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Studies on the Iron-Binding Site of Diphtheria Bacilli
1. Quantitative Binding of Iron by Iron-Deficient Cells of a Toxinogenic Strain of *Corynebacterium diphtheriae*[**]

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SUMMARY

Iron-binding by washed iron-deficient cells of a toxinogenic strain of *C. diphtheriae* was studied quantitatively and kinetically and the effects of pre-treatment of the bacilli by heating, of the hydrogen ion concentration in the reaction medium and of the incubation temperature were observed. Kinetic observations showed that iron-binding proceeded very rapidly and the rate was highest during the first ten minutes. Quantitative relationships were obtained between the amount of iron taken up by an unit of bacilli and the concentration of either the bacilli or of the external iron. The binding capacity was found to be rather heat stable but it was markedly affected by the hydrogen ion concentration of the medium. The optimal pH range for iron binding was between about 6.6 and 7.6. However, when maleate buffer was replaced by citrate buffer, only a very small amount of the external iron was taken by the bacilli even near the optimal pH. Iron-binding to the organism took place even at 2°C and appeared to be quite firm. Thus once iron was bound to the cells, no ordinary buffers at near neutral pH were unable to extract more than 5 per cent. Although about 40 to 50 per cent of the cell-bound iron was extractable with 10 per cent aqueous solutions of some organic acids such as malic, citric and tartaric acid, almost complete extraction of the iron could be obtained using 10 per cent ascorbic acid (pH 2.3) or even better using 10 per cent sodium hydrosulfite in M/40 maleate buffer at a final pH of 6.6.

INTRODUCTION

Since the report of Pappenheimer and Johnson (1936), the inhibitory effect of iron on diphtheria toxin production has been studied extensively by many workers and especially quantitatively by Pappenheimer and his associates (1947). Although few workers (Norin, 1943; Yoneda, 1957, 1958; Clark, 1958) observed iron-binding by diphtheria bacilli, much attention has been directed towards the quantitative relation between the yield of toxin and the exogenous iron in the medium. Such a relation is possible provided all the added iron is invariably and quantitatively associated with the extracellular liberation of the toxin. However, the relation of iron to the extracellular release of toxin by diphtheria bacilli appears not to be so simple. During growth, the organism seemed to take up only about half the iron added to the medium (Yoneda, 1956; Clark,

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1958). Furthermore, during investigations on the mode of action of iron on the extracellular liberation of toxin by a toxinogenic strain of *C. diphtheriae*, one of the authors (Yoneda, 1957) found that the rate of toxin production in an iron-free synthetic medium by a heavy inoculum of washed cells was entirely dependent upon the iron content of the medium used for harvesting the inoculum. Moreover, it was demonstrated that the yield of toxin in an iron-free medium decreased with increasing concentrations of the ferric citrate solution with which the organism had only been in contact for a very short time before washing with iron-free medium and inoculation. These observations suggest that the iron in the medium may be taken up very rapidly by iron-deficient diphtheria cells and only this cell-bound iron acts as a rate-limiting factor in the extracellular liberation of toxin. Therefore, it was necessary to re-examine the quantitative iron-toxin relation. Before doing this, however, a basic and quantitative knowledge of the iron-binding capacity of diphtheria bacilli was necessary. This was studied quantitatively and kinetically.

This paper describes results of experiments on the binding of iron by iron-deficient cells of a toxinogenic strain of *Corynebacterium diphtheriae* and on the release of cell-bound iron by several organic acids and by sodium hydrosulfite.

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 this study. The organism was subcultured on the synthetic AMC medium described previously (Yoneda, 1950, 1951, 1957),

2. *Preparation of iron-deficient diphtheria bacilli*

A small volume (about 3 ml) of a 20 hour old shaking culture of SM–1 strain in a test tube was inoculated into 100 ml aliquots of AMC medium containing 25 μg of $FeSO_4 \cdot 7H_2O$ in one liter Erlenmeyer flasks. The culture was then incubated at 34–35°C for 18 hours, shaking at 120 strokes per minute at an amplitude of 10 cm. After incubation, the organisms were recovered by centrifugation, washed twice with iron-free AMC medium by centrifugation at room temperature and then resuspended into 50 ml of a fresh iron-free AMC medium dispensed in a 500 ml Elrenmayer flaks. The optical density of the suspension at 590 mμ in a Coleman type spectrophotometer was usually 4–5.0. The suspension was then shaken at 34–35°C at the same stoke rate and amplitude as before for a further 15 hours. At the end of incubation, the optical density of the culture was usually 10.0 to 12.0 and the toxin content of the culture supernatant was 60 to 90 Lf/ml. The organism were recovered by centrifugation, washed three times with distilled water by centrifugation and then suspensed in M/40 of maleate buffer (pH 6.8). The nitrogen content of this iron-deficient cell suspension was determined by the micro-Kjeldahl-Nessler method as modified by Yokoi and Akashi (1955).

