Growth of *Staphylococcus aureus* in Media of Restricted and Unrestricted Inorganic Iron Availability

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SUMMARY

A new procedure for the removal of iron from complex media is given. With this method, the iron requirements for the growth of *Staphylococcus aureus* in casein hydrolysate medium were investigated. Trypticase medium (0.6-0.8 µg. Fe/ml.) was depleted of iron by treatment with the specific iron-chelator bathophenanthroline; on analysis it was shown to contain 0.01-0.02 µg. Fe/ml. Preliminary studies showed that *S. aureus* grew well in the iron-depleted medium. Further iron restriction of the already depleted medium was accomplished by the addition of conalbumin, a specific iron-chelating protein present in egg white. The initiation of growth, rate of growth, and total crop were all dependent on the concentration of free iron in the medium. The availability of iron was a function of the percentage iron saturation of the conalbumin.

INTRODUCTION

Of the several roles that inorganic compounds in nutrient media may play in the growth of micro-organisms Knight (1936) gave prominent place to the participation of 'elements required as catalysts, or in the manufacture of enzymes for essential growth processes'. Among the metals, iron is an essential element for this purpose in the vast array of micro-organisms whose metabolism involves the use of the cytochrome system for terminal oxidation, catalase, peroxidase, and other iron-containing catalysts. *Bacillus pycnoticus* (Ruhland, 1924), *Azotobacter vinelandii* (Horner & Burk, 1934), *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Serratia marcescens*, etc. (Waring & Werkman, 1943), *Escherichia coli* (Ratlidge & Winder, 1964) and *Nocardia opaca* (Webley, 1960) are among those organisms whose growth response to the concentration of available inorganic iron in the culture media have been studied in detail. For these studies, well defined simple salts media with sugars or glycerol as carbon and energy sources have generally served as culture media and have lent themselves to procedures for depleting the available iron (Donald, Passey & Swaby, 1952) to low values so that growth responses to measured iron additions could be examined.

Staphylococcus aureus is inhibited in growth in nutrient broth containing a measured amount of hen egg white (Schade & Caroline, 1944). The addition of inorganic iron to such a medium in an amount sufficient to saturate the iron-chelating egg-white protein conalbumin (Alderton, Ward & Fevold, 1946) over-

comes this inhibition completely. The growth-inhibitory properties of conalbumin through its specific iron chelation are paralleled by those of siderophilin (transferrin), the iron-binding β_1 globulin of human and other mammalian sera (Schade & Caroline, 1946; Laurell, 1947). Because of this capacity for specific iron chelation in the serum, siderophilin can function not only as a transporter of iron in the blood but also as a non-specific immunity factor (Schade, 1960). In the latter connexion it has been shown (Schade, 1963) that the rate of aerobic growth of *S. aureus* in human serum as a culture medium is a function of the percentage iron saturation of the siderophilin present.

To aid an examination of the growth and metabolism of *Staphylococcus aureus* grown in a readily prepared complex medium under conditions approximating those found in blood serum, we have used an iron-depleted casein digest medium supplemented with egg white as a serum substitute. To this medium we have added known amounts of inorganic iron to realize several degrees of iron restriction, for comparison with conditions of unrestricted availability of iron. The present paper gives a simple method for the removal of iron from a complex medium and reports the results of growth studies on the iron requirements of *S. aureus* grown in this medium.

METHODS

Organism. A penicillin-resistant, coagulase-positive strain of *Staphylococcus aureus*, phage type 80/81, was used throughout. Stock cultures on nutrient agar (Difco) slopes were refrigerated and subcultured every 2 months.

Media. The basal medium contained: pancreatic digest of casein (Trypticase, Baltimore Biological Laboratories.), 20 g.; biotin, 110 μ g.; nicotinic acid, 50 μ g.; thiamine hydrochloride, 50 μ g.; double glass-distilled water to 1 l., adjusted to pH 7.6 with NaOH, autoclaved (20 min, 121°). On final analysis (Schade, Oyama, Reinhart & Miller, 1954), this medium was found to contain 0.6–0.8 μ g. iron/ml. Iron-depleted medium was prepared with a trypticase solution (treated as described below) followed by the vitamin supplementation and adjustment of pH value to conform to the basal medium formula; the final iron concentration of this medium was 0.01–0.02 μ g./ml. A 30% (w/v) glucose solution sterilized by filtration was added as desired to media, to final concentration 2 g./l.

