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Studies on the Extracellular Protein of a Toxinogenic Strain of *Corynebacterium diphtheriae* 1. Effect of Iron on the Release on the Extracellular Protein**

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SUMMARY

Kinetic and quantitative studies were made on the effect of iron on the extracellular liberation of TCA precipitable protein and toxin by the SM-1 strain of *C. diphtheriae* in a chemically defined medium. From kinetic experiments, a great difference was found in the pattern of release of the extracellular non-toxin protein and that of toxin by the organism. Particularly, it was found that the release of the former continues even after the toxin production had stopped. Under certain experimental conditions, the organism seems to release into the external medium a nearly constant amount of non-toxin protein regardless of the concentration of iron in the medium. Toxin protein appears to comprise the major part of the extracellular protein whose production is completely inhibited by excess iron in the growing medium. The significance of the converting factor (μg protein N/Lf) in interpreting these results is discussed.

INTRODUCTION

It has been noted by several workers (Pappenheimer *et. al.*, 1948; Cohn *et. al.*, 1949; Pope *et. al.*, 1951) that strains of toxinogenic diphtheria bacilli produce a variety of non-toxin protein in addition to a specific toxin protein. According to Brandwijk, Tasman and Ramshorst (1951a, 1951b), the ratio of toxin to total extracellular protein changes considerably during the growth of a strain of *C. diphtheriae*. This suggests that the pattern of toxin production is different from that of the non-toxin protein release. Although neither the nature nor the mode of production of the non-toxin protein are as yet clear, it may be that the mode of liberation of the protein is essentially different from that of toxin. Since traces of iron in the medium affect the yields of diphtheria toxin greatly, the problem arises of whether iron also affects the production of the non-toxin protein by the organism. In other words, does iron has a selective inhibitory action on toxin production ? In this respect, it is of considerable significance to see the effect of iron on the extracellular release of the non-toxin protein by the organism. A

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differential influence of iron on the release, if it were existing on the toxin and other extracellular diphtherial proteins, could imply the presence of different mechanisms for their release. Therefore, studies on the effect of iron on the extracellular liberation of these proteins might reveal some of the features of diphtheria toxin formation.

In the present paper are described the results of a study on the extracellular release of protein in relation to the iron concentration by a variant of Park William's No. 8 strain in a simplified chemically defined medium.

MATERIALS AND METHODS

1. *Bacterial culture*

A variant strain (SM–1) of Park William's No. 8 Toronto Harvard strain, originally isolated in our laboratory, was used throughout the study. The organism was subcultured on the synthetic AMC medium described previously (Yoneda, 1950, 1951, 1957).

2. *Cultural methods*

A slightly modified AMC medium was used in this study. The formula per liter of the medium was as follows; L-asparagine 5.0 g, DL-methionine 0.2 g, L-cystine 0.4 g, KH_2PO_4 5.0 g, NaCl 4.0 g, sodium succinate 3.0 g, sodium lactate (60%, Merck Reagent) 3.0 ml $CaCl_2 \cdot 2H_2O$ (50% solution) 2.0 ml, deferrated maltose (50% solution) 40 ml, glucose (30% solution) 3.3 ml and a solution of II—vitamins and metals—(Mueller and Miller, 1941), 2 ml. Before the addition of these sugars and solution II, the medium was deferrated by the calcium phosphate gel method (Mueller and Miller, 1941; Yoneda, 1950). After the addition of solution II to this deferrated medium, the mixture was autoclaved at 10 lbs. for 10 minutes. Suitable amounts of sterile solutions of maltose and glucose were then added and the medium was dispensed aseptically in 30 ml aliquots in specially designed 300 ml flasks. Iron was added to this medium as a sterile solution of 0.01% $FeSO_4 \cdot 7H_2O$ in 0.1% HCl just before inoculation with tiny pericles from a four day old culture in a test tube. Cultures were grown at 32–33°C and aliquots were taken at intervals for measuring growth, toxin and TCA precipitable protein.

