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Effect of Metal Ions on Diphtheria Toxin Production

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The effect of several metal ions on the production of diphtheria toxin was tested. By using the gel immunodiffusion system for detecting toxin, a wide range of metal ion concentrations was conveniently surveyed. Five divalent cations, Fe^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+} inhibited toxin production within a range of concentrations that did not inhibit growth of the producing strain. Growth and toxin production were inhibited at identical concentrations by both Cd^{2+} and Zn^{2+}, whereas Al^{3+} and Sr^{2+} affected neither growth nor toxin production over the range of concentrations tested. The data showed that Fe^{2+} was the most effective inhibitor on an equivalence basis, followed by Cu^{2+}, Co^{2+}, and Ni^{2+} in descending order. All eight strains of *Corynebacterium diphtheriae* chosen from diverse ecological origins responded similarly to all metals at similar concentrations. A mutant strain which produces toxin at Fe^{2+} concentrations 500 times greater than are inhibitory for the parent strain had simultaneously acquired resistance to inhibitory concentrations of Cu^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+}. This suggests that there is at least one common point in the activity of all these metal ions, and that toxin may respond broadly to changes in metal ion concentrations in the environment.

Over the past 40 years, since the study of Pappenheimer and Johnson (10), attention has been focused on the role of iron in the production of diphtheria toxin. Low iron concentrations (0.1 to 0.2 μg/ml) are required for maximum yields of toxin, whereas at higher concentrations (1 μg/ml) toxin production is depressed or abolished. Although it seems clear that iron is critical in the regulation of toxin production, it is generally overlooked that other metal ions have also been implicated. Locke and Main (7) and Pope (11) showed that small amounts of copper increased toxin production. Pappenheimer and Johnson (10) showed that both iron and copper increased toxin yields up to an optimal concentration then depressed yields as the concentration was increased. In the case of copper, growth was simultaneously inhibited at concentrations inhibitory for toxin. Clarke (1) made the first broad study of the effect of metal ions on diphtheria toxin synthesis. Examining a variety of metals at a single concentration (5×10^{-5} M), he found that Ag^{2+}, Al^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} produced no significant change in toxin yields, but that Co^{2+} produced a decrease in toxin production disproportionate to its effect on growth. The effects of Mn^{2+} and Pb^{2+} were equivocal, whereas Cr^{2+} partially inhibited growth and toxin production at the test concentration and Cd^{2+} completely inhibited growth.

Recently, a regulatory role for iron in toxin synthesis has been proposed by Murphy et al. (8, 9) in which an iron-activated repressor inhibits toxin synthesis at the level of transcription. In view of increased interest in the regulation of toxin synthesis, we undertook to reexamine the inhibitory effect of metal ions on toxin production. Since it seemed desirable to study a number of metal ions over a wide range of concentrations, we used a simple test system, a modified gel immunodiffusion plate system (6) in which the effect of metal ions on growth and toxin production could be simultaneously assessed.

MATERIALS AND METHODS

Strains of bacteria. The toxinogenic strains of *Corynebacterium diphtheriae* studied were C7(β), four randomly selected pharyngeal isolates (1027, 1072, 1078, and 1044), and two isolates from cutaneous lesions (179 and 385), taken from our stock collection, and a PW8 strain supplied by J. Murphy. Some special strains were also studied. Strain C7(β)hm723 (4), a strain in which toxin production is highly resistant to iron, and its cured, nonlysogenic, nontoxinogenic derivative C7(−)hm723 were supplied by T. Uchida. Strain C7(β^{tox+}_{cr1}), a strain in which toxin production is slightly resistant to iron inhibition due to a phage mutation, was also supplied by J. Murphy. We also isolated C7(β^{tox+}_{cr1})hm723 for testing. Nontoxinogenic strain C7 was taken from our stock collection. All strains were grown overnight on heart infusion Agar (Difco Laboratories, Detroit, Mich.) at 35°C and then

maintained at refrigerator temperature and transferred monthly.

