The Effect of Cobaltous Ions on the Formation of Toxin and Coproporphyrin by a Strain of *Corynebacterium diphtheriae*

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SUMMARY: Several cations have been shown to affect protein and porphyrin formation by the G 12/6 strain of *Corynebacterium diphtheriae*. Cobaltous ion (Co^{++}), at concentrations only slightly inhibiting growth, caused a marked inhibition of toxin formation. This effect was not produced by vitamin B_{12} or annulled by increases of cysteine or histidine concentration. Only a small proportion of added Co^{++} , at concentrations above $0.2 \mu\text{g./ml.}$, was found in washed organisms, and the effect of Co^{++} was more marked than that of Fe^{++} when based on the weight of metal in the organisms. No appreciable proportion of the Co^{++} existed as a porphyrin complex. The growth inhibition caused by $5 \mu\text{g. Co}^{++}/\text{ml.}$ was annulled by $0.6 \mu\text{g. Fe}^{++}/\text{ml.}$ Co^{++} caused no increase in the cytochrome *b* content of the organism, nor did it affect the iron concentration required for maximum toxin formation. The combined toxin-inhibitory effect of $\text{Fe}^{++} + \text{Co}^{++}$ was considerably less than the sum of their independent effects. The bearing of these findings on the thesis that diphtheria toxin is the apoenzyme of cytochrome *b* is discussed.

A concentration of $0.8 \mu\text{g. ferrous ion/ml.}$ in the culture medium almost completely prevents the appearance of toxin in culture filtrates of the G 12/6 strain of *Corynebacterium diphtheriae* (Clarke, 1958). Of $0.4-0.5 \mu\text{g. iron/ml.}$ found in the organisms only 9% can be extracted as protohaemin from saline-washed organisms, most of which is derived from bacterial cytochrome *b* (Pappenheimer & Hendee, 1949; Rawlinson & Hale, 1949). Of the residual iron, a small fraction can be removed by extraction with citrate buffer, but the remainder is firmly bound in the organisms and only half of it is removable by extraction with HCl acetone. Since cytochrome *b* is the predominant respiratory enzyme of the *C. diphtheriae*, there is some difficulty in deciding the possible site and function of this remaining iron. Several enzyme systems known to be present in bacteria, e.g. peptidases and aldolase (Mohamed & Greenberg, 1945; Berger, Johnson & Peterson, 1958) are known to be activated by iron and in this role iron can be replaced by a number of other divalent cations. The metallic ions, however, in these enzymes are not firmly bound components, comparable with haem iron in cytochromes, and do not seem likely to remain in the organisms after washing with citrate solutions. Nevertheless, it seemed worthwhile to examine whether only iron inhibited toxin formation or whether this inhibition could be caused also by other cations. The present contribution reports the inhibition of diphtheria toxin formation by several other cations besides iron, and a detailed examination of the action of cobaltous ions; a preliminary account of this work has already appeared (Clarke & Clarke, 1953).

METHODS

The G 12/6 strain of *Corynebacterium diphtheriae*, the medium used throughout this investigation, and the methods for the determination of toxin, coproporphyrin, culture filtrate protein-N, bacterial-N, iron and haem were the same as previously described (Clarke, 1958).

