Inhibition of Bacterial Growth by Metal Salts

A SURVEY OF EFFECTS ON THE SYNTHESIS OF RIBONUCLEIC ACID AND PROTEIN

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(Received 5 June 1969)

Inhibition of the growth of *Escherichia coli* M.R.E. 600 by six different metal salts was accompanied by a greater decrease in the synthesis of RNA than in that of protein. The action of cobalt chloride was exceptional; inhibited cells made an excess of RNA to an extent depending on the concentration of Co^{2+} , the time of incubation and the concentration of Mg^{2+} in the medium. Preferential synthesis of RNA in the presence of cobalt chloride was not confined to *E. coli* but occurred to various extents in some, but not all, of the other micro-organisms that were tested. Possible reasons for the special effect of Co^{2+} are discussed.

This work derives from a study by Levy, Skutch & Schade (1949) on the effect of Co^{2+} on the nucleic acid content and growth rate of *Proteus vulgaris*. These authors noted that, when resting cells were inoculated into a meat-extract broth, a transient increase in the RNA content of the cells preceded changes in turbidity and dry weight; when cobalt sulphate was included in the medium there was little immediate effect on the synthesis of RNA, although growth was almost completely inhibited. This apparent dissociation of the production of RNA from that of other cell components in the presence of Co^{2+} is somewhat similar to the action of antibiotics such as puromycin, chloramphenicol and the tetracyclines, which, when added to exponentially growing cells of *Escherichia coli*, sharply decrease protein synthesis while permitting the continued production of RNA (Gale & Folkes, 1953; Takeda, Hayashi, Nakagawa & Suzuki, 1960; Holmes & Wild, 1966). However, Bernlohr & Webster (1958) found that Co^{2+} caused an almost equal inhibition of the synthesis of protein and RNA when added to growing cultures of *Azotobacter agilis*.

We have used exponentially growing cultures of a range of micro-organisms and studied their inhibition by a number of salts of metals including those of cobalt. In some, but not all, bacteria Co^{2+} causes considerable preferential synthesis of RNA; the nature of this RNA is considered in the paper following (Blundell & Wild, 1969). None of the other metal ions tested led to the accumulation of RNA by inhibited cultures.

EXPERIMENTAL

Organisms. Laboratory stocks of the following organisms were used: *Escherichia coli* strain B, *E. coli* 3/62 (a methionine-requiring auxotroph of the Oxford 518 strain), *Enterobacter cloacae*, *Bacillus megaterium* strain KM and *Alcaligenes* sp. Other bacteria were *Escherichia coli* strain M.R.E. 600 (Cammack & Wade, 1965), *E. coli* 58-161 (a methionine-requiring 'relaxed' auxotroph of strain K12; Borek, Ryan & Rockenbach, 1955), *Arthrobacter simplex* (N.C.I.B. 8929), *Micrococcus denitrificans* (N.C.I.B. 8944) and *Pseudomonas oxaliticus* (N.C.I.B. 8642). The bacteria were maintained by subculture on nutrient-agar slopes that were stored at 5°.

Media and growth conditions. Bacteria were grown at 37° in a shaking-incubator. Extinctions of cultures were measured at 450 nm. with a Unicam SP. 500 spectrophotometer and cuvettes of 1 cm. light-path. A minimal medium (Kjeldgaard, 1961) was used, adjusted to pH 7.4 with conc. HCl and containing (per l.): tris, 12.1 g.; KCl, 2 g.; NH_4Cl , 2 g.; Na_2HPO_4 , 142 mg.; Na_2SO_4 , 50 mg.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 500 mg.; $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 2 mg.; glucose, 2 g. For the growth of some organisms, this medium was supplemented with 2 g. of Difco vitamin-free casamino acids (casein hydrolysate)/l.

Cultures were often grown in a similar minimal medium but containing 5 mg. of glucose/l.: growth was then limited at E_{450} about 0.01 by exhaustion of the glucose. After growth overnight, 2 g. of glucose/l. was added (together with the casamino acids where appropriate); growth then resumed without appreciable lag and experiments were started when the cultures had E_{450} 0.065–0.090. Bacteria that grew poorly or not at all in the minimal medium (*A. simplex*, *Ps. oxaliticus* and *Alcaligenes* sp.) were first incubated overnight in the supplemented medium: a portion of this culture was then diluted with fresh medium

to an extinction of about 0.01 and experiments were started after about three generations of growth.