Egg white was removed aseptically from fresh eggs and added as required to media at 1% (v/v) final concentration. One ml. egg white, through its content of conalbumin, bound approximately 20 μ g. iron (Schade *et al.* 1954). A solution of ferrous ammonium sulphate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, Fisher: C. P.), 0.1% (w/v) with respect to iron, was prepared in 0.06 N-HCl containing 0.006% (w/v) ascorbic acid, sterilized by filtration, and added to the iron-depleted medium in measured amounts as required. Media were dispensed in 50 ml. volumes in 300 ml. EDTA-rinsed, iron-free, sterile DeLong culture flasks (Bellico Glass, Inc., Vineland, N. J.).

Conditions of growth. Inocula were prepared from *Staphylococcus aureus* grown at 37° in 2% iron-depleted trypticase medium without glucose or egg white for 12 hr. The resultant suspension had an extinction (600 m μ ; E_{600}) of 0.3; this was roughly equivalent to 7×10^8 cocci/ml. This suspension was diluted 1/1000 in sterile saline and 0.3 ml. of dilution used as the inoculum/50 ml. medium. The culture flasks were incubated at 37° and shaken in a gyratory-incubator shaker (Model

G 25, New Brunswick Scientific Co., New Brunswick, N.J.) at a shaking rate of 270 rev./min. with an amplitude of 1 in. Growth was followed by periodic measurement of the extinction E_{600} of the culture with a model DU Beckman spectrophotometer. Cultures containing egg white were passed through a coarse sintered-glass filter before making the extinction measurements.

Procedure for iron removal. Methods of preparation of de-ironized media for microbiological purposes (Donald *et al.* 1952) have in general used inorganic precipitates, e.g. CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$, as trace element absorbers; specific iron-chelating chromogenic agents followed by separation of stable metal complexes by solvent extractions; biological agents, e.g. *Aspergillus niger*, as iron scavengers (Foster, 1949). The methods which use iron-chelating compounds followed by solvent extraction are the most desirable because of the specificity of the metal removed. However, when the media to be treated are complex (e.g. beef or yeast extract, peptones, brain heart infusion) the usual solvent extraction procedure with 8-hydroxyquinoline and chloroform (Waring & Werkman, 1942) becomes laborious and time-consuming (Rubbo, Albert, & Gibson, 1950). We recommend for reliability and simplicity the following procedure which we used to prepare an iron-depleted pancreatic digest of casein medium for these studies.

For the preparation of 4 l. of 2% (w/v) iron-depleted pancreatic digest of casein (Trypticase) solution the following steps were used.

(1) Trypticase (82 g.) was dissolved in double glass-distilled water to 820 ml. and adjusted to pH 3.3 with 22 ml. concentrated HCl.

(2) A stock solution of bathophenanthroline reagent was prepared by adding 500 mg. 4,7-diphenyl-1,10-phenanthroline to 25 ml. ethanol made 0.1 N with respect to HCl; to this was added 500 mg. ascorbic acid and the solution diluted to 100 ml. with water.

(3) To the acidified Trypticase solution (1) was added, while stirring and heating to 100°, the bathophenanthroline reagent solution in an amount about 120% of that required to bind all the ionic iron present (18 mg. bathophenanthroline will bind 1 mg. iron). Preliminary iron analysis of the concentrated Trypticase solution by any suitable method (e.g. Schade *et al.* 1954) indicated the number of ml. reagent solution needed.

(4) The treated mixture was cooled to room temperature and filtered with suction through 3 layers of Whatman no. 42 paper.