3. *Assay methods*

At the end of incubation, the cultures were harvested and centrifuged at 4,000 rpm for 30 minutes in the cold. The precipitated cells were then washed three times with physiological saline before measuring the bacterial nitrogen by the micro-Kjeldahl method modified by Parnas. The volume of the supernatant was measured and then the solution filtered through the Toyo No. 50 filter paper. After recentrifugation at the same speed for 30 minutes, the final clear supernatants were used to determine toxin and total TCA precipitable protein.

Toxin was determined by the flocculation test and expressed as micrograms per milliliter assuming that 1 Lf is equivalent to 0.39 μg N (Pappenheimer and Yoneda, 1957). Antitoxin, containing 1416 flocculating units per ml, was kindly supplied by Dr. H. Hata in the Kitasato Institute.

Total extracellular protein was precipitated from the culture supernatants by addition of 10% trichloroacetic acid to a final concentration of 5%. The precipitate was washed twice with 5% TCA and then the nitrogen was measured by the modified micro-Kjeldahl method.

RESULTS

1. *Kinetic observations on toxin and total extracellular protein production by SM-1 strain in relation to the effect of iron*

Two types of experiments were carried out in this study. In each case, 14 flasks, each containing 30 ml of the complete medium, were employed. In one experiment, 1.0 ml of sterile 0.001 % $FeSO_4.7H_2O$ solution was added to each flask and the same volume of 0.01 % $FeSO_4.7H_2O$ solution, in the other experiment. These media were then inoculated with a tiny pericle from a four day old culture of SM-1 strain and incubated at 32–33°C. Duplicate flasks were taken at day intervals in both experiments for measuring the growth, toxin and total TCA precipitable protein. Since evaporation took place during the incubation, the final volume of each aliquot was carefully measured before centrifugation so that errors could be corrected. The results are illustrated in Tables 1 and 2 respectively.

Table 1. EXtracellular release of total TCA precipitable protein and toxin during the growth of SM-1 strain of C. *diphtheriae* on AMC medium with iron deficiency.*

Incubation days	Total Growth	Toxin **		TCA precipitable protein	
	mg bacterial nitrogen	Total Lf	μg protein N /mg bacterial N.	Total μg protein	μg protein /mg bacterial N.
0	—	0	—	—	—
1	—	0	—	—	—
2	0.22	0	0	(trace)	(trace)
3	6.23	110	6.7	255	40.9
4	14.72	864	22.9	1500	102.0
5	15.20	1458	37.3	2106	138.5
6	15.22	1485	38.1	2412	158.4

* 2.0μg Fe/31 ml of medium.
** Using the factor, 0.39 μg protein nitrogen per Lf.

Table 2. Extracellular release of total TCA precipitable protein and toxin during the growth of SM-1 strain of C. *diphtheriae* on AMC medium with excess iron.*

Incubation days	Total Growth	Toxin		TCA Precipitable protein	
	mg bacterial nitrogen	Total Lf	μg protein N /mg bacterial N.	Total μg protein N.	μg protein N /mg bacterial N.
0	—	0	—	—	—
1	—	0	—	—	—
2	0.25	0	0	(trace)	(trace)
3	7.18	0	0	244	34.0
4	15.08	0	0	1282	79.6
5	16.35	0	0	1505	92.1
6	16.20	0	0	1911	118.0

* 20.0 μg Fe/31 ml of medium.

Although the growth of the organism in these experiments was good, toxin production was rather low snggesting the medium was not completely deferrated.

From Table 1, it can be seen that toxin can be detected on the 3rd day and the yield is nearly maximal on the 5th day when the growth of the organism had almost ceased. The total TCA precipitable protein in the culture filtrate however, increased continuously even after termination of growth and, on the final day of incubation, it was approximately 15 milligrams. If the amount of the TCA precipitable protein/mg bacterial nitrogen is plotted against incubation time, it is found that there is a rather sharp break in the curve on between the 4th and 5th day when no further increase of toxin was seen. The ratio of toxin to total extracellular protein (Lf/mg protein N) changed considerably during the incubation period and the maximal ratio (705 Lf/mg protein N) was obtained in this experiment on the 5th day when both growth and toxin production had almost stopped.

