Carbohydrate Metabolism and Production of Diffusible Active Substances by *Staphylococcus aureus* Grown in Serum at Iron Levels in Excess of Siderophilin Iron-saturation and Below

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SUMMARY

Results of studies are reported on the carbohydrate metabolic capabilities of cells of *Staphylococcus aureus* grown in human serum at varied percentage iron-saturations of its contained siderophilin; on the production by such cells of coagulase, hyaluronidase, staphylokinase, and haemolysins; and on the effectiveness of some antibiotics as growth inhibitors under similar *in vitro* growth conditions. Cocci grown in serum at a normal serum iron level and percentage of iron-saturation show a significant reduction in their endogenous respiration, in their capacity to attack lactate, a complete loss of ability to oxidize formate, and a several-fold increase in anaerobic glycolytic capacity as compared to those cocci grown in serum containing unchelated free ionic iron. These metabolic changes, in conjunction with an observed increased rate of acid production and rate of glucose utilization by the growing culture of 'low-iron' cells *versus* the 'high-iron' cells, indicate an increased dependence upon glycolytic rather than oxidative energy production processes by the iron-restricted cells. The strain of *S. aureus* employed in these studies, although characterized by conventional methods as a producer of coagulase, hyaluronidase, haemolysins, and staphylokinase, did not elaborate any of these pathogenesis-linked factors at detectable levels when grown in normal serum. Coagulase, alone, was found at 'high-iron' levels. The amount of dimethoxyphenyl penicillin required to inhibit growth of our *S. aureus* strain grown in serum increased with increasing percentage of iron saturation of the siderophilin while the effectiveness of a given inhibitory concentration of kanamycin and chloramphenicol was indifferent to any variation in the iron-saturation value.

INTRODUCTION

When *Staphylococcus aureus* is grown in human serum under 5 % CO₂-air, the growth rate is a function of the percentage iron-saturation of its contained plasma siderophilin (Schade, 1963). If trypticase plus conalbumin of eggwhite is substituted for serum, similar growth-rate control is exercised by the percentage iron-saturation of the added conalbumin (Theodore & Schade, 1965*a*). A study of the relative oxidative capabilities and carbohydrate metabolism of resting *S. aureus* grown in glucose-trypticase-conalbumin media with and without iron restriction showed that iron-restricted cells were severely limited in their oxidative ability to attack glucose and not

at all capable of metabolizing L-lactate, D-lactate, pyruvate, or formate as compared with the unrestricted cells (Theodore & Schade, 1965*b*).

As iron is added to sera at physiological pH values and at levels below the plasma siderophilin saturation value, the metal is rapidly and firmly bound. No free iron can be detected by addition of α, α -dipyridyl or bathophenanthroline to such sera (Schade, 1961). The magnitude of the binding constants is very high, the estimates for the pK_1 and pK_2 at pH 7.4 being 27.7 and 30.3 respectively (Davis, Saltman & Benson, 1962). The iron in the complex is trivalent and bound with essentially ionic bonds (Ehrenberg & Laurell, 1955).

The present investigation was undertaken to determine the carbohydrate metabolic capabilities of cells of *Staphylococcus aureus* grown in serum at low and high iron concentrations; the production by such cells of coagulase, hyaluronidase, staphylokinase, and haemolysins; and the effectiveness of some antibiotics as growth inhibitors under similar *in vitro* growth conditions.

METHODS

Organism. A penicillin-resistant strain of *Staphylococcus aureus*, phage type 80/81, was used throughout. Stock cultures on nutrient agar slopes were kept in the cold and subcultured every 2 months.

Media. Pooled, blood-bank serum was employed. In any given experiment in which varied percentage iron-saturations of the serum's siderophilin were required, the same serum was used throughout. For the growth of bacteria employed in the metabolic studies, glucose was added to the serum to a final concentration of about 0.30 % (w/v); otherwise, the sera were glucose-fortified by the addition of 1 mg./ml. serum. To obtain conditions of restricted and unrestricted iron availability, iron as ferrous ammonium sulphate was added to the serum as required. All sera were equilibrated with 5 % CO₂ in air at 37° before incubation and maintained in this gas atmosphere during culturing. Seed cultures were grown in iron-restricted serum or in iron-depleted trypticase medium (Theodore & Schade, 1965*a*) for 16 hr at 37°.