(5) The filtrate was twice extracted with 150 ml. of a mixture of isoamyl alcohol + benzene (50 + 50, by vol.) followed by two extractions with 150 ml. volumes of benzene.

(6) The extracted medium was filtered once through three layers of Whatman no. 42 filter paper and adjusted to desired pH value with saturated NaOH.

(7) The adjusted medium was diluted with double glass-distilled water to 4 l. This was used as the supply of 2% (w/v) Trypticase iron-depleted for further preparation of the media detailed above.

RESULTS

Growth of Staphylococcus aureus in iron-rich and iron-depleted Trypticase media

The growth characteristics of *Staphylococcus aureus* in Trypticase medium before and after iron depletion was first examined, growth in nutrient broth being used as a standard for comparison. Representative results are shown in Fig. 1. *S. aureus* grew well in the iron-rich Trypticase medium ($0.67 \mu\text{g. Fe/ml.}$) after a lag period of

Fig. 1

Fig. 2

Fig. 1. Growth of *Staphylococcus aureus* in nutrient broth ($0.274 \mu\text{g. Fe/ml.}$), ▲; 2% iron-rich Trypticase medium ($0.67 \mu\text{g. Fe/ml.}$), ●; and 2% iron-depleted Trypticase medium ($0.016 \mu\text{g./ml.}$), ○.

Fig. 2. Same as Fig. 1, except 0.2% glucose added to the media.

Fig. 3. Growth of *Staphylococcus aureus* in iron-rich and iron-depleted 2% Trypticase media + 1% (v/v) egg white, with and without added glucose (0.2%). Iron-rich Trypticase + egg white ($0.46 \mu\text{g. Fe/ml. excess}$), ●; iron-rich Trypticase + egg white + glucose ($0.46 \mu\text{g. Fe/ml. excess}$), ○; iron-depleted Trypticase + egg white ($0.037 \mu\text{g. Fe/ml. excess}$), △; iron-depleted Trypticase + egg white + glucose ($0.037 \mu\text{g. Fe/ml. excess}$), ▲.

about 10 hr. In iron-depleted medium (0.016 $\mu\text{g. Fe/ml.}$) the lag period of growth was similar, but the rate of logarithmic growth and total crop were smaller. Cultures grown in nutrient broth showed a rate of logarithmic growth comparable to that found in iron-rich Trypticase medium, but there was a lag period of only 7 hr. This shortened lag period probably reflected the presence of some readily available carbon and energy sources in the nutrient broth. To test this the above experiments were repeated with 0.2% (w/v) glucose added to the several media. The results (Fig. 2) show that glucose addition did decrease the lag period in the iron-rich Trypticase medium to that found with the nutrient broth, but did not affect the lag in the iron-depleted medium. The logarithmic growth rates were increased in all three of the glucose-supplemented media. Thus, the lag period and logarithmic growth rate of *S. aureus* in the Trypticase media were dependent on the available iron concentration and the presence of a readily utilizable carbon and energy source such as glucose. It appeared probable that under conditions of limited iron the initial synthesis or activation of iron-requiring enzymes was depressed, with consequent delay in growth response. Further decrease of available iron through the addition to Trypticase medium of an iron chelator should result in increase of the lag period and depression of the logarithmic growth rate.

Growth on iron-depleted Trypticase medium + egg white

To restrict further the amount of nutritionally available iron, the iron-depleted Trypticase medium with 1% (v/v) egg white was added as a source of the specific iron-binding conalbumin. To this supplemented medium, measured amounts of iron were added to give known degrees (%) of saturation of the conalbumin present. The resulting conditions were similar to those used by Schade (1963) to study the growth of *Staphylococcus aureus* in human serum at different degrees of iron saturation of siderophilin.