Cobalt determination. The cobalt content of washed organisms and cellular fraction was determined by a modification of the method of Marston & Dewey (1940). This method, which allowed the determination of 2 $\mu\text{g.}$ cobalt/10 g. plant material, involved the extraction of cobalt with diphenyl thiocarbazono to prevent the interference of the iron present in so large a quantity of material. This procedure proved unnecessary in the present work on the analysis of bacteria grown in the presence of cobalt, since the concentrations of iron and cobalt were of the same order. The cultures were centrifuged for 1 hr. at 3000 rev./min. in Pyrex test tubes (19 $\times$ 150 mm.) and the supernatant fluid was poured off and kept for toxin, coproporphyrin and protein analysis. The organisms were emulsified in 25 ml. ice-cold 1 % (w/v) NaCl warmed for 1 min. in a water bath at 45°, left at room temperature (*c.* 18°) for 30 min. and re-centrifuged. This washing was repeated twice more, and the organisms then dried at 150° for 1 hr. Combustion was effected in 2 ml. of a mixture of redistilled nitric acid (80 ml.) + A.R. sulphuric acid (3 ml.) + A.R. perchloric acid (5 ml.), the tubes being heated until nitric oxide was evolved; the mass of organisms was then broken up and left at room temperature for final slow combustion to near dryness the following day. When the tubes had cooled 1 ml. of 0.2N-citric acid was added to each, and the contents transferred quantitatively to silica dishes, 1 ml. of 0.2 % nitroso-R-salt added with stirring and the reaction adjusted to pH 8.0 with buffered NaOH by using a pH meter. After well washing the electrodes, the dish was boiled for 1 min. on a small flame, 1 ml. of conc. nitric acid added with stirring and the solution boiled for a further 1 min. After cooling for 1 min. 0.5 ml. of 0.2N-bromine water was added with stirring to decolorize the excess nitroso-R-salt, and the solution allowed to stand for 5 min. The excess bromine was removed by boiling for 1 min., and on cooling the volume was made up to 10 ml. with glass-distilled water. The colour was compared with similarly treated standards by using a Hilger spectrophotometer (blue-green filter); blank determinations, which showed no detectable cobalt content, were made up by treating 2 ml. of combustion mixture exactly as the samples to be analysed. Added cobalt was found to be recovered quantitatively from 1 to 10 $\mu\text{g.}$ /sample in the presence of a bacterial suspension of twice the strength of that encountered in the analyses, and at this concentration 15 $\mu\text{g.}$ iron caused no deflexion from the standard curve.

RESULTS

Preliminary testing of the action of different cations

The basal partially-defined medium on which the G 12/6 strain produced a maximum yield of toxin, namely, one containing 0.20–0.24 $\mu\text{g.}$ iron/ml. did not allow detectable toxin formation when a further 0.6 $\mu\text{g.}$ iron/ml. (to give

a concentration of 1.25×10^{-5} M- $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was added. Any significant effect of the salts of the other metals should, therefore, be detectable at a concentration of 5×10^{-5} M, which was used to test a variety of cations for their ability to inhibit the formation of toxin. In some instances the addition of this concentration of salt caused a decrease in the pH of the medium, in which case this was readjusted before autoclaving. Duplicate cultures were grown for each cation tested, and were harvested by centrifugation after 7 days of incubation at 34° .

In assessing the results shown in Table 1, the best estimate of growth, i.e. total cellular synthesis, is given by the sum bacterial-N + filtrate protein-N. This is a reasonable measure since there was no evidence that any appreciable proteolysis occurred in the cultures up to 7 days. Where the figures for bacterial-N were lower than those in the control cultures but the sum of bacterial-N + filtrate protein-N was similar to that in the control cultures, it was assumed that the added cation had accelerated lysis of the organisms. The validity of this assumption was confirmed by the fact that in such cases the toxin and coproporphyrin concentrations in the filtrate were usually comparable with those of the controls.

Table 1. *The effect of different cations on the growth, lysis, toxin and coproporphyrin of cultures of Corynebacterium diphtheriae, strain G 12/6*

G 12/6 strain of *C. diphtheriae* was grown for 7 days at 34° in 30 ml. of partially-defined medium. Salts were added to 5×10^{-5} M with adjustment when required, before autoclaving.