3. *Assay methods*

Unless otherwise specified, the iron-binding capacity of the iron-deficient diphtheria bacilli was measured by the following procedure; a cell suspension containing 200–240 μg. becterial nitrogen per ml was dispensed in 5 ml aliquots in test tubes and a equal volume of a freshly prepared ferric citrate solution-(about 4–5 μg. Fe/ml of distilled water) was quickly added to each. The iron-cell suspension was then incubated at 37°C for 120 minutes to complete the iron binding by the cells. As a control, the ferric citrate solution was diluted twice with M/40 of maleate buffer (pH 6.8) and incubated at 37°C for 120 minutes. After incubation, both the iron-cell suspension and the control iron solution were centrifuged at 4,000 rpm for 30 minutes in the cold, and then the iron content of the clear supernatants and of the precipitated cells was determined.

Quantitative determination of iron in the supernatant was performed as follows; a 6.5 ml aliquot was mixed with 0.7 ml of 10 per cent ascorbic acid solution and 0.8 ml of 0.1 per cent *o*-phenanthroline solution. After complete colour development by incubating the mixture at 40°C for 60 minutes, the colour intensity was measured at 510 mμ in a Coleman type spectrophotometer. The molar extinction coefficient was found to be $8.4 \times 10^6 \times \mathrm{Mol}^{-1}$ and thus the optical density of the colour was converted to μg. of iron by using the factor, 6.55 μg Fe/O.D.

The iron content of the precipitated cells was determined either by direct analysis or by using the figures calculated by subtracting the amount of iron in each test supernatant from that in the control. However, since the calculated figures were always in good agreement with those obtained by the direct iron analysis, they were usually regarded as the quantities of the iron taken up by the cells. Cell-bound iron was analyzed directly as follows; the cells were first digested completely by heating in a small amount of aqueous sulfuric acid (50 per cent) with three drops of concentrated hydrogen peroxide (30 per cent). The digested material was then neutralized with saturated NaOH in the cold and the excess ammonia removed by heating over a small flame. After cooling, 1.4 ml of 5 per cent L-ascorbic acid solution and 0.8 ml of 0.1 per cent *o*-phenanthroline solution were added to the almost dried residue thus obtained. The final volume was then made up to 8.0 ml with distilled water. The mixture was incubated at 40°C for 60 minutes and the colour was measured as described above. The iron content of the same amount of the reagents used for digestion was also determined.

4. *Extraction of iron*

Quantitative extraction of iron from iron-bound cells was performed by the following method; the cells having bound iron after removal from the iron solution were washed twice with M/40 of maleate buffer (pH 6.8), centrifuged at 4,000 rpm for 30 minutes and then resuspended in 10 ml of the same buffer containing 10 per cent sodium hydrosulfite. Cell-bound iron was extracted by incubating the suspension at 37°G for 30 minutes. The iron content of the clear supernatant, obtained by centrifugation at 4,000 rpm for 60 minutes, was determined by the *o*-phenanthroline method described above.

RESULTS

Relation of the iron-binding by iron-defficient diphtheria bacilli to the concentration of iron in the medium.

A suspension of iron deficient diphtheria bacilli in M/40 of maleate buffer was prepared according to the method described in the preceding section. The concentration of the bacterial suspension was 226.8 μg bacterial N/ml. 5 ml of the suspension was quickly added to equal volume of solutions containing the following varying amounts of ferric citrate; 0.0, 0.493, 0.718, 0.858, 1.350, 1.764, 2.790, 3.670 and 4.220 μg Fe/ml. After incubation at 37°C for 120 minutes, the iron-cell mixtures were cooled and centrifuged at 4,000 rpm for 30 minutes, and iron contents of the clear supernatants were determined. Ferric citrate solution in M/80 maleate buffer (pH 6.8) containing corresponding amounts of iron to the test iron-cell suspensions was treated similarly.

Table 1. Relation of the iron-binding capacity of iron-deficient diphtheria cells to the concentration of iron

Iron conc. in medium. (μg/ml)	Iron bound to cells. (μg Fe/mg Bact. N)	Ratio of cell-bound iron/external iron	f *
0.493	2.04	0.47	(2.13)
0.718	2.87	0.45	(2.20)
0.853	4.43	0.58	1.70
1.350	6.82	0.57	1.74
1.764	8.41	0.54	1.84
2.790	12.50	0.45	1.97
3.670	14.30	0.39	2.26
4.220	13.80	0.33	2.69

* f was calculated according to the equation (3).