Media. KL virulence agar (Difco) plus the supplement described by Hermann et al. (3) was used as the base medium for testing metal ion effects on toxin production. The same lot of medium was used throughout these experiments. To ensure proper mixing of the metal ions, an appropriate amount of the metal salt solution was first pipetted into sterile tubes held in a 49°C water bath followed by 14 ml of supplemented KL virulence agar. After mixing, the agar was poured into petri dishes, swirled, and then allowed to harden and dry. A control plate to which no metal was added was included in each series.

Reagents. The metal ions tested and the molecular weights of their salts were: $Al_2(SO_4)_3 \cdot 18H_2O$ (666.4); $CdSO_4$ (208.5); $CoSO_4 \cdot 7H_2O$ (281.1); $CuSO_4$ (249.71); $FeSO_4 \cdot 7H_2O$ (278); $MnSO_4 \cdot H_2O$ (169); $NiCl_2 \cdot 6H_2O$ (237.7); $Sr(NO_3)_2$ (211.6); and $ZnSO_4 \cdot 7H_2O$ (287.6). Stock solutions containing 14,000 and 1,400 μg/ml were made up in distilled water. All solutions were autoclaved except the iron salt, which was acidified with 1 drop of concentrated H_2SO_4 per 200 ml and sterilized by filtration to avoid excessive oxidation. It should be noted that the contamination of the salts of Co^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+} by iron was not a factor in their inhibition of toxin formation. The iron level, as specified, was at least 20 times lower than the minimum concentration that would have independently inhibited toxin formation at the critical concentrations of the other metal ions.

The toxin and antitoxin preparations used in the gel immunodiffusion tests were obtained from Connaught Laboratories Ltd., Ontario, Canada.

Testing for toxinogenesis. A previously described modification of the standard in vitro gel immunodiffusion tests for toxinogenesis (6) was used. Two filter paper strips (approximately 6 by 0.6 cm) saturated with antitoxin (200 to 400 antitoxin units/ml) were placed in parallel on the surface of the plate containing supplemented KL virulence agar. Each strip was approximately 1 cm from the center of the plate. A template was used to facilitate placement and ensure uniformity. Strains of *C. diphtheriae* were grown overnight on heart infusion agar plates. Inocula were lifted from these plates on the tip of sterile applicator sticks (0.2 cm in diameter) and were spotted about 0.7 cm from one of the antitoxin-containing strips. Seven inocula could be conveniently placed on each side of the strip for a total of 28 per plate. A nontoxinogenic control of C7 and a toxinogenic control of C7(β) were always included for each strip. Replicate inocula of each strain were made to check the reproducibility of the test. After inoculation the plates were placed, inverted, in plastic bags containing a moist paper towel and incubated at 35°C for up to 3 days. Plates were examined at 24-h intervals for growth and for toxin-antitoxin precipitin lines.

A control plate without added metal ions performed two functions. First, it established the ability of the medium to yield a positive test for toxinogenesis. Second, it provided the standard to determine whether growth in the presence of the metal ion was normal. A regulatory role for a metal ion in toxin production can only be inferred if toxin production is affected at concentrations that do not affect growth. In practice, inhibition of growth was easily detected, though small differences from the control could be missed. However, the distinction was unequivocal in cases where inhibition of toxin production as distinct from growth actually occurred.

RESULTS

The effects of the various metal-containing compounds on toxin production by C7(β) were initially tested at salt concentrations of 5, 100, and 500 μg/ml, and subsequently the test concentrations were refined on the basis of these data. A representative set of results is given in Table 1. The compounds are arranged in groups according to their effect. Similar results were obtained in repetitive experiments.

These data show that Co^{2+}, Cu^{2+}, Fe^{3+}, and Ni^{2+} all inhibited toxin production by C7(β), as detected in the gel immunodiffusion test, at concentrations that permitted normal growth. The Mn^{2+} ion was unique in having a broad zone of concentrations in which the results were variable, followed by a concentration at which the first complete inhibition of toxin production was accompanied by growth that was slightly below normal. Inhibition of toxin production by Cd^{2+} and Zn^{2+} appeared to coincide with inhibition of growth. Over the range of concentrations tested, Al^{3+} and Sr^{2+} had no effect on either growth or toxin production.