RNA and protein synthesis. To cultures were added 15 μ g. of uracil/ml. and 0.1 μ C of [5-³H]uracil/ml. and the incorporation of radioactivity into material insoluble in 5% (w/v) trichloroacetic acid at 0° was used as a measure of synthesis of RNA. Protein synthesis was followed in the same cultures. For those growing in the minimal medium, about 70nc of carrier-free [³⁵S]sulphate/ml. was added; alternatively 50 μ g. of L-methionine/ml., together with about 70nc of L-[³⁵S]methionine, was used. The same quantity of radioactive methionine was added without carrier to media supplemented with casamino acids.

For the determination of radioactivities, samples (1.0 ml.) were pipetted into 1 ml. of 10% (w/v) trichloroacetic acid at 0°. After standing for at least 15 min., the precipitates were collected by filtration on Whatman GF/B glass-fibre circles and washed with ice-cold 5% (w/v) trichloroacetic acid. The filters were then transferred to scintillation vials and dried at 80° for 1 hr. in a vacuum oven; 5.0 ml. of scintillator solution was added to each vial and the radioactivity of the samples was counted in a Beckman liquid-scintillation counter, usually to a minimum of 10000 counts for each isotope. The scintillator solution contained; toluene, 11; 5-(4-biphenyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD), 5g.

For cultures growing exponentially, the ratio of newly synthesized RNA to newly synthesized protein should be constant as should therefore be the ratio of radioactivity incorporated into RNA (c.p.m./ml. of culture)/radioactivity incorporated into protein (c.p.m./ml. of culture). It was noted that with some organisms a constant ratio was not established immediately after the addition of the radioactive compounds. The procedure used was therefore to add carrier uracil (and methionine if appropriate) about three generations before the start of an experiment and radioactive material about one generation before. The radioactivities incorporated at the beginning of the experiment were then subtracted from subsequent values. The ratio of radioactivity incorporated into RNA/radioactivity incorporated into protein was then constant throughout an experiment in the untreated control culture.

Many of the observations were concerned with the relative syntheses of RNA and protein during the inhibition of growth caused by the addition to the media of metal salts. Oversynthesis of RNA (or protein) during inhibition will result in a value for the ratio (radioactivity incorporated into RNA/radioactivity incorporated into protein) higher (or lower) than the value in a control culture. A useful index of preferential synthesis (the 'IC ratio') is then obtained by dividing the ratio for the inhibited culture by that for the control; values greater than unity, for example, measure oversynthesis of RNA relative to protein.

Chemicals. The metal salts used to inhibit growth were A.R. grade. Radioactive chemicals were from The Radiochemical Centre, Amersham, Bucks. Chloramphenicol was from the Sigma (London) Chemical Co., London S.W.6, and butyl-PBD from CIBA (A.R.L.) Ltd., Duxford, Cambs.

RESULTS

Inhibition of E. coli M.R.E. 600 by salts of metals.

Table 1 shows the results of a number of different experiments carried out with cultures of *E. coli*

Table 1. *Inhibition of E. coli M.R.E. 600 by metal ions*

The general experimental methods are described in the text; portions (50 ml.) of cultures (*E*₄₅₀ 0.07–0.09) growing exponentially in minimal medium were used. Synthesis of RNA and protein was followed by the radioactivities incorporated (c.p.m./ml.) from [³H]uracil and [³⁵S]sulphate respectively into material insoluble at 0° in 5% trichloroacetic acid. The increases in the radioactivities and in *E*₄₅₀ in the presence of the additions after incubation for 1 hr. are given as percentages of the increases in the corresponding control, whose mean generation time was about 55 min. The IC ratio, also computed after inhibition for 1 hr., is a measure of the preferential synthesis of RNA and is explained in the Experimental section. For the experiment involving inhibition by CoCl₂ in the presence of 2g. of casamino acids/l., the amino acid mixture was added about three generations before the start of the experiment and L-[³⁵S]methionine was used to follow protein synthesis.