Cation added	Toxin titre (Lf/ml.)	Bact.-N ($\mu\text{g.}/\text{ml.}$)	Filtrate	
			Protein-N ($\mu\text{g.}/\text{ml.}$)	Coproporphyrin ($\mu\text{g.}/\text{ml.}$)
No addition	76	300	100	5.4
Cu^{++}	78	300	89	5.8
Ag^{++}	78	320	112	4.6
Zn^{++}	85	247	196	5.9
Cd^{++}	Growth only just detectable			
Al^{++}	78	323	95	5.6
Pb^{++}	63	334	81	5.2
Cr^{++}	48	185	116	5.8
Mn^{++}	58	272	137	4.7
Co^{++}	34	272	108	4.6
Ni^{++}	80	320	99	5.3

The effects resulting from the addition of different cations show a marked variation. Cd^{++} was so toxic that only a barely detectable pellicle of organism appeared, and no analyses were made. Cr^{++} caused a moderate inhibition of growth and a decrease in toxin yield, though the coproporphyrin concentration in the culture filtrate was unaffected. Cu^{++} , Ag^{++} , Al^{++} , Ni^{++} all caused no significant deviation in growth, lysis or toxin titre from the controls, though Ag^{++} caused a decrease in the coproporphyrin concentration. Mn^{++} caused no growth inhibition as judged by total protein-N found, though lysis was accelerated, and the toxin and coproporphyrin concentrations were decreased. Co^{++} showed the most marked decrease in toxin yield, though growth was not inhibited appreciably. Zn^{++} , unlike any of the other cations, caused an

overall increase in bacterial protein, reflected in increased toxin and coproporphyrin yields, and a marked increase in bacterial lysis. Pb^{++} caused no growth inhibition but showed a decrease in toxin and coproporphyrin yield.

Spectroscopic examination of the culture filtrates revealed an alkaline absorption spectrum (four-banded) of free porphyrin in every case except those cultures with added Zn in which there were typical two-banded metalloporphyrin spectra with peaks at 570 and 536 m μ . Thus in all other cases not more than a small fraction of the porphyrin was found in combination with the added cation, and the method of coproporphyrin analysis used would have resulted in the whole of the acid-labile zinc coproporphyrin complex being determined as free porphyrin. However, the possibility was not excluded that some of the small decreases of coproporphyrin found in the case of other metals which form acid-stable porphyrin complexes might have been due to a fraction of the coproporphyrin being held as unextractable metalloporphyrin. The results confirmed the observations of Mueller (1941) in respect of Mn^{++} , and also indicated that the effects of Co^{++} , Zn^{++} and Pb^{++} might merit further investigation. Zinc salts were found in a subsequent experiment to be active at much lower concentrations than those used here ($1 \times 10^{-5}\text{M}$), but since their effect appeared to be solely one of accelerating lysis and they did not lead to any decrease in toxin titre, this effect was not followed up. The medium used in this experiment was deficient in Zn and Co when growth was in Pyrex flasks, since maximum titres of 90–100 Lf units/ml. were obtained by the addition of these two ions at 0.1 $\mu\text{g.}/\text{ml.}$ or by the use of soda-glass vessels for growth.

The effect of increasing concentrations of Co^{++}

Since the effect of Co^{++} on toxin formation appeared to be less than that of Fe^{++} at similar concentrations, the cobalt content of the organisms was investigated. It had been found previously that about half the Fe present in the medium at any concentration up to that necessary to inhibit toxin formation was to be found in the saline-washed organisms. The centrifuged organisms from a culture with added cobalt contained a large proportion of easily removable cobalt as demonstrated by the results shown in Table 2. Since the free culture medium contained in the plugs of centrifuged organism was

Table 2. *Removal of cobalt from Corynebacterium diphtheriae by washing with buffered 1 % (w/v) NaCl solution*

Organisms from 30 ml. of culture of *C. diphtheriae*, strain G 12/6, containing 10 $\mu\text{g. Co}^{++}/\text{ml.}$ were washed with ice-cold 1 % (w/v) NaCl buffer (pH 7.0). After warming in a 45° water bath for 1 min. and 30 min. at room temperature the organisms were centrifuged down at 3000 rev./min. for 1 hr.; the volume of deposited organisms was 0.6–0.8 ml.