The effect of the addition of excess iron on the liberation of extracellular protein by the organism is shown in Table 2. It will be seen that the addition of 20 μg iron to 31 ml medium completely inhibits the production of toxin. However, the organism released appreciable amounts of TCA precipitable protein under these condition and the content gradually increased as a function of incubation time. The production curve of the TCA precipitable protein appears to be similar in pattern to that of the protein obtained with iron deficiency, but no break is seen in the former curve. Further, it was found that the quantity of total TCA precipitable protein released by mg nitrogen of the organism under this condition was much less than in an iron deficient medium. Thus, as seen in Table 2, the TCA precipitable protein content of the culture filtrate on the 3rd,

Table 3. Comparison of the decrease in total TCA precipitable extracellular protein with excess iron* and the actual quantities of toxin produced with iron deficiency.**

Incubation days	Toxin μg protein N/mg *** bacterial N.	Decrease in total TCA**** precipitable protein μg protein N/mg bacterial N.
0	—	—
1	—	—
2	0	—
3	6.7	6.9
4	22.9	22.4
5	30.3	46.4
6	38.1	40.4

* 20 μg Fe/31 ml of the medium.
** 2.0 μg Fe/31 ml of the medium.
*** Using the factor, 0.39 μg protein nitrogen per Lf.
**** (Total TCA precipitable protein produced with iron deficiency.) — (Total TCA precipitable protein produced with excess iron.)

4th, 5th and days of incubation were 34, 79.6, 92.1 and 118.0 μg N/mg bacterial N respectively. On the other hand, the corresponding values for TCA precipitable protein released in an iron deficient medium were 40.9, 102.0, 138.5 and 158.4 μg N (see in Table 1). The former values are 20 to 35 per cent less than the latter. In the iron deficiency, the organism also produced 6.7, 22.9, 37.3 and 38.1 μg N toxin on the 3rd, 4th, 5th and 6th day respectively.

Of particular interest is a comparison of these toxin values with the decrement in total TCA precipitable protein obtained by the addition of 20 μg iron to the medium. As can be seen in Table 3, the decrements in total TCA precipitable protain with excess iron were 6.9, 22.4, 46.4 and 40.4 μg N on the 3rd, 4th, 5th and 6th days respectively. Addition of the actual quantity of toxin to the corresponding values of the total TCA precipitable protein in the iron-excess medium resulted in the following values; 40.7, 102.5, 129.4 and 156.1 μg N.

2. *Effect on the toxin and total TCA precipitable protein production by the SM-1 strain of varying quantities of iron*

As described in the preceding section, kinetic studies showed that the TCA precipitable protein released by unit amount of organism in an iron deficient medium was significantly larger than that of the protein obtained when the medium contained sufficient iron to completely inhibit toxin production. The increment in total TCA precipitable protein appeared to be similar to the actual quantities of toxin produced. These results then lead us to think that release of toxin protein might be selectively inhibited by iron. In other words, iron may not exert any influence on production of non-toxin protein. Therefore, the effect of varying amounts of iron on the yield of toxin and total TCA precipitable protein in the culture filtrates was studied.

A typical result was as follows: Sixteen flasks each containing 30 ml of a deferrated complete AMC medium were employed in this experiment. Just before the inoculation of a four-day old pericle, varying amounts of a sterile iron solution in 1 ml were added to the medium in each flask as follows; 0, 0.5, 1.0, 2.0, 3.0, 4.0, 10.0 and 20.0 μg respectively. After four-days incubation* at 32–33°C, the contents of duplicate flasks were thoroughly mixed. The final volumes of the mixed cultures was then measured. The cells obtained by centrifuging each mixture at 4,000 rpm for 30 minutes were washed twice with physiological saline prior to the determination of the bacterial nitrogen. The supernatants were filtered through Toyo No. 50 filter paper and again centrifuged at the same speed for 30 minutes. Toxin and total TCA precipitable protein were estimated in the clear supernatants.