$f=m/x.y$, where m is μg Fe/ml (Iron concentration used for contact),

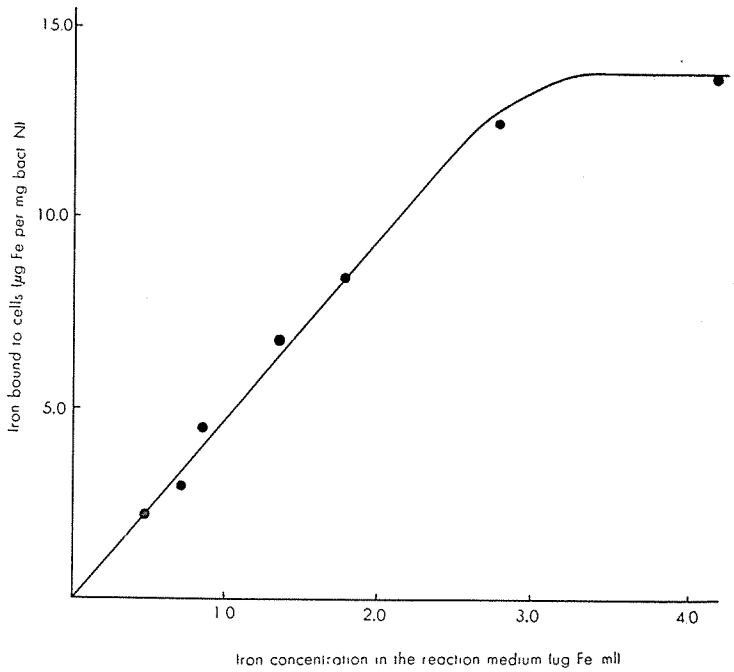
x, μg Fe/mg bacterial N,

y, μg bacterial N/ml.

Bacterial concentration, 113.4 μg N/ml; Incubation at 37°C for 120 minutes.

The result is shown in Table 1. The more iron present in the surrounding medium, the more iron was taken up by the organism. Iron-binding reached a plateau when the concentration of the external iron became more than 3 μg/ml. The maximal amount of iron taken up by mg bacterial nitrogen was approximately 14 micrograms. The ratio of cell-bound iron per external iron was 0.47, 0.45, 0.58, 0.57, 0.54, 0.45, 0.39 and 0.33 with increasing iron concentrations in the contact medium. Therefore, between 0.718 and 1.764 μg external Fe/ml, the mean value of the ratio was 0.56 indicating that diphtheria bacilli bind about 50 to 60 per cent of the iron in contact with them. Figure 1 illustrates the quantitative relation of the iron-binding to the concentration of the external iron used for contact.

Fig. 1. Relation of the iron-binding capacity of iron-deficient diphtheria cells to the concentration of iron



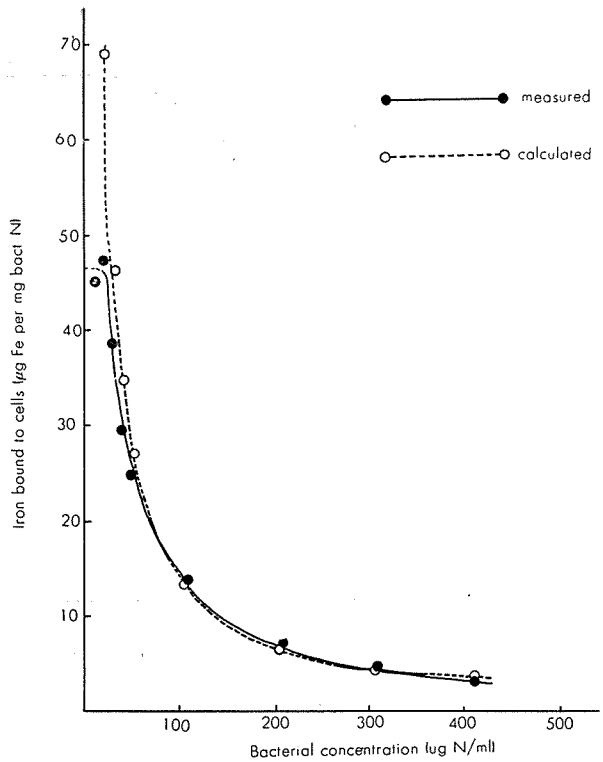
Bacterial concentration used, 113.4 µg N/ml; Incubation at 37°C for 120 minutes

Iron appears to be taken up linearly by unit weight of organisms until the external iron concentration reaches nearly 3 µg Fe/ml and a plateau appaers to be formed with higher concentrations of iron. It must be noted here however, that the first two values, 2.04 and 2.87 µg Fe/mg bacterial N, might be inaccurate since concentrations below these points are somewhat too low for the iron-determination method used in this study. Thus precise information on iron-binding at very low iron concentrations must await analysis with radio-active iron.

Quantitative relation between the bacterial concentration and iron-binding

Varying amounts of diphtheria bacilli were brought into contact with a constant amount of iron. Thus a bacterial suspension containing 816.4 µg of bacterial N/ml was first diluted appropriately with M/40 maleate buffer (pH 6.8) to the following concentrations; 408.2, 306.2, 204.1, 102.1, 51.0, 40.8, 30.6, 20.4 and 10.2 µg N/ml. 5 ml aliquots of these suspensions were quickly added to an equal volume of ferric citrate solution (5 µg Fe/ml). The cell-iron mixtures were then incubated at 37°C for 120 minutes and the iron determined in the clear supernatants obtained by centrifugation at 4,000 rpm for 30 minutes.