Analytical procedures. Serum iron and iron-binding capacity of the serum were determined by the methods of Schade, Oyama, Reinhart & Miller (1954). Standard Warburg manometric techniques were used for measuring O₂ uptake and CO₂ production by resting cell suspensions. At harvest, the cultures, grown for 14 hr on a rotary shaker at 37°, were centrifuged at 4° at 4000 *g* for 20 min. and washed twice in 0.05 M-phosphate buffer (pH 7.4). For catalase (Schade, 1963) and O₂ uptake measurements, the washed cocci were resuspended in the same 0.05 M-phosphate buffer; for the measurement of glycolytic activity they were taken up in 0.03 M-bicarbonate buffer (pH 7.4). All suspensions of cocci in their respective salt solutions were diluted to an extinction at 600 m μ appropriate to their intended use. Preliminary analytical studies established that an $E_{600\text{ m}\mu}^{1.0\text{ cm}}$ of 1.0 was equivalent to about 320 μ g. dry weight organisms/ml. and 2×10^9 cocci/ml. of the suspension measured.

Glucose was determined by glucostat (Worthington Biochemical Corp., Freehold, New Jersey); pyruvate as the 2,4-dinitrophenylhydrazone, with benzene extraction (Friedemann & Haugen, 1943); lactate by the method of Barker & Summerson (1941); acetoin by the method of Westerfeld (1945); and acetate by the method of Soodak (1957).

For the determination of the production of soluble coagulase, we employed the qualitative method of Fisk (1940) and the quantitative procedure of Rogers (1954); for hyaluronidase estimation, the quantitative method of Swyer & Emmens (1947). The plate method of Elek & Levy (1950) served to define the haemolysins produced by our *Staphylococcus aureus* strain, while Jackson & Little's (1957) procedure was employed for quantitative estimation of haemolysin present in culture supernatants. The qualitative method of Christie & Wilson (1941) and the quantitative procedure of Tillet & Garner (1933) were used for staphylokinase determinations.

Antibiotic sensitivity discs on brain-heart infusion agar served preliminarily to determine growth inhibition of the cocci. More quantitative data with effective antibiotics were obtained through growth studies using serum as medium at given antibiotic concentrations.

EXPERIMENTAL

Carbohydrate metabolic activities of Staphylococcus aureus grown in high and low iron sera

The observation that the aerobic growth rate of *Staphylococcus aureus* in serum is governed by the serum iron concentration and the percentage iron saturation of the iron-binding protein, plasma siderophilin (Schade, 1963), suggests a restriction upon the energy-yielding oxidative metabolic systems of the organism involving iron as a functional constitutive element such as occurs, for example, in the cytochromes. To determine some relative oxidative capacities of cocci grown in serum at an iron level in excess of its binding capacity and at a percentage iron-saturation level found in normal serum, we cultured *S. aureus* in serum under both conditions in 5 % CO₂-air on a rotatory shaker at 37°. The harvested cocci were then examined for their oxidative

Table 1. *Rates of oxidation of various substrates, anaerobic glycolysis, and catalase activity of Staphylococcus aureus grown in serum at 105 % and 23 % iron saturation*

For oxidation rates, Warburg vessels contained 3.2 mg. dry wt cocci in 2.5 ml. 0.05 M-phosphate buffer (pH 7.4) in air at 37° plus 50–100 μ moles substrate; for glycolysis, 1.6 mg. cocci in 0.03 M-sodium bicarbonate buffer (pH 7.4) in 90 % N₂ + 10 % CO₂ at 37° plus 7 μ moles glucose; for catalase determinations with the high-iron and low-iron cocci, 0.05–0.1 mg. and 0.8–1.6 mg. cocci, respectively, in 0.055 M-phosphate buffer (pH 6.6) in air at 16° plus 36 μ moles H₂O₂. Q_{O₂} values represent μ l. O₂ consumed or produced/hr/mg. dry wt. cocci; Q_{CO₂} values signify μ l. CO₂ similarly produced. Q values were calculated for the maximum rate of gas consumption or production following a 10 min. initial equilibration period uncorrected for the endogenous rate where applicable.

Substrate	Serum iron saturation			
	105 % sat.	23 % sat.	105 % sat.	23 % sat.
	Q _{O₂} values		Q _{CO₂} values	
Endogenous	19	3	—	—
Glucose	44	38	22	83
L-Lactate	136	63	—	—
Pyruvate	0	0	—	—
Acetate	0	0	—	—
Succinate	0	0	—	—
Formate	85	0	—	—
H ₂ O ₂ (catalase)	13,500	1,150		

activity on selected substrates as well as their glycolytic and catalase activities. Table 1 summarizes typical data from such an experiment.