The results are summarized in Table 4. It will be seen that iron affects not only the yield of toxin but of the total TCA precipitable protein produced by the organism. Thus, the amount of the total TCA precipitable protein released

* The incubation period is one of the most important factors for obtaining quantitative results. Thus the culture should be harvested before the termination of the growth. In our experience, when samples were taken after growth had ceased, the quantity of total TCA precipitable protein released with excess iron was frequently much higher than expected.

Table 4. Effect of the addition of varying amounts of iron to the medium on the yield of total TCA precipitable extracellular protein and toxin of SM-1 strain of *C. diphtheriae*.*

Iron added	Growth	Toxin		TCA precipitable protein non-toxin protein***		
μg Fe/31 ml of medium.	mg bacterial N.	Total Lf	μg protein N** /mg bacterial N.	Total μg N.	μg protein N /mg bacterial N.	μg protein N /mg bacterial N.
0	16.30	1620	39.4	1744	106.9	67.5
0.5	17.76	1402	31.4	1844	103.8	72.4
1.0	16.84	1352	31.9	1786	106.0	75.1
2.0	19.01	1188	24.8	1731	91.1	66.3
3.0	18.80	1199	25.3	1747	92.9	67.6
4.0	16.82	598	14.1	1330	79.1	65.0
10.0	16.38	80	2.0	1089	66.4	64.4
20.0	16.87	0	0	1080	63.9	63.9

* Grown on AMC medium at 32–33°C for 4 days.

** Using the factor, 0.39 μg protein nitrogen per Lf.

*** $\left(\begin{matrix}\text{Total TCA precipitable protein} \\ \text{in the culture filtrate}\end{matrix}\right) - \left(\text{toxin}\right) = \text{non-toxin protein}$

per mg bacterial nitrogen decreased gradually from 106.9 to 63.0 μg N with increasing iron concentrations up to 20 μg per 31 ml of the medium. Maximal toxin production was obtained in this experiment in a medium containing no iron. The value was 39.4 μg N/mg bacterial N. Inhibition of toxin production by iron was as expected and no toxin was detectable in the medium containing 20 μg of added iron. To study the quantitative effect of iron on the yields of either toxin or non-toxin protein, the following calculations were made; for every iron concentration, the quantity of toxin (μg N/mg bacterirl N) was subtracted from the corresponding value of total TCA precipitable protein. The difference represents the non-toxin protein released per unit of organism. Thus the differences were 67.5, 72.4, 75.1, 66.3, 67.6, 65.0, 64.4 and 63.9 at the iron concentration of 0, 0.5, 1.0, 2.0, 3.0, 4.0, 10.0 and 20.0 μg/31 ml. The mean value was then 67.8 μg N/mg bact. N. Therefore, with the exception of two values, 72.4 and 75.1 obtained at lower concentrations of iron, the amount of non-toxin protein produced per unit of organism is similar regardless of the iron content of the medium. There may be a slight increase in the value at low iron concentrations. Whether these two high values are, in fact, of significance or were due to some experimental errors is uncertain.

Table 5 shows the result of another calculation. In this case, the amount of non-toxin protein (63.9 μg protein N/mg bact. N) produced at the highest iron concentration was subtracted from those of the total TCA precipitable protein released at various other iron concentrations. When the mean value (67.8 μg N) was employed instead of 63.9, the differences were then (−2.9), (−1.4), 11.3, 25.1, 23.3, 38.2, 36.0 and 39.1 μg N.

As anticipated from previous work, increasing amount of toxin were produced with decreasing concentrations of iron. Thus the toxin contents were 0.0, 2.0, 14.1,

Table 5. Increase in total TCA precipitable extracellular protein and actual toxin produced with decrease in iron content.

Iron added.	Increment in total TCA precipitable protein*		Toxin
μg Fe/31 ml of medium.	μg protein N/mg bacterial N.		μg protein N** /mg bacterial N.
	I ***	II ****	
0	43.0	39.1	39.4
0.5	39.9	36.0	31.4
1.0	42.1	38.2	31.9
2.0	27.2	23.3	24.8
3.0	29.0	25.1	25.3
4.0	10.0	11.3	14.1
10.0	2.5	(−1.4)	2.0
20.0	0.0	(−2.9)	0.0

* (Total TCA precipitable protein at various iron concentrations.) − (Total TCA precipitable protein produced with 20 μg Fe/31 ml of medium.)