Fig. 2. Relation of the iron-binding capacity of iron-deficient diphtheria cells to the bacteral concentration



Iron concentration, 2.5 μg Fe/ml; incubation at 37°C for120 minutes

The results are summarized in Figure 2. The amount of iron taken up per mg of bacterial nitrogen is plotted against the bacterial concentration used for the contact. The iron-binding capacity of the organism decreases with increasing bacterial concentrations and the rate of decrease was found to be particularly rapid when the bacterial concentration was below about 100 μg N/ml. Maximal iron-binding was observed when less than 20.4 μg N/ml of the organism was in contact with 2.5 μg Fe/ml of the ferric citrate solution and the binding value was approximately 46 micrograms Fe/mg bacterial N. The ratios of cell-bound iron/external iron in this experiment were 0.18, 0.38, 0.47, 0.48, 0.51, 0.56, 0.59, 0.57 and 0.54 at bacterial concentrations of 10.2, 20.4, 30.6, 40.8, 51.0, 102.1, 204.2, 306.2 and 408.2 μg N/ml respectively.

The curve in rhis figure expresses the change in iron-binding capacity with change in the bacterial concentration and should be hyperbolic. Thus, actual total amounts (T) of cell-bound iron can be calculated by the following equation;

$$T = x \cdot y, \qquad (1)$$

where x represents the μg iron taken up per mg bacterial N and y, the mg bacterial nitrogen in the total cells used for the contact. Since only a part of

the external iron used for contact is taken up by the organism,

$$T=\frac{m}{f} \qquad (2)$$

where m represents the total iron (μg) used for contact with the cells and f, a factor concerning the iron-binding capacity of the organisms. Therefore, from (1) and (2), one can depict the following equation;

$$x=\frac{m}{f\cdot y} \qquad (3)$$

which should express the curve in this figure and represent the overall iron-binding capacity of the organism in relation to the bacterial concentration used.

The value, f, calculated from the actual amounts of x, y and m in this experiment according to equation (3) was 5.41, 2.57, 2.11, 2.06, 1.96, 1.78, 1.68, 1.77 and 1.85 at bacterial concentrations of 10.2, 20.4, 30.6, 40.8, 51.0, 102.1, 204.2, 306.2 and 408.2 μg N/ml respectively. It will be seen that, when 2.5 μg Fe/ml of iron was in contact with the organism at a concentration of more than 100 μg N/ml, these values are nearly constant regardless of the bacterial concentration and the mean value is 1.77. Using this value, the amount of cell-bound iron (μg Fe/mg bacterial N) was recalculated and compared with that of the iron actually measured. As is seen in Table 2 and Figure 2, calculated and measured

Table 2. Relation of the iron-binding capacity of iron-deficient diphtheria cells to the bacterial concentration. Comparison of the measured amount of the iron bound to the cells with that calculated from the mean value for f.

Bacterial conc. (mg N/ml)	Iron bound to cells (μg Fe/mg bact. N)	
	measured	calculated * **
0.4082	3.30	3.45
0.3062	4.63	4.63
0.2041	7.26	6.92
0.1021	13.82	13.81
0.0510	24.90	27.77
0.0408	29.66	34.72
0.0306	38.56	46.29
0.0204	47.55	69.44
0.0102	45.09	138.88

* Assuming that f is 1.77.

** Calculated from equation (3).

Iron concentration, 2.5 μg Fe/ml; Incubation at 37°C for 120 minutes.

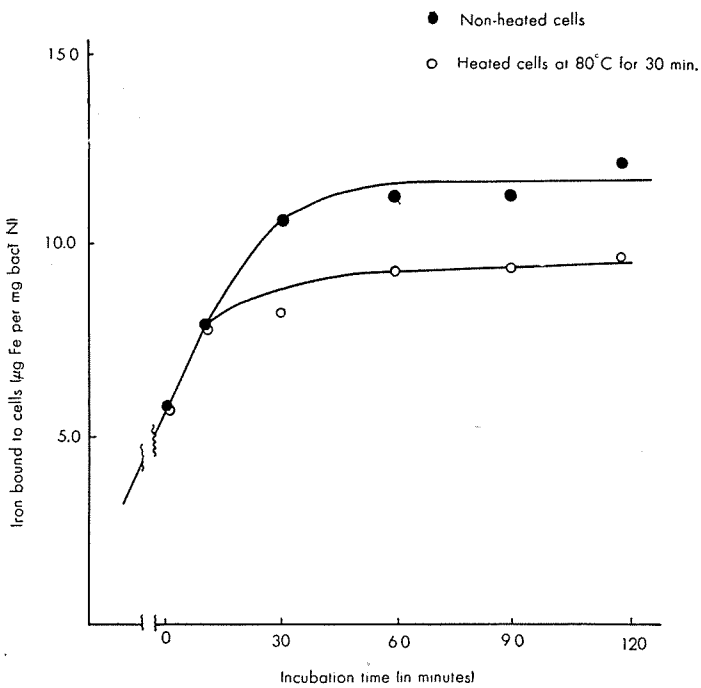
values agreed well at bacterial concentrations of more than 100 μg N/ml. However the exact value of f must be determined from further similar experiments.