Addition	Concn. (μ M)	% Increase in			IC ratio
		<i>E</i> ₄₅₀	RNA	Protein	
CdCl ₂	1.5	57	47	59	0.80
	2.5	38	22	40	0.56
	3.0	27	15	29	0.51
Zinc acetate	100	37	34	51	0.62
	200	23	16	34	0.47
	500	16	6	21	0.29
(NH ₄) ₆ Mo ₇ O ₂₄	10	45	36	48	0.75
	30	27	16	23	0.68
CuCl ₂	300	81	71	82	0.88
	600	66	57	69	0.82
	1000	49	38	56	0.66
HgCl ₂	0.2	80	79	83	0.95
	0.3	32	28	44	0.66
	0.4	13	6	16	0.36
NiCl ₂	25	38	34	55	0.63
	40	23	9	24	0.38
	50	18	3	16	0.22
CoCl ₂	30	63	68	56	1.20
	100	21	36	17	2.08
	300	12	23	4	6.26
CoCl ₂ (+ casamino acids)	600	49	77	50	1.54
	1000	28	45	20	2.30
	3000	9	14	3	4.12

M.R.E. 600 growing exponentially in minimal medium and in which the effects of different metal ions on extinction and the synthesis of RNA and protein were investigated. With the exception of cobalt chloride, results with the different salts are very similar although the concentrations that were needed to cause comparable inhibitions varied by over 1000-fold from the very toxic mercuric and cadmium salts to the much less effective cupric and zinc salts. In general, although the synthesis of both protein and RNA was retarded by these metal ions, the formation of RNA was inhibited more than that of protein; as a consequence, the IC

ratios (described in the Experimental section and measured after inhibition for 1 hr.) were less than unity. As the concentration of a toxic metal salt was increased this effect became more pronounced and the IC ratio fell still further; for example, in cultures inhibited by 50 μ M-nickel chloride, the amount of RNA synthesized per unit protein was less than one-quarter of that in the corresponding control.

The action of cobalt chloride was exceptional. Synthesis of RNA was affected less than that of protein, so that inhibited cultures accumulated RNA. Although the extent of the synthesis of both components decreased with increasing concentrations of cobalt chloride, the oversynthesis of RNA was more pronounced at higher concentrations; during inhibition by 300 μ M-cobalt chloride about six times as much RNA was made per unit protein as in an uninhibited culture. Experiments not reported here confirmed that these effects of cobalt chloride were those of the Co^{2+} ion; solutions of cobalt sulphate produced a response that was indistinguishable from that caused by equimolar concentrations of the chloride.

Preferential synthesis of RNA during inhibition by Co^{2+} was not confined to cells that had been grown in the minimal medium. Table 1 also gives the results of an experiment in which organisms growing in medium supplemented with casamino acids were incubated with cobalt chloride. The concentrations of this salt required to produce inhibition were about ten times those producing a comparable response in minimal medium, perhaps because of chelation of Co^{2+} by the amino acids, particularly histidine (Schade, 1949), that were present. However, at these higher concentrations the effects of cobalt chloride were similar to those in minimal medium: there was preferential synthesis of RNA and the IC ratio increased with increasing concentration of cobalt chloride.

Fig. 1 illustrates two further aspects of the effects of cobalt chloride on *E. coli* M.R.E. 600 growing in minimal medium. Fig. 1(a) shows change in extinction and preferential synthesis of RNA during inhibition by different concentrations of cobalt chloride. The IC ratios fell with increasing time of inhibition, except at the highest concentration used (300 μ M), where protein synthesis was completely inhibited within 15 min. Fig. 1(b) shows an experiment in which cells were incubated for 2 hr. in media containing a constant concentration of cobalt chloride (100 μ M) and three different concentrations of Mg^{2+} . Abelson & Aldous (1950) observed that the toxic effects of a number of metal ions, including Co^{2+} , were decreased in the presence of high concentrations of Mg^{2+} , which served to diminish their uptake by the cells. Fig. 1(b) shows that an increase in the Mg^{2+} concentration

Fig. 1. Inhibition of *E. coli* M.R.E. 600 by CoCl_2 . (a) Portions (50 ml.) of a culture growing exponentially in the minimal medium containing [^3H]uracil and [^{35}S]sulphate (as described in the Experimental section) were incubated without CoCl_2 ($\circ$) and with CoCl_2 at concentrations 0.03 mM ($\bullet$), 0.1 mM ($\square$) and 0.3 mM ($\blacksquare$). Growth (E_{450}) and preferential synthesis of RNA as given by the IC ratio (see the Experimental section), were measured. (b) A portion (12.5 ml.) of a culture that had been grown in the minimal medium that lacked MgCl_2 . The final concentration of Mg^{2+} in this medium was then 0.1 mM. [^3H]uracil and [^{35}S]sulphate were added (as described in the Experimental section). After growth to E_{450} 0.065, portions (50 ml.) were incubated with the addition of MgCl_2 alone to give a final concentration of 2.5 mM ($\circ$), or 0.1 mM- CoCl_2 and MgCl_2 to give final concentrations of 0.25 mM ($\blacksquare$), 0.75 mM ($\triangle$) and 2.5 mM ($\blacktriangle$). Growth and the IC ratio were measured.

decreased the inhibition of growth and preferential synthesis of RNA as measured by the extinction and IC ratios respectively; the latter again decreased with increasing time of inhibition. These effects are discussed below.