Treatment			
Unwashed	Washed $\times$ 1	Washed $\times$ 2	Washed $\times$ 3
Co^{++} organisms from 30 ml. culture ($\mu\text{g.}$)			
13.3	4.5	3.2	3.4
13.2	4.8	3.5	3.5
12.9	5.0	3.5	3.4

considerably less than their volume of 0.6–0.8 ml. ($\equiv 6\text{--}8\ \mu\text{g. Co}^{++}$), the 10 $\mu\text{g. Co}^{++}$ which was removed by 3 washings must have contained some Co^{++} from the organisms themselves. This concentration of Co^{++} in the organisms is probably related to that found in yeasts (Nickerson & Zerahn, 1949).

Cobalt concentrations added, up to 6.4 $\mu\text{g./ml.}$ medium were tested in duplicate cultures, being harvested after 7 days growth at 34°. The results in Table 3 show that, especially at the higher concentrations of Co^{++} , only a small proportion of the metal was in the organisms, and confirm that the decrease in toxin titre was disproportionately greater than the inhibition of growth and coproporphyrin formation. The basal medium, apparently, contained a suboptimal concentration of Co^{++} , since the addition of 0.05 $\mu\text{g./ml.}$ stimulated growth and also toxin and coproporphyrin formation. When related to the concentration of metal in the organisms Co^{++} appears to have a greater effect on toxin formation than iron (see Clarke, 1958).

Table 3. *Effect of cobalt concentration in medium on cobalt content of organisms grown therein*

Cultures (80 ml.) of *Corynebacterium diphtheriae* strain G 12/6 were grown for 7 days at 34°. The organisms were centrifuged down and washed three times with 1% (w/v) NaCl buffered pH 7.0 before Co determination.

Co added ($\mu\text{g./ml.}$)	Toxin titre (Lf/ml.)	Bacterial-N ($\mu\text{g./ml.}$)	Filtrate protein-N ($\mu\text{g./ml.}$)	Bacterial Co ($\mu\text{g./ml.}$)	Copropor- phyrin ($\mu\text{g./ml.}$)
—	74	360	71	0.005	5.2
0.05	86	393	78	0.027	5.5
0.4	68	347	60	0.047	4.5
1.6	39	295	52	0.080	4.3
3.2	14	—	—	0.107	3.8
6.4	10	233	81	0.083	2.2

The effect of cysteine and histidine on the activity of cobalt

Schade (1949) reported that cobalt at low concentrations inhibited the growth of many micro-organisms. He investigated its effect on the growth of *Proteus vulgaris* in particular, and found that the toxicity depended on the composition of the medium. Histidine reversed the inhibition competitively in the ratio of 2 molecules for every atom of Co present. This was interpreted as being due to the formation of a Co-histidine complex (Burk, Hearon, Caroline & Schade, 1946), which is formed at 37° and pH 7.5, and is stable under these conditions. Cobalt is also known to form complexes with cysteine (Michaelis, 1929; Schubert, 1931). It was considered that the presence of appreciable concentrations of histidine and cysteine in the medium used here might account for the fact that only a small proportion of the cobalt added to the medium was, apparently, taken up by the organisms during growth. The effect of the addition of each amino acid was therefore investigated (Table 4). It would appear that the amino acid-cobalt complexes are not stable under the conditions of growth, since the preformed complex with histidine was not less active than CoCl_2 , and neither cysteine nor histidine caused any decrease in the activity of Co^{++} .

Table 4. *The effect of histidine and cysteine on the activity of cobalt*

Cultures of *Corynebacterium diphtheriae* strain G 12/6 (30 ml.) were grown for 7 days at 34°. The amino acids and cobalt were added before autoclaving, and the sterile histidine-cobalt complex after autoclaving of the medium.