Experiments to test the effect of added egg white itself on the growth of *Staphylococcus aureus* in Trypticase medium before and after treatment for iron removal, with and without added glucose, are summarized in Fig. 3. The iron in the untreated medium was sufficient to saturate the conalbumin of the 1% egg white and to leave an excess of 0.46 $\mu\text{g. iron/ml.}$; to treated medium, iron was added (as ferrous ammonium sulphate) to provide an excess of 0.037 $\mu\text{g. Fe/ml.}$ Comparison of the appropriate growth curves in Figs. 1 and 2 indicates that no inhibition of growth was occasioned by egg white in any of the four media when iron was present in excess. On the contrary, in the media without added glucose, some enhancement of the logarithmic growth rates resulted and the lag period of about 10 hr in Trypticase medium alone (Fig. 1) was decreased to 5 hr. This decrease in lag period might have been due in part to the glucose introduced into the medium by the egg white; on analysis (Glucostat, Worthington Biochemical Corp., Freehold, N.J.), this was found to contain 10 mg. glucose/ml. egg white. The logarithmic growth rates of the cultures in treated and untreated media with egg white were comparable.

Having established the suitability of egg white-supplemented iron-depleted Trypticase as a growth medium for *Staphylococcus aureus* when iron was added to excess, the growth was examined when the absolute iron concentration was less than that required to exceed the iron-binding capacity of the conalbumin content.

Table 1 shows the concentration of inorganic iron in the media prepared and the various resultant percentage iron saturations of conalbumin. With the exception of the medium used as the control in which the conalbumin was saturated to excess (118 %), the only iron available to the organism was that which was present as free iron in equilibrium with the iron + conalbumin complex,

Conalbumin has an extremely high affinity for iron and forms a very stable complex; the dissociation constant of the complex is not precisely known but has been estimated to be in the range of 10^{-28} to 10^{-30} M at pH 7.6 (Warner & Weber, 1951). The relative amounts of free ionic iron available in the several media at different percentage iron saturations can be calculated by use of an equation based on conalbumin having two dependent iron-binding sites (Schade, 1963). Table 1, last column, gives the values when the 8 % saturation value is used as a baseline for comparison with the other percentage saturation values.

Table 1. *Egg white-supplemented, iron-depleted 2 % Trypticase media at different percentages iron saturation of conalbumin*

Absolute Fe concentration ($\mu\text{g. Fe/100 ml. medium}$)	% Fe saturation of conalbumin (20.7 $\mu\text{g. Fe/100 ml. medium} = 100\%$)	Relative amounts of available Fe (8 % saturation as base)
1.6	8	1
3.7	18	1.59*
5.74	28	2.12
11.95	58	4
18.16	88	9.2
20.23	98	23.8
24.37	118	∞

* As $[\text{Fe}] = \sqrt{\left(K \frac{[\text{conalbumin} \cdot \text{Fe}_2]}{[\text{conalbumin}]}\right)}$, then by substitution for 18 % saturation,

$$[\text{Fe}] = \sqrt{\left(K \frac{18\%}{82\%} \text{ or } 0.468 \sqrt{K}\right)}.$$

$$\text{For 8 \% saturation, } [\text{Fe}] = \sqrt{\left(K \frac{8\%}{92\%} \text{ or } 0.295 \sqrt{K} \cdot \frac{0.468 \sqrt{K}}{0.295 \sqrt{K}} = 1.59\right)}.$$

The results of growth studies with the above media are shown in Fig. 4. The character of the growth patterns observed was markedly affected by increasingly restricted amounts of iron available to *Staphylococcus aureus* through the dissociation of the iron + conalbumin complex at decreasing percentages of its iron saturation. In the media where the conalbumin was 98, 88, and 58 % iron-saturated, almost normal growth curves were obtained, with increasingly longer lag periods and lower logarithmic growth rates associated with the lower saturation values. When the percentage saturation values were 28, 18, and 8, the cultures did not show a normal growth pattern, but grew at constant, increasingly smaller rates as the percentage iron-saturation of conalbumin decreased. Considering that the growth of *S. aureus* in the medium whose conalbumin was 88 % saturated approached that in medium containing an excess of iron (118 % saturation), and that the concentration of available iron in the former case is probably of the order of only 10^{-28} M, it is