** Using the factor, 0.39 μg protein nitrogen per Lf.

*** Calculated on the basis of actual amount (63.9 μg N/mg bacterial N) of non-toxin protein.

**** Calculated on the basis of the mean amount (67.8 μg N/mg bacterial N) of non-toxin protein.

25.3, 24.8, 31.9, 31.4 and 39.4 μg N/mg bacterial N at the iron concentration of 20.0, 10.0, 4.0, 3.0, 2.0, 1.0, 0.5 and 0.0 μg per 31 ml of the medium respectively. Therefore, when the quantity of extracellular protein/mg bacterial N is plotted against the iron concentration, the slope of toxin curve coincides to some extent with that of the difference in protein contents described above.

Finally, we must note here that the effect of iron on bacterial growth was somewhat unexpected. As is seen in Table 4, the growth at more than 4.0 μg iron was considerably less than that at 2.0 or 3.0 μg iron. The reason for this decrease is not clear.

DISCUSSION

From the results described in the preceding sections, it is concluded that 1) The pattern of the extracellular release of non-toxin protein differs greatly from that of toxin by SM-1 strain of *Corynebacterium diphtheriae*. Particularly, the release of the former continues even after toxin has stopped. 2) Provided calculations were made on the basis of the converting factor, 0.39 μg protein N/Lf, the organism seems to release into the medium nearly constant amounts of non-toxin protein regardless of the concentration of iron in the medium. 3) Toxin protein appears to comprise the major part of the extracellular protein whose production can be completely inhibited by excess iron.

As mentioned previously, Brandwijk *et. al.* (1951a, 1951b) reported a change

in ratio of toxin to total extracellular protein during toxin production of diphtheria bacilli. The result of the present kinetic studies on the extracellular liberation of protein by SM-1 strain in a synthetic medium was in line with their finding, and indicates further that production of toxin differs from that of the other extracellular protein. The latter can be released in the presence of enough iron to completely inhibit the toxin production, and it virtually represents the non-toxin protein released by the diphtheria bacilli.

From Tables 1 and 2, it can be calculated that the non-toxin protein is released linearly with time and continues even after cessation of toxin production. Comparison of the production curves of non-toxin protein and total TCA precipitable protein including toxin obtained in the absence of the excess iron, shows that the slope of the latter is considerably steeper than that of the former. It also appears possible to calculate the latter curve from the curves for toxin and non-toxin protein. As Table 3 shows, the decrements in total TCA precipitable protein due to excess iron are similar to the actual quantities of toxin produced. It seems therefore that the rate of the production of non-toxin protein is not influenced only by the presence of a concentration of iron sufficient to completely inhibit toxin production and, accordingly, the rate of toxin production can alter the shape of the production curve of the total extracellular protein in iron deficiency.

The continuous increase of non-toxin protein even after the termination of bacterial growth suggests autolysis of the cells. Thus we have sometimes observed that incubation periods of over 6 days always result in a considerable decrease in bacterial growth with a concomitant increase of total TCA precipitable protein in the culture filtrate. However, from preliminary experiments, insignificant nucleic acid is detectable in the TCA precipitates when the culture was harvested before the cessation of growth. This shows that the autolysis or phage lysis could play only a minor part tn the extracellular release of protein during the active growth of the organism (Yoneda and Pappenheimer, 1957). More evidence is, however, required to settle this point.