CoCl ₂ added (M × 10 ⁵)	Histidine- HCl added (M × 10 ⁵)	Co-histidine complex added (M × 10 ⁵)	Cysteine- HCl added (M × 10 ⁵)	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)	pH value
—	—	—	—	400	87	7.62
5	—	—	—	360	35	7.66
5	10	—	—	357	35	7.75
—	10	—	—	417	87	7.20
—	—	5	—	333	35	7.39
80	—	—	—	162	5	7.51
80	160	—	—	159	3	6.81
—	160	—	—	420	88	7.48
—	—	80	—	187	3	6.52
—	—	—	—	360	74	7.38
—	—	—	160	363	78	7.26
10	—	—	—	233	10	6.72
10	—	—	160	236	10	7.26

The effect of vitamin B₁₂

Since cobalt is a component of the vitamin B₁₂ molecule, 7-day cultures with and without added cobalt were analysed for this growth factor, the assays being carried out in Glaxo Laboratories by kind premission of Dr W. F. J. Cuthbertson. A culture grown on medium to which no cobalt had been added was found to contain 0.0097 μg. vitamin B₁₂/ml. (5 % of it in the organisms), and one from medium containing added Co⁺⁺ at 8 μg./ml. contained 0.15 μg. vitamin B₁₂/ml. (83 % in the organisms). Since organisms grown in this concentration of cobalt contained 0.1 μg. cobalt/ml. and vitamin B₁₂ contains 4 % Co, only 6 % of that cobalt was present in the vitamin B₁₂ molecule. It was confirmed that vitamin B₁₂ was not responsible for the decrease in toxin formation in medium to which cobalt was added, since, while having a slight growth-augmenting effect, addition of this vitamin had no effect on toxin formation at least in concentrations up to 1.6 μg./ml. (Table 5).

Table 5. *The effect of adding vitamin B₁₂ on toxin formation by Corynebacterium diphtheriae*

Vitamin B₁₂ was added as 'Cytamen 50' after sterilization to 30 ml. cultures of *C. diphtheriae* strain G 12/6, which were grown for 7 days at 34° and the organisms then harvested.

Culture	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)
Control (i), no added vitamin B ₁₂	407	84
Control (ii), no added vitamin B ₁₂	410	87
0.05 μg. vitamin B ₁₂ added	443	84
0.20 μg. vitamin B ₁₂ added	423	85
0.80 μg. vitamin B ₁₂ added	407	90
1.60 μg. vitamin B ₁₂ added	410	89

The effect of cobalt on the iron concentration for maximum growth and toxin formation

Since it was found previously that only half the iron present in medium at any concentration up to 0.86 $\mu\text{g./ml.}$ was found in the organisms, it was thought possible that the effect of adding cobalt might be that of combining with metal-complexing molecules in the medium, and thus allowing all the iron

Table 6. *The effect of cobalt on the iron concentration of maximum growth and toxin formation by Corynebacterium diphtheriae strain G 12/6*

Cultures (80 ml.) were incubated for 7 days at 34°. FeSO_4 and CoCl_2 were added before autoclaving the medium.

Iron added ($\mu\text{g./ml.}$)	Cobalt concentrations							
	Nil		0.1 $\mu\text{g./ml.}$		0.4 $\mu\text{g./ml.}$		2 $\mu\text{g./ml.}$	
	Toxin (Lf/ml.)	Bacterial-N ($\mu\text{g./ml.}$)	Toxin (Lf/ml.)	Bacterial-N ($\mu\text{g./ml.}$)	Toxin (Lf/ml.)	Bacterial-N ($\mu\text{g./ml.}$)	Toxin (Lf/ml.)	Bacterial-N ($\mu\text{g./ml.}$)
Nil	57	260	57	235	47	207	23	186
0.03	63	327	65	333	56	305	22	293
0.06	80	413	80	447	63	377	38	338
0.09	80	413	85	480	69	468	46	367
0.12	77	457	77	480	69	480	46	386
0.15	68	467	69	480	55	487	38	391
0.20	53	450	53	463	46	473	26	420
0.30	28	437	25	443	22	457	14	357