Kinetic observations on iron-binding by iron-deficient diphtheria bacilli

140 ml of a bacterial suspension containing 230 μg N/ml in M/40 maleate

buffer (H 6.8) was divided pinto two parts. One part was heated at 80°C for 30 minutes. Both the heated and non-heated suspensions were then mixed with 70 ml of ferric citrate solution (4.86 μg Fe/ml) and incubated at 37°C. A 10 ml aliquot was taken from each at various intervals and it was immediately centrifuged at 4,000 rpm for 30 minutes. Iron was determined in the clear supernatants thus obtained.

Fig. 3. Relation of incubation time to the iron binding capacity of iron-deficient diphtheria cells



Bacterial concentration, 115 μg N/ml; Iron concentration, 2.43 μg Fe/ml; Incubation at 37°C

The results are shown in Figure 3. Although the cell-iron mixture was centrifuged as quick as possible after the incubation, it took several minutes before most cells were precipitated, so that the organism was still in contact with iron during a part of the centrifugation period. Therefore, in this figure, the amount of iron taken up per mg bacterial nitrogen is plotted arbitrarily against the incubation time at 37°C. As is seen from the figure, the organism bound iron very rapidly and approximately 47 per cent and 87 per cent of the total bound iron was taken up at the arbitrary zero-time and after 30 minutes incubation respectively. On further incubation, iron-binding proceeded at a very slow rate and, even after 120 minutes incubation, the organism gained less than 15 per cent of the iron already taken up. A similar pattern for iron-binding was obtained with organism heated at 80°C for 30 minutes. In this case, how-

ever, the amount of iron bound to the cells after 120 minutes incubation was 9.62 μg Fe/mg bacterial N : a value about 23 per cent lower than that of iron (12.17 μg Fe/mg bacterial N) bound to the non-heat treated organism. It is of interest that the amount of iron teken up at the arbitrary zero-time both by heated and non-heated cells was identical. It is thus apparent that heat-treatment affects iron-binding by the organism only during incubation at 37°C. It may also be possible that there are two different kinds of iron binding sites, that is, heat stable and heat labile sites.

Effect of heat-treatment on iron-binding capacity of the cells

In the preceding experiment it was shown that the intial rate of iron-binding by iron-deficient diphtheria bacilli was unaltered, but the iron-binding capacity was reduced consideraby by the heat treatment at 80°C for 30 mintes. The change in iron-binding capacity were therefore furthere studied using different temperatures for the heat-treatment.

50 ml of the bacterial suspension containing 226 μg N/ml was divided into five parts and 10 ml aliquots of suspension were heated at 37, 55, 70, 80 and 100 °C for 30 minutes. After cooling, each suspension was quickly mixed with an equal volume of 4.86 μg Fe/ml ferric citrate solution and the cell-iron mixtures incubated at 37°C for 120 minutes.

Table 3. Effect of the heat-treatment of cells on the iron binding capacity.

Temperature of heat-treatment (C)	Iron bound to cells (μg Fe/mg bamt. N)	Reduction in the iron-binding capacity. (%)	f *
37	12.26	0	1.75
55	10.97	11	1.95
70	10.38	15	2.07
80	9.73	21	2.21
100	9.57	22	2.23

* Calculated from equation (3)

Bacterial concentration, 113.0 μg N/ml; Heat-treatment for 30 minutes; 2.43 μg Fe/ml; Incubation at 37°C for 120 minutes.

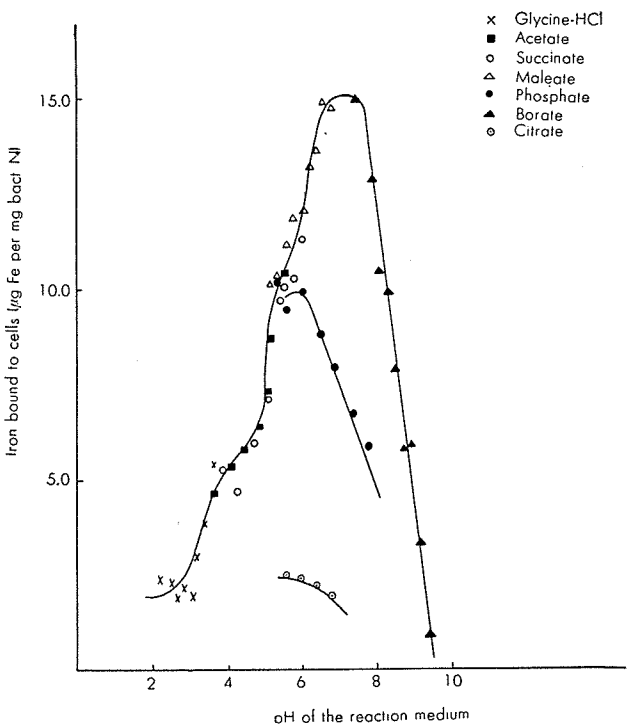
Table 3 illustrates the effect of heat-treatment of the organisms at various temperature on the iron-binding capacity. The binding capacity was apparently somewhat affected by the type of heat-treatment and decreased almost linearly with increased temperature to 80°C. The reduction, however, was not great and the amount of iron taken up by 100°C–heat treated cells was only about 22 per cent lower than that taken up by 37°C–treated cells.