clear that very low iron concentrations satisfy the normal nutritional needs of this organism. It is remarkable that, under the specific growth conditions used, the 88% iron-saturated conalbumin medium offered so critical a concentration of available iron that an additional fourfold decrease in available iron concentration (28% iron-saturation) effectively altered the normal growth pattern and the growth rate. Additional evidence that the availability of iron from the iron + conalbumin complex is a function of the percentage iron-saturation of conalbumin was given by Schade (1963), who showed that *S. aureus* grown in given media in which the conalbumin was 10, 50, and 95% iron-saturated contained 0.97 , 2.24 and 12.7×10^4 iron atoms, respectively, per coccus.

Fig. 4

Fig. 5

Fig. 4. Growth of *Staphylococcus aureus* in 1% (v/v) egg white-supplemented, 2% Trypticase iron depleted media at different percentages iron-saturation of conalbumin.

Fig. 5. Same as Fig. 4, except 0.2% glucose added to the media.

Table 2. Measurements of pH values of cultures of *Staphylococcus aureus* grown in 1% egg white-supplemented, iron-depleted 2% Trypticase media, with and without glucose, at different percentages iron-saturation of conalbumin

		Time (hr)							
		8	9	10	11	12	13	14	15
		pH value							
With glucose	% Fe-saturation								
	8	7.45	7.62	7.39	7.53	7.22	6.74	5.89	5.46
	18	7.45	7.38	7.58	7.57	7.36	7.02	6.16	5.5
	58	7.4	7.21	7.03	6.62	6.07	5.77	5.85	5.97
	98	7.17	6.55	5.87	5.57	5.61	5.67	5.73	5.82
	118	7.03	6.23	5.67	5.57	5.63	5.69	5.76	5.83
Without glucose	8	7.7	—	7.6	—	—	—	7.61	—
	118	7.55	—	7.67	—	—	—	8.1	—

When 0.2% glucose was added to several of the media given in Table 1 (8, 18, 58, 98, 118% iron-saturated) inoculated with *Staphylococcus aureus*, and incubated at the same time as those without added glucose, the results shown in Fig. 5 were obtained. Glucose addition increased the growth rates in all the cultures but most markedly in those in which the conalbumin was 58, 98, and 118% iron-saturated; of particular note is the delayed increase in growth (12 hr) in the 18% iron-saturated

medium. The explanation for this delay is probably related to the change in pH value which accompanied growth. Since the Trypticase medium was poorly buffered, acid production from added glucose can effect a decrease in pH value and so increase the amount of free iron in the medium available for growth. Schade & Caroline (1944) showed that below pH 7.0 iron was increasingly available as a result of the shift in equilibrium of the iron+conalbumin complex. Measurements of pH value of the cultures were made during the incubation period (Table 2). A rapid decrease in pH value occurred in the 8 and 18 % iron-saturated conalbumin cultures between 12 and 15 hr. During this period the cocci in the 18 % iron-saturated culture were capable of responding with increased growth to the greater concentration of available iron until a pH value was reached which halted further development. The 8 % iron-saturated culture lacked this capability. No decrease of pH value occurred in the cultures without glucose.

DISCUSSION

Although there have been reported many studies on the iron requirements of bacteria, few have been done with Gram-positive organisms grown in a complex medium depleted of iron. Schade (1968), in an approach to *in vivo* conditions, used as a growth medium for *Staphylococcus aureus* human serum whose siderophilin acts as a naturally-occurring specific iron chelator. In this iron-restricted medium, the growth of the pathogen was responsive to the additions of known amounts of iron and to the percentage saturation of the chelator. To have a readily available iron-chelating complex medium approximating to human serum, we have used an iron-depleted Trypticase medium supplemented with conalbumin (as from egg white). The results of growth studies with this medium were similar to those obtained with serum and illustrated the profound growth regulatory effects of changes in the availability of iron at extremely low concentrations of it. The use of this medium made possible the harvesting of sufficiently large amounts of *S. aureus* grown in conditions of iron restriction for use in studies of their metabolic activities, to be reported later.

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