to remain freely available to the organisms. A series of cultures was therefore grown in which the effect of iron in the presence of increasing cobalt concentration was determined. If the action of cobalt was that of allowing the organisms to take up a higher percentage of the iron present in the medium, then the iron concentration required for maximum growth might decrease as the cobalt concentration was increased. The results in Table 6 show that the reverse occurred, allowing for the fact that in the presence of excess iron, cobalt itself was growth-limiting up to a concentration of 0.1 $\mu\text{g./ml.}$ The iron concentration required for maximal toxin yield was unaffected by the addition of cobalt.

The effect of cobalt on the cytochrome b and total iron content of diphtheria bacilli

Although cobalt does not act passively in providing more available iron in the medium it seemed possible that it might cause more of the iron of the organisms to be directed to the system responsible for inhibiting toxin formation; if Pappenheimer's suggestion were correct, this would be cytochrome *b*. It was therefore considered relevant to see whether adding cobalt caused any increase in cytochrome *b*, and also whether cobalt was present in the organisms as a haem complex which might have an action similar to haemin. Iron coproporphyrin III and protoporphyrin IX (haemin) complexes are capable of causing a decrease of toxin titre, though they are not as active as FeSO_4 in this respect (Clarke, 1958). The cobaltous complexes of coproporphyrin III

and protoporphyrin IX (prepared by heating the free porphyrin in dilute NH_4OH solution with the theoretical quantity of CoCl_2) when similarly tested were found to be inactive up to a concentration of $1\text{ }\mu\text{g. cobalt/ml.}$, at which concentration CoCl_2 caused a 40% decrease in toxin titre. The inactivity of the coproporphyrin complex, which is readily formed on autoclaving medium containing both components, was demonstrated by the fact that while cobalt and coproporphyrin both readily inhibited growth of *Corynebacterium diphtheriae* (coproporphyrin probably by combination with essential trace elements), added together in equimolar concentration they caused no growth inhibition.

When organisms grown in a medium containing $10\text{ }\mu\text{g. cobalt/ml.}$ were examined spectroscopically in the visible spectrum, in pyridine and reduced with sodium dithionite there appeared to be no qualitative or quantitative difference between the absorption bands of these organisms and those from control cultures, indicating that the addition of cobalt had not led to a conversion of more iron to haem enzymes, nor was there any appreciable cobalt porphyrin complex present. Washed organisms suspended in 0.85% (w/v) NaCl solution containing cobalt coproporphyrin III complex took up this complex readily, though the uptake was inhibited by adding culture medium.

To determine whether cobalt was present in the organisms as porphyrin complexes, a batch of organisms grown in a medium containing $10\text{ }\mu\text{g. Co}^{++}/\text{ml.}$ was washed three times with 1% (w/v) NaCl solution buffered at pH 7.0 and extracted by the method of Rawlinson & Hale (1949). The flow-sheet (Table 7) shows the method used and distribution of cobalt between the fractions. There appeared to be no appreciable cobalt present as unbound porphyrin complex (ethanol soluble) or as bound complex removable from the

Table 7. *The extraction and fractionation of cobalt from Corynebacterium diphtheriae strain G 12/6*

organisms by HCl+acetone, nor was any visible by spectroscope as pyridine haemochromogen.

The lack of detectable increase in the absorption spectrum of organisms containing cobalt when viewed with a Hartridge reversion spectroscope indicated no increase in cytochrome *b* content, but this required confirmation. The cytochrome *b* content of these organisms and of control organisms, both from cultures containing only 0.2 $\mu\text{g. iron/ml.}$, was not high enough to allow accurate determination to be made on ultrasonic disintegrated organisms as has been used for those grown in a higher iron concentration, but the extraction procedure with HCl+acetone could be used. Cultures were grown in rectangular soda-glass bottles, duplicate bottles containing 400 ml. of the basal partially-defined medium being harvested as follows:

(1) Optimum iron concentration (0.2 $\mu\text{g./ml.}$)—(a) with no added cobalt, (b) with 5 $\mu\text{g. Co}^{++}/\text{ml.}$ (added as CoCl_2).