Effect of the hydrogen ion concentration on the iron-binding capacity

Using six different buffers, the effect of the hydrogen ion concentration of the medium used for iron-cell contact was observed between pH 2.3 and 9.2. The buffers used in this experiment were glycine–HCl (pH 2.2–3.6), acetic acid–

Na acetate (pH 3.6–5.6), succinic acid–NaOH (pH 3.8–6.0), citric acid–Na citrate (pH 5.4–6.4), maleic acid–NaOH (pH 5.2–6.8), Na_2HPO_4–KH_2PO_4 (pH 5.6–8.0) and borax–HCl (pH 7.6–9.2). All buffers used were M/20. 2.5 ml of bacterial suspension containing 550 μg N/ml of distilled water was made up to 5 ml with the buffers mentioned above and then mixed with 5 ml of ferric citrate solution containing 6.0 μg Fe/ml.

Fig. 4. Effect of the hydrogen ion concentration in the reaction medium on the iron binding capacity of iron-deficient diphtheria cells



Bacterial concentration. 110 μg N/ml; Iron concentration 3.0 μg Fe/ml; Incubation at 37°C for 120 minutes

The result is shown in Figure 4. Iron-binding by the organism is dependent upon the hydrogen ion concentration in the reaction medium. The optimal pH range was rather narrow (pH 6.6 to 7.6). Little or no iron binding was seen when the pH was below 3.0 or above 9.0. A small but rather sharp peak was seen at pH 5.4 and 3.8. It is as yet uncertain whether or not these latter are of significance. The optimal pH for the iron binding appeared to shift to around 6.0 in phosphate buffer and the iron-binding capacity decreased markedly on decreasing the hydrogen ion concentration of the buffer. Of particular interest is the finding that very little external iron was taken up by organisms even near the optimal pH when maleate was replaced by citrate buffer.

Table 4. Comparison of the iron binding capacities of iron-deficient diphtheria cells in maleate and citrate buffers.

pH of the reaction medium	Iron bound to cells (μg Fe/mg bact. N)	
	M/80 maleate buffer	M/80 citrate buffer
5.4	15.15	2.76
5.8	15.15	2.76
6.2	15.82	2.56
6.6	16.92	2.20

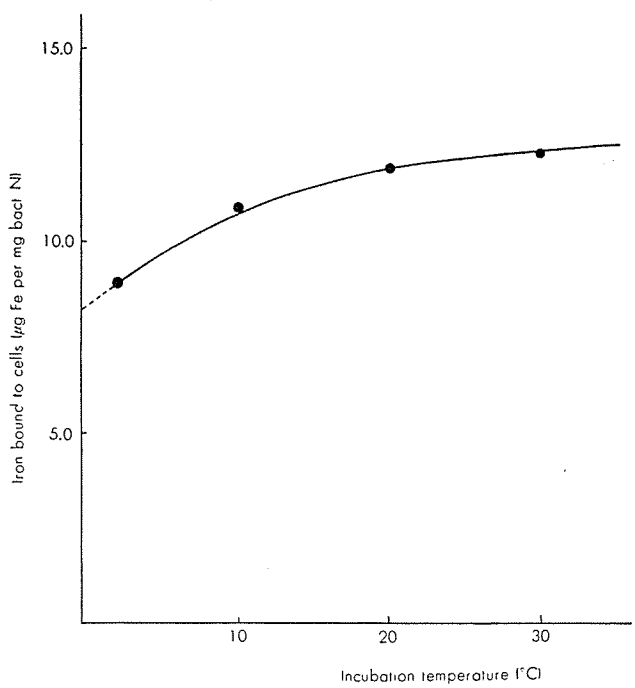
3.0 μg Fe/ml; Bacterial concenfrttion, 100 μg N/ml; Incubation at 37°C for 120 minutes.

As is seen from Table 4, in citrate buffer at pH 6.6, the organisms were found to bind only about 13 per cent of the iron taken up in maleate buffer at the same pH.

The effect of the temperature during the incubation of the iron-cell mixture

20 ml of bacterial suspension containing 220 μg N/ml of M/40 maleate buffer

Fig. 5. Effect of incubation temperature on the iron-binding capacity of iron-deficient diphtheria cells



Bacterial concentration, 110 μg N/ml; Iron concentration, 2.43 μg Fe/ml; Incubation for 120 minutes

(pH 6.8) was divided into five parts and 10 ml aliquots of the suspension were incubated at 2, 10, 20 and 37°C. After equilibration, each suspension was mixed with an equal volume of 4.86 μg Fe/ml ferric citrate solution at the corresponding temperatures. These cell-iron mixtures were then incubated at the same temperatures for 120 minutes.

Figure 5 shows the effect of the incubation temperature on the iron-binding capacity of the organism. Iron-binding decreases slightly at lower incubation temperatures but the decrease is not great since, at 2, 10 and 20°C, the organism can bind about 72, 88 and 96 per cent of the iron which was bound to the cells at 37°C.

