed enzymatic activity of GAL, in the presence of the four heavy metals tested, was studied. The results emphasize that all tested metals interact with GAL-activity. Table 4 shows that the inhibitory effect is higher for the Hg and Cu ions, than for those of Cd and Zn. It also appears that Zn has no effect on GAL-biosynthesis, while the induction of this enzyme is diminished by Cd, Hg and Cu.

Table 4. Influence of heavy metals both on ß-galactosidase induction and activity.

METAL	Final concn (µM)	Influence on	E.U.[a]	S.A.[b]	% S.A.
Control[c]	-	Induction	43.2	929	100
		Activity	86.4	882	100
Mercury	10	Induction	7.5	202	21.7
		Activity	7.5	76	8.6
Cadmium	80	Induction	15.3	365	38.3
		Activity	27.9	285	32.3
Zinc	25	Induction	43.1	1002	107
		Activity	52.6	537	60.8
Copper	200	Induction	1.0	24	2.6
		Activity	3.6	37	4.2

[a] One Enzymatic Unit (E.U.) was defined as the amount of enzyme producing 1 nmol of o-nitrophenol in 1 min.
[b] Specific Activity (S.A.) expressed as E.U./Proteins (mg).
[c] Control data represent E.U.s and S.A.s in the absence of metal.

The results described above on the behaviour of E. coli cells in the presence of the four heavy metals (Hg,Cd,Zn,Cu) tested as regards some enzymatic activities (TDH and GAL), viability and injury and recovery or repair can be summarized as follows. TDH-inhibition is probably the result of both the direct interation of metal with the enzymatic activity and of the metal's ability to bind the cellular envelope. The relatively low toxicity of the Zn ion, as deduced from growth studies (Morozzi et al. 1982), is strongly enhanced in TDH-assay. This is probably due to the much higher ability of the Zn ion, with respect to other ions tested, to bind the bacterial cell envelope. At chemical analysis the cellular envelope fraction revealed among other components the presence of succinic dehydrogenase (Beveridge and Kowal 1981).

Moreover TDH-activity in the presence of heavy metals, generally lower in whole cells than in disrupted cells, could indicate that the membrane bound enzymes are more sensitive than the cytoplasmic enzymes. Only in the case of zinc are the differences (between whole and disrupted cells) significant ($p < 0.01$) in all doses tested. In addition for the zinc ion, TDH-activity of disrupted cells is independent of metal concentration. This is probably due to the slight metal incorporation in cell envelopes disrupted by sonication. In the case of the copper ions TDH-activity is unchanged for whole and disrupted cells, perhaps owing to its high affinity for cytoplasmic particles.
Neither Zn nor Cd ions, at the same dose which causes a strong TDH-inhibition, influence viability. This could indicate that alternative metabolic pathways are possible in the presence of the above mentioned ions. In fact, as discussed by Roller et al. (1980) the dehydrogenation of sugar substrates could be essential to the life of the cells if no shunt pathway replaces it.
Contrary to the results of Mitra et al. (1975), for cells exposed to Cd, neither Zn nor Cd produces injury or death in E.coli, so there is only a bacteriostatic effect. On the contrary Hg and Cd produce metabolic stress in E.coli. There are, some hypothesis on the causes of this stress. Sjogren and Gibson (1981), for instance indicate a strong dependence of the stress due to a dilute environment on energy yielding processes, our data support the opinion that the cellular interaction with Hg and Cu is chiefly expressed at the peptidoglycans and cell wall synthesis level. Metal-injured cells, in fact, exhibit an inability to recover in the presence of D-cycloserine, while a certain degree of recovery is shown when 2,4-Dinitrophenol is present. In this connection it was found that the heavy metals Hg and Cu, which produce injury in E.coli strongly interact with both the biosynthesis and enzymatic activity of the ß-galactosidase, of the same microorganism, while a lower inhibitory effect on the ß-galactosidase activity was observed for Zn and Cu ions which do not produce injury.

The data presented in this study, in which pure E.coli cultures were used, underlines the complexity of metal-bacteria interactions. The results therefore emphasize that the binding of heavy metals to bacterial envelopes may play an important role in environments polluted with heavy metals. Consequently the possibility of an understimation of load determined by direct plating in selective media should be taken into account every time the enumeration of organisms occurs in the presence of heavy metals.

REFERENCES

Anderson IC, Rhodes M, Kator H(1979) Sublethal stress in Escherichia coli: a function of salinity. Appl Environ Microbiol 38: 1147-1152

A.P.H.A., A.W.W.A., W.P.C.F.(1980) Standard Methods for the Examination of Water and Wastewater. 15th Ed Washington: A.P.H.A.

Beveridge TJ, Kowal SF(1981) Binding of metals to cell envelopes of Escherichia coli K-12. Appl Environ Microbiol 42: 325-335

Bissonette GK, Jereski JJ, Mc Feters GA, Stuart DG(1977) Evaluation of recovery methods to detect coliforms in water. Appl Microbiol 33: 590-595

Gadd GM, Griffiths AJ(1978) Microorganisms and heavy metal toxicity. Microbial Ecology 4: 303-317

Liu D, Thomson K(1983) Toxicity assessment of chlorobenzenes using bacteria. Bull Environ Contam Toxicol 31: 105-111

Mc Feters GA, Cameron SC, Le Chevallier MV(1982) Influence of diluents, medium and membrane filters on detection of injured waterbone coliform bacteria. Appl Anviron Microbiol 43: 97-103

Mitra RS, Gray RH, Chin B, Bernstein IA(1975) Molecular mechanism of accomodation in Escherichia coli to toxic levels of Cd^{2+} J Bacteriol 121: 1180-1188

Morozzi G, Cenci G, Caldini G(1982) The tolerance of an environmental strain of Escherichia coli to some heavy metals. Zbl Bakt Hyg, I Abt Orig B 176: 55-62

Pardee AB, Jacob f, Monod J(1959) The genetic control and cytoplasmic expression of "inducibility " in the synthesis of ß -galactosidase by E.coli. J Mol Biol 1: 165-178

Przybliski KS, Witter LD(1979) Injury and recovery of Escherichia coli after sublethal acidification. Appl Environ Microbiol 37: 261-265

Roller SR, Olivieri VP, Kawata K(1980) Mode of bacterial inactivation by chlorine dioxide. Water Res 14: 635-641

Sjogren RE, Gibson MJ(1981) Bacterial survival in a dilute environment. Appl Environ Microbiol 41: 1331-1336

Tobin RS, Dutka J(1977) Comparison of the surface structure, metal binding, and fecal coliform recoveries of nine membrane filters. Appl Environ Microbiol 34: 69-79

Zaske SK, Dockins WS, Schillinger JE, Mc Feters GA(1980a) New methods to assess bacterial injury in water. Appl Environ Microbiol 39: 656-658

Zaske SK, Dockins WS, Mc Feters GA (1980b) Cell envelope damage in E.coli caused by short-term stress in water. Appl Environ Microbiol 40: 386-390

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