(2) Iron concentration of 0.8 $\mu\text{g./ml.}$ —(a) with no added cobalt, (b) with 5 $\mu\text{g. Co}^{++}/\text{ml.}$ (added as CoCl_2).

The organisms were harvested after 5 days of incubation at 34°, since it had been found previously that maximum growth was reached in this time and that longer incubation led to lysis, particularly of cultures containing higher iron and cobalt concentrations. Saline-washed organisms were analysed for cobalt, nitrogen, haemin, iron and cobalt, and coproporphyrin and toxin determinations were made on the culture supernatant fluids.

Table 8. *The effect of cobalt on cytochrome b content of Corynebacterium diphtheriae strain G 12/6*

The batches (2 $\times$ 400 ml.) of each different culture were harvested after 5 days of incubation at 34°. The haemin values given are those from extraction with HCl+acetone from defatted organisms. Values are in terms of units given/ml. culture.

Fe concn. in medium ($\mu\text{g.}$)	Co added ($\mu\text{g.}$)	Bacterial- N ($\mu\text{g.}$)	Bacterial- Fe ($\mu\text{g.}$)	Bacterial- haemin ($\mu\text{g.}$)	Bacterial- Co ($\mu\text{g.}$)	Toxin (Lf)	Copro- porphyrin ($\mu\text{g.}$)
0.2	Nil	476	0.135	0.044	—	76	3.6
0.2	5	371	0.185	0.037	0.39	22	2.7
0.8	Nil	555	—	0.46	—	11	0.90
0.8	5	550	—	0.31	0.23	2-3	1.70

The growth inhibition caused by cobalt in medium with a low iron concentration was prevented by the increase of iron concentration to 0.8 $\mu\text{g./ml.}$ (Table 8). This concentration of cobalt, however, caused a decrease in the haemin content of the culture. Since the haemin content/unit bacterial-N of the low-iron cultures was unaffected by adding cobalt, it is possible that cobalt caused a decrease in cytochrome *b* synthesis which in an iron concentration of 0.2 $\mu\text{g./ml.}$ was growth-limiting, but in an iron concentration of 0.8 $\mu\text{g./ml.}$ was not.

From the results of the iron and cobalt analyses on these organisms it would seem that the presence of cobalt in the medium enhanced the total uptake of iron by the organisms though their haem content was not increased,

whereas the higher the iron content of the medium the smaller the proportion of cobalt taken up. (The methods of analysis used for these two metals were insensitive to alterations in concentration of the other.) To understand whether the uptake of these two metals is in any way connected would involve a more detailed investigation. The decreases in toxin titre caused by 0.6 $\mu\text{g.}$ iron/ml. and 5 $\mu\text{g.}$ cobalt/ml. were not additive when both were present in the same culture. This was confirmed by a further experiment, where incubation for 7 days allowed maximum toxin production (Table 9). It is clear that the toxin decrease caused by either metal was dependent on the concentration of the other present in the medium, which is evidence of relationship between their modes of action.

Table 9. *The mutual dependence of the action of iron and cobalt on toxin formation and growth of Corynebacterium diphtheriae strain G 12/6*

The cultures were incubated for 7 days at 34°. Values are in terms of units given/ml. culture.