Of the metal ions inhibiting toxin production during normal growth, Fe^{2+} was unique in at least two ways. First, Fe^{2+} was from 60 to 750 times as effective on an equivalent weight basis as Co^{2+}, Cu^{2+}, or Ni^{2+} (Table 2). Second, Fe^{2+} could be increased to much higher concentrations relative to its toxin-inhibiting level than Co^{2+}, Cu^{2+}, or Ni^{2+} without affecting growth.

A number of other toxinogenic strains of *C. diphtheriae* were tested for the effect of metal ions on toxin production and growth. Included were the five pharyngeal isolates, among them PW8, and two isolates from cutaneous lesions. The results were identical with those recorded for C7(β), with the minor variation that the two cutaneous strains were slightly more sensitive to Fe^{2+} and Ni^{2+} than were the pharyngeal strains.

Three additional strains of *C. diphtheriae*, C7(β)hm723, C7(β^{tox+}_{ct1}), and C7(β^{tox+}_{ct1})hm723, of special interest to the question of regulation were also tested for toxin production in the presence of various concentration of Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and Mn^{2+}. The results (Table 3) show that the iron-resistant host mutant C7(β)hm723 simultaneously acquired significant resistance to Co^{2+}, Cu^{2+}, Ni^{2+}, and Mn^{2+}. Strain C7(β^{tox+}_{ct1}) containing the phage mutation may have simultaneously acquired a small level of resistance to Co^{2+}, but for the most part it was indistinguishable from C7(β). Finally, the strain combining

both the host and phage mutations to iron resistance, C7($\beta^{tox^+}_{cI^1}$)hm723, responded as if the host mutation were dominant.

A control was run to test whether the metal iron effects were due to interference with the precipitin reaction on the immunodiffusion plates rather than toxin production. Filter paper disks were soaked in diphtheria toxin containing

TABLE 1. *Effect of metal ions on toxin production by C. diphtheriae*

Metal ion	Concn (μg/ml)		Growth[a]	Toxinogenicity tests[b]	Effect on toxin production
	Compound[c]	Ion			
Co^{2+}	40	8	++	4/4	No effect
	60	12	++	4/4	
	80	17	++	25/25	
	100	21	++	26/29	Variable
	120	25	++	6/21	
	150	31	++	0/4	Inhibited
	200	42	++	0/4	
	300	63	++	0/4	
	400	84	+ → ++	0/4	
	500	105	−	0	Not recorded
Cu^{2+}	5	1.3	++	17/17	No effect
	10	2.6	++	14/14	
	20	5.2	++	14/14	
	30	7.6	++	0/14	Inhibited
	40	10	+ → ++	0/4	
	50	13	+ → ++	0/17	
	100	26	+	0	Not recorded
	500	128	−	0	
Fe^{2+}	0.01	0.002	++	17/17	No effect
	0.1	0.02	++	31/31	
	0.5	0.1	++	18/35	Variable
	0.8	0.16	++	0/4	Inhibited
	1	0.2	++	0/4	
	10	2	++	0/4	
	50	10	++	0/4	
Ni^{2+}	100	25	++	8/8	No effect
	200	50	++	17/17	
	250	62	++	17/17	
	300	75	++	17/21	Variable
	350	88	++	0/4	Inhibited
	500	125	+ → ++	0/17	
	1,000	250	−	0	
Mn^{2+}	5	1.6	++	21/21	No effect
	80	26	++	4/4	
	100	32	++	31/33	Variable
	200	64	++	20/21	
	300	97	++	3/4	
	400	129	++	1/4	
	500	161	+ → ++	0/21	Inhibited
Al^{3+}	5	0.4	++	17/17	No effect
	100	8	++	33/33	
	500	40	++	33/33	
	800	64	++	16/16	
	1,000	80	++	16/16	
Sr^{2+}	5	2	++	17/17	No effect
	100	42	++	17/17	
	500	208	++	33/33	
	800	333	++	16/16	
	1,000	416	++	16/16	