Inhibition of other organisms by cobalt chloride. A number of other bacteria were examined for their ability to effect some preferential synthesis of RNA during inhibition by cobalt chloride. The results are summarized in Table 2 and show that a spectrum of behaviour exists. The three strains of *E. coli* that were examined (B, 3/62 and 58-161) were similar to *E. coli* M.R.E. 600; appreciable preferential synthesis of RNA also occurred in *M. denitrificans*, *E. cloacae* and *A. simplex*. With *B. megaterium* KM and *Ps. oxaliticus*, IC ratios were generally less than unity and only occasionally could some slight preferential synthesis of RNA be demonstrated. The *Alcaligenes* sp. synthesized

Table 2. *Inhibition of micro-organisms by cobalt chloride*

The general methods are described in the text. Portions (50 ml.) of cultures (E_{450} 0.07–0.09) growing exponentially in either minimal medium (M) or the same supplemented with casamino acids (CAA) were used. Synthesis of RNA and protein was followed by the increase in radioactivities (c.p.m./ml.) incorporated from [^3H]uracil and either [^{35}S]sulphate or L-[^{35}S]methionine into material insoluble at 0° in 5% trichloroacetic acid. Preferential synthesis of RNA is expressed as the IC ratio (see the Experimental section), determined after inhibition for 1 hr.; the increase in E_{450} in this time is given as a percentage of the increase in the corresponding control without CoCl_2 .

Organism	Medium	Mean generation time of control (min.)	^{35}S label for protein synthesis	Concn. of CoCl_2 (mM)	% Increase in E_{450}	IC ratio
<i>E. coli</i> B	M	64	Sulphate	0.03	32	1.47
				0.06	26	1.56
				0.1	16	1.88
<i>E. coli</i> 3/62	M	53	Methionine	0.03	77	1.34
				0.1	37	4.30
				0.3	21	4.25
<i>E. coli</i> 58-161	M	57	Methionine	0.03	80	1.18
				0.1	56	1.91
				0.3	24	4.87
<i>E. cloacae</i>	M	47	Sulphate	0.06	58	1.44
				0.1	41	2.26
				0.2	17	5.00
<i>M. denitrificans</i>	M	91	Sulphate	0.06	52	1.52
				0.1	45	2.44
				0.3	71	0.90
<i>B. megaterium</i>	M	69	Sulphate	0.06	61	0.93
				0.03	79	1.01
				0.06	49	1.23
<i>A. simplex</i>	CAA	150	Methionine	0.3	100	1.34
				0.6	56	2.17
				1.0	50	3.49
<i>Ps. oxaliticus</i>	CAA	81	Methionine	0.3	68	0.88
				0.6	35	0.94
				1.0	27	1.06
<i>Alcaligenes</i> sp.	CAA	60	Methionine	0.3	84	0.80
				0.6	70	0.75
				1.0	42	0.58

Table 3. *Inhibition of an Alcaligenes sp. by chloramphenicol*

The experiment used two portions (each of 50 ml.) of a culture (E_{450} 0.07) growing exponentially in minimal medium supplemented with casamino acids, [^3H]uracil and [^{35}S]methionine. At zero time 10 μg . of chloramphenicol/ml. was added to one portion. Results are given as the increases in the radioactivities (c.p.m./ml.) incorporated from zero time into material insoluble at 0° in 5% trichloroacetic acid.

Time (min.)	Radioactivity incorporated (c.p.m./ml.)			
	Control		+ Chloramphenicol	
	RNA	Protein	RNA	Protein
30	538	396	374	6
60	1120	696	617	47

excess of protein over all time-intervals and with all concentrations of cobalt chloride that were tested.

Thus the inhibition of RNA and protein synthesis caused by Co^{2+} in this organism is similar to that brought about in *E. coli* M.R.E. 600 by salts of metal ions other than Co^{2+} (Table 1). However, this response of the *Alcaligenes* sp. was not accompanied by an inability of this strain to make an excess of RNA under other circumstances. Table 3 shows that, during incubation with 10 μg . of chloramphenicol/ml., protein synthesis was inhibited much more than was the synthesis of RNA.