Extraction of cell-bound iron by various organic acids and sodium hydrosulfite

60 ml of bacterial suspension containing 230 μg N/ml of M/40 maleate buffer (pH 6.8) was added to an equal volume of 6.0 μg Fe/ml ferric citrate solution and the mixture was divided into six parts. 20 ml aliquots of the iron-cell suspension were then incubated at 37°C for 120 minutes. After incubation, each suspension was centrifuged at 4,000 rpm for 30 minutes and iron was determined in the supernatant. The precipitated cells were washed three times with M/80 maleate buffer (pH 6.8) and centrifuged at the same speed. The organism at this stage was found to bind 15.21 μg Fe/mg bacterial nitrogen. The washed organisms were resuspended in 10 ml of 10 per cent *L*-ascorbic acid solution or in 10 per cent sodium hydrosulfite in M/40 maleate buffer (pH 6.8). In each case triplicate samples were employed. These suspensions were then incubated at 37°C for 30 minutes and the iron released was determined colorimetrically in the supernatants obtained by centrifugation at 4,000 rpm for 30 minutes. The mean iron content of each group was 11.38 μg Fe/ml in the ascorbic acid solutions and 17.50 μg Fe/ml in the hydrosulfite solutions. Therefore, extraction of cell-bound iron was complete with sodium hydrosulfite, while only 65 per cent of the iron was extracted with ascorbic acid solution. However, when the extraction was repeated twice with the latter solution, 96 per cent of the iron was removed from the cells. For complete extraction, the temperature during extraction seems to be important. Thus, when cells were extracted at 2°C instead of at 37°C, the efficiency was very low and 24 and 57 per cent of the cell-bound iron were extractable with the ascorbic acid and hydrosulfite solutions respectively.

Using the same batch of iron-bound cells (15.21 μg Fe/mg bacterial N) and a similar procedure, the efficiency of several other organic acids and various buffers for extraction of iron from the cells was studied. Extractions were performed at 37°C for 30 minutes. The results are summarized in Table 5.

Almost no iron was extractable with buffers such as maleate, phosphate and borate at above pH 6.0. On the other hand, appreciable iron was extractable with succinate, acetate and glycine–HCl buffers and the efficiency appeared to increase with increasing hydrogen ion concentration. Iron was also extractable with 10 per cent aqueous solutions of several organic acids and the efficiency was found to increase in order in acetic, succinic, malic, citric and tartaric acids. No significant difference was found at different pHs of these solutions.

Table 5. Extraction of cell-bound iron in solutions of organic acids, sodium hydrosulfite and buffers of varying pH.

Solvents used for extraction		pH	Cell-bound iron (μg Fe/ml)		Efficiency (%)
			extracted	non-extracted	
0.9% saline *		6.8	< 1	> 16.5	—
buffers used (M/40) *	borate	9.0	< 1	> 16.5	—
		8.5	< 1	> 16.5	—
		8.0	< 1	> 16.5	—
	phosphate	7.5	< 1	> 16.5	—
		7.0	< 1	> 16.5	—
	maleate	6.5	< 1	> 16.5	—
		6.8	< 1	> 16.5	—
	succinate	5.5	2	15.5	11.4
		5.0	2.5	15.0	14.2
	acetate	4.5	3.8	13.7	11.3
		4.0	4.5	13.0	25.7
	glycine-HCl	3.5	5.5	12.0	31.4
		3.0	5.7	11.8	32.5
		2.5	6.7	10.8	38.2
10% organic acid	acetic	2.43	5.06	12.44	28.9
	succinic	2.85	4.80	12.70	27.2
	malic	2.36	7.39	10.11	42.2
	citric	2.52	7.80	9.70	44.5
	tartaric	2.45	9.76	7.74	55.7
	ascorbic	2.30	12.52	4.98	71.5
10% sodium hydrosulfite in M/80 maleate buffer.		6.6	17.50	0.0	100.0
No extraction		—	—	17.50	—

Extraction at 37°C for 30 minutes.

* Extraction at 37°C for 30minutes was repeated twice

DISCUSSION

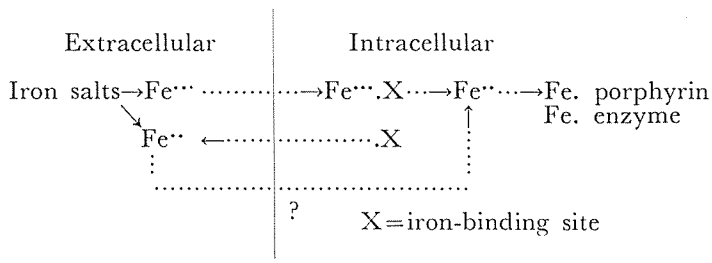
If one were to assume the existence of specific sites for binding iron atoms in the cell of this diphtheria organism, these would be million. It was demonstrated in the present study that organisms could bind as much as 46 micrograms of iron per milligram of bacterial nitrogen. Thus, assuming that one mg bacterial N is equivalent to 3.2×10^{10} cells (Barksdale and Pappenheimer, 1954), it will be clear that one diphtheria cell could bind about 1.6×10^{7} atoms of iron, that is about a hundred times the amount of iron necessary to inhibit toxin production.