TABLE 1—*Continued*

Metal ion	Concn (μg/ml)		Growth[a]	Toxinogenicity tests[b]	Effect on toxin production
	Compound[c]	Ion			
Cd^{2+}	0.005	0.0027	++	4/4	No effect
	0.01	0.0054	++	4/4	
	0.05	0.027	++	4/4	
	0.1	0.054	+ → ++	4/4	
	1	0.54	+	0	Not recorded
	5	2.7	+	0	
	100	54	−	0	
	500	270	−	0	
Zn^{2+}	5	1.1	++	17/17	No effect
	100	23	+ → ++	24/24	
	140	32	+ → ++	4/4	
	160	36	+	2/2	
	180	41	−	0	Not recorded
	200	45	−	0	

[a] ++, Normal growth equal to the control; +, slight growth; −, no growth; + → ++, change from slight to normal growth over the 3-day period of observation.
[b] Number of positive tests/total number of tests; 0, no reading because of poor growth.
[c] Compounds used are given in Materials and Methods.

TABLE 2. *Comparison of metal ions inhibiting toxin production by C. diphtheriae*

Metal ion	Atomic wt	Lowest inhibitory concn		Inhibition ratio[a]
		μg/ml	Relative equivalent wt[b]	
Fe^{2+}	55.84	0.16	1	>62
Cu^{2+}	63.57	7.6	60	3–17
Co^{2+}	58.94	31	260	3–4
Ni^{2+}	58.69	88	750	1–3

[a] Minimum concentration (in micrograms per milliliter) of metal ion inhibiting growth completely/minimum concentration inhibiting toxin production.
[b] Ratio of the lowest inhibitory concentration of each metal ion relative to iron expressed in equivalent weights.

TABLE 3. *Relative resistance of selected C. diphtheriae mutants to metal ion inhibition of toxin synthesis*[a]

Metal ion	*C. diphtheriae* strain			
	C7(β)	C7($\beta^{tox^+}_{cti^+}$)	C7(β)hm723	C7($\beta^{tox^+}_{cti^+}$)hm723
Fe^{2+}	1	2	500	500
Co^{2+}	1	1.5	5–10	5
Cu^{2+}	1	1	3–15	3–15
Ni^{2+}	1	1	4	4
Mn^{2+}	1	1	8–10	8–10

[a] Relative resistance was calculated as the ratio of minimum concentration of the metal ion required to inhibit toxin production relative to the minimum concentration required to inhibit toxin production by C7(β).

5, 50, 100, and 500 Lf (flocculating) units/ml and placed on plates containing filter paper strips with the usual concentration of antitoxin as well as critical concentrations of Co^{2+}, Cu^{2+}, Fe^{2+}, and Ni^{2+} which inhibited toxin production without affecting growth. A control plate without added metal ions was also run. Positive precipitin reactions were observed at the same concentrations on both sets of plates for all metal ions. The range of toxin concentrations tested yielded strong to very faint precipitin reactions and would have detected significant effects of metal ions on the precipitin reaction.

One potential of the present system is its use in screening bacterial strains for the production of diffusible chelating agents. For example, since *Escherichia coli* produces compounds that chelate iron (2), we tested the ability of a strain of *E. coli* to promote toxin production by C7(β) on plates containing inhibitory concentrations of Fe^{2+}. The method used was to inoculate the strain of *E. coli* directly behind the C7(β) inoculum on the test plate. When this was done, C7(β) produced toxin at normally inhibitory concentrations of 0.16 and 0.2 μg of Fe^{2+} per ml. A similar effect by *E. coli* was noted at two normally inhibitory concentrations of Cu (7.6 and 10 μg/ml) and at inhibitory concentration of Co^{2+} (25 and 31 μg/ml). Although these effects are not necessarily due to chelation, the results showed that the method and variations of it could be used to screen for bacterial strains with this potential.