E. coli strain 58-161 is a methionine-requiring auxotroph that also carries the 'relaxed' mutation (Stent & Brenner, 1961); unlike 'stringent' organisms, it is therefore able to continue to make RNA for some considerable time when the cells are incubated in a growth medium lacking methionine (Borek *et al.* 1955). Table 4 shows that under these conditions the accumulation of RNA in the absence of net protein synthesis was appreciably inhibited by Co^{2+} in the concentration range (30–300 μM)

Table 4. *Accumulation of RNA by E. coli 58-161 in the presence of cobalt chloride*

A culture (E_{450} 0.09) growing exponentially in a minimal medium containing [^3H]uracil and 50 μg . of L-methionine/ml. was filtered through a 5 cm. Oxoid filter. The cells were washed with medium lacking methionine and resuspended (at zero time) in the original volume of medium, again without methionine. Portions (50 ml.) were then immediately incubated further in the presence and absence of CoCl_2 . Results are given as the increase in radioactivities (c.p.m./ml.) incorporated from zero time into material insoluble at 0° in 5% trichloroacetic acid.

Concn. of CoCl_2 (mM)	Radioactivity incorporated (c.p.m./ml.)	
	At 30 min.	At 60 min.
0	444	826
0.03	462	725
0.1	394	569
0.3	304	—

used with growing cells in the experiments of Table 2.

DISCUSSION

Table 1 shows that the normal response of *E. coli* to the presence of inhibitory concentrations of salts of metal ions involves a greater decrease in the rate of synthesis of RNA than in that of protein. Although this could be a reflection of a greater sensitivity of key enzymes concerned in nucleic acid metabolism than of events more directly concerned in protein synthesis, inhibited cultures comprise cells that have recently undergone an abrupt decrease in growth rate whose consequences in other circumstances often include a considerable decrease in the rate of synthesis of RNA (Neidhardt & Fraenkel, 1961). A somewhat similar decline in the synthesis of RNA relative to protein was observed during the more specific inhibition of *E. coli* by analogues of amino acids (Turnock & Wild, 1966).

The action of cobalt chloride on *E. coli* is unlike that of the other salts that were tested: protein synthesis is inhibited more than the synthesis of RNA. This effect becomes more pronounced at higher concentrations of cobalt chloride, although total synthesis is then less. In this respect, inhibition of *E. coli* by Co^{2+} is somewhat similar to that brought about by chloramphenicol, puromycin or the tetracyclines; as mentioned above, although these antibiotics, and streptomycin (Anand & Davis, 1960), strongly inhibit protein synthesis, the synthesis of nucleic acids is less affected by them so that inhibited cells accumulate RNA. In particular,

Holmes & Wild (1967) found that increasing concentrations of chlortetracycline produced a progressive decrease in the synthesis of both RNA and protein, but a greater preferential synthesis of RNA.

The antibiotics that cause the accumulation of RNA appear to have in common a direct effect on the reactions concerned in protein synthesis, which they inhibit in cell-free systems. Puromycin acts by virtue of its structural similarity to aminoacyl-transfer RNA (Yarmolinsky & de la Haba, 1959); chloramphenicol may function as an inhibitor of the peptidyltransferase reaction (Monro & Marcker, 1967) and the tetracyclines interfere with the binding of aminoacyl-transfer RNA to ribosomes (Hierowski, 1965; Suarez & Nathans, 1965). It is therefore possible that, like these antibiotics, Co^{2+} has some specific effect on the active protein-synthesizing unit whose consequences include a continued synthesis of RNA.

The results of Fig. 1 and Tables 2 and 4 suggest, however, that this 'antibiotic-like' effect of Co^{2+} is accompanied by an inhibition of RNA synthesis and a general response of the cells similar to that caused by the other inhibitory salts. The decrease in preferential synthesis of RNA with time of incubation is probably a reflection of these opposing effects. Moreover, a different set of sensitivities of these processes to Co^{2+} might well lead to a situation in which there is little accumulation of RNA (*B. megaterium* and *Ps. oxaliticus*) or, as in the *Alcaligenes* sp., to one in which Co^{2+} inhibits the formation of protein less than the synthesis of RNA and so produces a response similar to that of other inhibitory salts on *E. coli*.

The authors thank Miss Anne Cooper for skilled technical assistance. M.R.B. is grateful to the Medical Research Council for a research studentship.

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