Therefore this organisms has a tremendous capacity to concentrate iron in or onto the cell from the surrounding medium. Assimilation or uptake of iron by various other species has been reported previously by Waring and Werkman (I943) and Pappenheimer and Shaskan (1944) in growing cultures. The characteristic feature of the binding of iron by diphtheria bacilli appears to be that the cells bind only a part of the external iron with which they were in contact. As mentioned previously, the iron-binding capacity (x μg Fe/mg bacterial N), of a given organism (y mg bacterial N) in the medium containing a given amount of iron (m μg) may be expressed by the equation; $x=m/f \cdot y$, where f is a factor expressing the change in the iron binding capacity. Therefore, from this equation, the value of f is one provided that all the iron in the medium is taken up by the organism. This may be so with *Clostridium welchii* reported by Pappenheimer aud Shaskan (1944). Thus, the value for f calculated from their data on the iron content of the organism is close to 1. However, in the present experiments on the iron binding of non-growing iron-deficient diphtheria bacilli, f was around 1.7–1.8 and never lower than 1.68. In other words, under no conditions, did the diphtheria bacilli bind more than 60 per cent of the iron with which they were in contact. Iron uptake by diphtheria bacilli during growth appears to be much the same as that described above. Yoneda (1957) and Clark (1958) observed that organisms took up only about the half of the iron added to the growing medium. According to Clark (1958), only 50–60 per cent of the iron present in the medium, at all concentrations tested, could be recovered from the diphtheria cells. Consequently, f in his experiments was between 1.5 and 2.0. Therefore, iron-uptake by diphtheria organisms may be essentially the same in both growing or non-growing cells, and there may be a different mechanism involved in the process in this organism and in others such as *Clostridium welchii.*

Although neither the intrinsic mechanisms involved in the iron binding nor the chemical nature of the sites of this particular organism are yet known, data presented in this paper reveal several properties of the binding of iron. In the first place, iron binding may be irreversible under normal physiological conditions. Thus, once iron is bound to the cell, no ordinary buffers or saline at near neutrality are able to release it. Secondly, an enzymatic reaction may not be involved, at least in the initial step of the iron binding and the sites on the cell for binding iron seem to be quite heat stable. As seen in Table 3, only 22–23 per cent reduction in the iron binding capacity was obtained by heating the cells at 80–100°C for 30 minutes. Moreover, idential amounts of iron were bound at an arbitrary zero-incubation time by both heated and non-heated cells. In addition, it was observed that iron binding by non-heat treated cells proceeded even at 2°C and the increase in binding was not so great (about 28 per cent) on raising the incubation temperature to 37°C. However, it is still uncertain whether or not the 22–23 per cent reduction in binding on heating, or the 28 per cent increase on raising the incubation temperature from 2 to 37°C is due to the presence of some heat labile binding sites or assimilation process. Thirdly, ionization of the iron salts used for contact experiments may be necessary before binding occurs and the iron is presumably in the ferric form. Thus, when M/

80 citrate buffer was used, the organism bound only 13 per cent of the iron even at near the optimal pH. Considering the molar ratio of iron/citrate in this reaction medium and the iron-chelating activity of citrate, it may be assumed that a very slow dissociation of ferric citrate takes place. A similar phenomenon may occur in phosphate buffer. Thus as was seen in Figure 4, the amount of iron bound to the organism in the M/80 phosphate buffer of pH 7.6 was about 39 per cent of that in M/80 borate buffer at the same pH. In this case, however, the slow dissociation and the insolubility of the iron-phosphate complex may limit the binding of iron. This may partially account for the increasing iron binding capacity with increasing acidity in phosphate buffer. When the reaction medium contained sodium hydrosulfite, iron was not bound to the organism even at neutrality. This strongly suggests that only the ferric iron can be bound. Conversely, bound iron is released completely in sodium hydrosulfite and, almost completely in ascorbic acid. Iron bound to the site must probably be in the ferric form, like ferrichrome (Nielands, 1957) or of ferritin (Granick, 1951).* The true state of the diphtheria cell-bound iron will be known further from studies on the isolation and chemical nature of the iron-binding sites.

Finally, from these properties and the quantitative nature of the iron binding by the organism, it may be that the binding sites serve as reservoirs for a large amount of iron and the pooled ferric iron may become available for haem synthesis or for the other iron enzymes after being reduced to the ferrous state in the cytoplasm.



The above tentative scheme illustrates such a possibility. Considering that haem iron comprises only about 9 per cent of the total iron content of the diphtheria bacilli (Clark, 1958) and that the organism with a little bound iron can grow in an iron-free synthetic medium (Yoneda, 1957 and unpublished data), the above speculation seems not improbable.

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* According to a personal communication from Dr. A. M. Pappenheimer, Jr., an iron-containing coloured substance has been isolated from a mutant strain of *C. diphtheriae* in his laboratory.

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