DISCUSSION

The results of this investigation show that over certain concentration ranges, the divalent cations Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and probably Mn^{2+} depress the production of diphtheria toxin

without affecting growth. This suggests that all of these metals act as regulators of toxin production. Over the range tested, Al^{3+} and Sr^{2+} had no effect on either growth or toxin production, whereas Cd^{2+} and Zn^{2+} depressed toxin production only at concentrations that inhibit growth. The general validity of these results is shown by the fact that a number of toxinogenic strains from disparate sources responded in a similar manner to these metal ions and responded over very similar concentration ranges.

Although the role of Fe^{2+} in regulating diphtheria toxin synthesis has been established by a number of investigators, Clarke (1) extended this work to include the effect of a number of metal ions on toxin production in broth cultures of *C. diphtheriae*. He concluded that Co^{2+} and Mn^{2+} also depressed toxin production without affecting growth. It is likely that the depressant effects of Cu^{2+} and Ni^{2+} were missed because the study was limited to a metal ion concentration of 5×10^{-5} M. Interestingly, Van Heyningen (12) showed that Ni^{2+} as well as Fe^{2+} and Co^{2+} affected the production of "neurotoxin" by *Shigella shigae* though the effect of Ni^{2+} was quite variable.

The data in Table 2 indicate that on an equivalence basis, Fe^{2+} is by far the most efficient regulator of toxin production. However, some caution must be used in interpreting these results, since Clarke (1) has shown that very little additional Co^{2+} ion is found intracellularly when the extracellular levels are increased above 0.2 μg/ml. In fact, he found that Co^{2+} was a more efficient regulator than Fe^{2+} based on the intracellular concentrations.

The interrelationship between Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} in the regulation of toxin synthesis is supported by our findings with strain C7(β)hm723, a host mutant in which toxin production is resistant to levels of Fe^{2+} at least 625 times that of wild-type C7(β). In addition to its resistance to Fe^{2+}, toxin production by this strain is simultaneously resistant to previously inhibitory concentration of the other four metal ions. The results suggest a common point of function for all these metal ions, but whether this involves transport, interaction with or activation of a repressor, or some other step in repression of toxin synthesis remains to be determined. The increase in iron resistance in C7(β)hm723 far exceeds that of the other metal ions, but this reflects the sensitivity of toxin production to extremely low concentrations of iron relative to the level at which growth is inhibited. Although the effect of iron seems dominant, these data suggest that toxin synthesis may be representative of a broader phenomenon, such as the response of this particular extracellular protein or perhaps a class of extracellular proteins to changing metal ion concentration in the environment.

The results with C7(β^{tox+}_{cI1})hm723, the strain carrying both the host and phage mutations to iron resistance, are also of interest in relation to the mechanism of regulation. In this strain, the host mutation is dominant; i.e., the strain is highly resistant to iron even though the phage mutation imparts only slight resistance. This indicates that the mechanism by which phage acquires partial resistance is in some way dependent on or related to the host regulatory system as proposed by Murphy et al. (8).

The gel immunodiffusion system used in this study has proved a convenient and reasonably sensitive method for screening a wide range of metal iron concentrations for their effect on toxin production. It detected alterations in the toxin reaction resulting from a change of 10 to 15 μg/ml in the critical concentrations of Co^{2+}, Cu^{2+}, Fe^{2+}, and Ni^{2+}. In addition, the level at which these ions effect these changes are comparable to those previously reported in studies using deferrated broth (1, 5, 10, 11). Nevertheless, some limitations and precautions in interpreting these results are necessary. The absolute and even relative quantitative reproducibility of these results may be affected by the lot of KL virulence agar used, since the base-line metal ion concentrations may vary. The variable reactions seen with some concentrations of metal ions also indicate that the test is sensitive to subtle differences perhaps in inoculum size, placement of the inoculum, or even metal ion distribution in the medium. The visual estimate of growth is admittedly crude, and the basic observations now require refinement. A more quantitative method for estimating growth, as in broth cultures, is obviously required. Finally, the gel immunodiffusion test can only detect a decrease in toxin production below the level required for a positive precipitin reaction. It does not measure the total extent to which toxin synthesis or release has been depressed. It is for these various reasons that we have used the term "inhibition of toxin production" rather than synthesis.

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