Fe concn. in medium ($\mu\text{g.}$)	Co added ($\mu\text{g.}$)	Toxin titre (Lf)	Bacterial-N ($\mu\text{g.}$)
0.2	Nil	95	577
0.2	2.0	37	498
0.4	Nil	16	480
0.4	2.0	11	540

DISCUSSION

In addition to iron, low concentrations of cobalt, lead or manganese depress the formation of toxin without materially affecting growth; cobalt and manganese also cause a slight decrease in coproporphyrin formation. The addition of 0.05 $\mu\text{g.}$ cobalt/ml. causes an increase of growth and toxin and coproporphyrin formation. At higher concentrations of cobalt in the medium, growth is inhibited as well as toxin formation provided the iron content is low. When, however, the iron concentration is increased to 0.8 $\mu\text{g./ml.}$, cobalt inhibition is prevented. Cobalt causes a decrease in haem content of the organisms at both iron concentrations, but, since the haem content is not the growth-limiting factor at the higher iron concentration, there is no growth inhibition. Preliminary observations of iron content of organisms in such cultures indicate that there is no overall decrease in iron content, but cobalt causes a change in metabolism resulting in a decrease in the amount of iron bound to the porphyrin nucleus.

There is further evidence that the effects of iron and cobalt are related in that the toxin decrease caused by either metal is dependent on the concentration of the other in the medium. Evidence has already been presented (Clarke, 1958) which indicates that the effect of iron in decreasing the formation of toxin by the G 12/6 strain of *Corynebacterium diphtheriae* is not associated quantitatively with the formation of cytochrome *b*. No serological relationship between diphtheria toxin and diphtherial cytochrome *b* has been obtained (Pappenheimer & Hendee, 1947). Neither has it been found possible to show any interference in cytochrome *b* activity in toxæmic muscle, though the

effect of toxin in the silkworm appears to implicate the cytochrome system (Pappenheimer & Williams, 1952).

No assessment of the role of iron in the formation of diphtheria toxin is complete without consideration of the reports of a similar phenomenon in the case of other exotoxins though it may seem unlikely that these toxins are in any way similar in their activities. When *Clostridium tetani* was grown on a casein hydrolysate medium (Mueller & Miller, 1943) the concentration of iron had to be decreased to 0.08 $\mu\text{g./ml.}$ for maximum toxin yield. Rogers & Knight (1946) found that in the presence of protein-free 'toxigenic factor' the maximum yield of α -toxin by *C. welchii* was obtained on a medium containing 0.3 $\mu\text{g./ml.}$ of iron. The addition of 0.08 $\mu\text{g./ml.}$ above this concentration resulted in a 50% decrease in toxin yield. No cytochrome system has been demonstrated in Clostridia. Van Heyningen investigated the neurotoxin of *Shigella shigae*, and found results which appear to be comparable with those found with *Corynebacterium diphtheriae*. When the organism is grown on a medium deficient in iron, the metal is almost quantitatively converted into haem; the addition of more iron leads to an increase of haem and neurotoxin formation. When, however, the iron concentration is increased further, the metal is taken up and is converted into a form in which it reacts with 8-hydroxyquinoline (i.e. is loosely bound in the cell) and the yield of neurotoxin falls (van Heyningen, 1955*a*). Moreover, this effect of iron, which is apparently not related to haem formation, is reproduced by cobalt and nickel. These two metals do not affect the respiration of the organisms but iron does (van Heyningen, 1955*b*).

Lascelles (1956) showed that cobalt inhibited the synthesis of iron protoporphyrin from α -aminolaevulinic acid by suspensions of *Rhodopseudomonas spheroides*. This supports the view that the decrease of iron protoporphyrin in cultures of *Corynebacterium diphtheriae* containing added cobalt may be a direct result of the inhibition of haem synthesis. It seems a reasonable working hypothesis that this action of cobalt releases more of the total iron of the culture medium to be available for an iron system responsible for the non-appearance of toxin in the culture filtrate. The total possible toxin inhibition by cobalt in any medium is governed by the total iron content, the degree of inhibition of haem synthesis and the proportion of the non-haem iron which is directed into this specific system.

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