Of particular interest is the quantitative effect of iron on the extracellular release of TCA precipitable protein by the diphtheria bacilli. As the result in Table 4 shows, the higher the concentration of iron in the medium, the smaller the amount of the TCA precipitable protein liberated by the organism. If the amount of protein released per mg of bacterial nitrogen is plotted against the iron concentration, there is good agreement between the curves for toxin and the total TCA precipitable protein. What does this imply ? Since toxin comprises a part of the total TCA precipitable protein, the good agreement between the curves indicates, on one hand, that the slope of the latter curve is largely due to toxin production. On the other hand, it suggests that the extracellular protein other than toxin, produced per unit of organism, is constant regardless of the iron concentration of the medium. Calculations show that this could be so. As can be seen in Table 5, although two values at the low level of iron were found to be rather high, the other six values for the amount of non-toxin protein were between 53.9 and 67.6 μg N/mg bacterial N. Therefore, the yield of non-toxin protein seems to be unaffected by iron and, if iron has an effect, it must be very minor. From kinetic

studies, the rate of non-toxin production seems unchanged by the iron in the medium, and it seems that iron does not exert any influence or, at most only a a slight influence on the extracellular release of non-toxin protein. This is a sharp contrast to the effect on toxin where release is completely inhibited by excess iron. Supposing that iron has no influence on the non-toxin protein production, the increment in total extracellular protein obtained with decreasing iron contents must be mostly due to toxin protein. The result in Table 5 shows that this may be the case. From the result, it can be calculated that toxin comprises at least more than 75 per cent of the increment in total TCA precipitable protein. Assuming a mean value (67.8 μg N/mg bacterial N) of non-toxin protein, more then 85 per cent of the increment become due to by toxin protein. However, it must be emphasized that, in these calculations, the factor used for converting flocculating units of toxin to micrograms of protein nitrogen is of great significance. In other words, the calculated percentage of toxin in the increment entirely depends on the factor. Accordingly when one uses 0.46 μg N/Lf (Pappenheimer, 1937) as the factor, the values for the toxin content are 0.0, 2.3, 16.6, 29.8, 29.2, 37.6, 37.0 and 46.5, and thus those of non-toxin protein are 63.9, 64.1, 62.5, 63.1, 61.9, 68.4, 66.8 and 60.4 μg N/mg bacterial N respectively, from which one may conclude that iron does not exert any influence on the yield of non-toxin protein while toxin protein comprises the total increment in total TCA precipitable ptotein obtained with decreasing iron contents. On the contrary, on the basis of a factor, 0.30 μg N/Lf (Pope *et. al.*, 1953; Katsura *et. al.*, 1957), the values for the toxin content become 0.0, 1.5, 10.8, 19.5, 19.1, 24.5, 24.1 and 30.3 μg N/mg bacterial N. In this case, the toxin content in the increment in total TCA precipitable protein comprises at least 58 per cent. It is possible to suppose that the non-toxin protein which comprises less than 42 per cent of the increment may be denatured toxin which loses its activity to combine with antitoxin. Recently crystalline diphtheria toxin protein has been obtained by Pope *et. al.*, (1953, 1958); Katsura *et. al.* (1957), Relyveld (1959) and one of the autors (Yoneda, 1958, unpublished) and the specific activity (Lf/mg protein nitrogen) is found to be around 3300–3400. Assuming that the crystalline protein represents the purest toxin and that the toxin is the sole component in culture filtrates which is detectable by flocculation, it is reasonable to employ the factor, 0.30 μg N/Lf for converting the floccuation units to μg of protein nitrogen. Unfortunately, however, it can not be assumed with certainty that the flocculation unit of a crude culture filtrates must represent the absolute weight of the toxin with a specific activity of 3300 Lf/mg protein N. In other words, there could be another toxin protein with lower specific activity than 3300 Lf, so that the flocculation unit of a crude culture filtrate might represent that of these toxin mixture. True answers may be given by further immunochemical analyses of extracellular protein components in the diphtheria culture filtrates.

Very recently Clark (1958) has reported that toxin production of *Corynebacterium diphtheriae* can be inhibited by the addition of cobalt ion to the medium its effect of iron. Although his data indicate that bacterial growth is also crucially influenced by cobalt ion* resulting in the reduction of toxin production, it may

still be of interest to see if this metal has any effect on the yield of non-toxin protein by the organism.

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* The result of our recent investigation suggests that cobalt reduces the growth of *C. diphtheriae,* and this eventually results in a great decrease of the yields of toxin.