The cocci used here were from a culture (low-iron) in pooled fresh normal human serum with a serum iron value of 80 $\mu\text{g. \%}$ and a total iron-binding capacity of 346 $\mu\text{g. \%}$ or 23.1 % saturation, and from a culture (high-iron) in the same serum made 105 % iron-saturated by addition of ferrous ammonium sulphate. Both media were brought initially to 0.27 % (w/v) glucose concentration level and equilibrated at pH 7.4–7.5 with 5 % CO_2 in air. The inocula were cocci grown through 2 overnight transfers in the glucose-fortified 23 % iron-saturated serum on a shaker at 37° in 5 % CO_2 -air. One ml. of such a culture was used to inoculate 200 ml. of the 23 % iron-saturated serum; 0.1 ml. served for the inoculum of 200 ml. of 105 % iron-saturated medium. Both sera were put in tightly screw-capped 1 l. Erlenmeyer flasks gassed with 5 % CO_2 -air and shaken at moderate speed on a rotatory shaker at 37°. Since the pH of the low-iron serum had previously been observed to fall below 7.0 towards the end of the 14 hr growth period and the glucose concentration to fall more precipitously than in the high-iron culture, precautions were taken to maintain the pH above 7.2 at all times through the growth period by addition of appropriate amounts of 10 % NaHCO_3 solution to the low-iron medium. Such lowering of the pH must be avoided if the siderophilin-iron-chelate is expected to maintain its integrity and keep the ionic iron level in the medium at its low value. Further, the cocci must be harvested before the glucose has been completely removed from the medium, since the metabolic capacities of *Staphylococcus aureus* suspensions are significantly affected by the presence or absence of glucose in their growth environment (Theodore & Schade, 1965*b*). The initial pH of both media was 7.4–7.6; at harvest the high-iron culture had a pH of 7.0 and the low-iron culture 7.28. A total of 5 ml. 10 % NaHCO_3 solution (w/v) had been added to the low-iron culture and 5 ml. physiological saline to the high-iron culture over the growth period. The initial glucose concentration in both media was 2.7 mg./ml.; at harvest, the low-iron culture contained 0.6 mg./ml. and the high-iron culture 1.3 mg./ml. The final $d_{600}^{1.0 \text{ cm}}$ value of the high-iron 14 hr. culture was 4.0, while that of the low-iron culture was 2.0. The lower turbidity of the culture grown in the normal 23 % iron saturated serum compared to that grown in the same serum at 105 % saturation, despite the ten-fold greater inoculum, is illustrative of the depressed growth rate due to iron chelation by the plasma siderophilin.

The summarized results (Table 1) of oxidative studies with the cocci grown in serum at a normal serum iron level show a significant reduction in their endogenous respiration, in their capacity to attack lactate and a complete loss of ability to oxidize formate as compared with those cocci grown in serum containing unchelated free ionic iron. The rates of oxidation of glucose by both populations, however, are comparable as are their common failure to attack acetate and succinate. The latter deficiency apparently is due to the glucose repressive effect on acetate and tricarboxylic acid cycle intermediates oxidation previously observed in cocci cultured in glucose-containing semi-synthetic media (Collins & Lascelles, 1962; Theodore & Schade, 1965*b*). That pyruvate is not oxidized by the high-iron serum grown cocci was unexpected since trypticase/glucose/high-iron-grown cocci possess this capability (Theodore & Schade, 1965*b*). When analyses of the end-products of oxidation of glucose and lactate were made for acetate, lactate, pyruvate, acetoin, and CO_2 , the

main products found were pyruvate and small amounts of CO_2 . Traces of acetate were found in all cases. Acetoin was detected only in the supernatants of the glucose oxidation experiments.

The reduction in the oxidative capacity of *Staphylococcus aureus* grown at normal serum iron levels is accompanied by an approximately fourfold increase in the anaerobic glycolytic capacity of the cocci. These concomitant metabolic shifts, in conjunction with the observed increased rate of acid production and rate of glucose utilization by the growing culture of low-iron cocci *versus* the high-iron cocci, indicate an increased dependence upon glycolytic rather than oxidative energy production processes by the iron-restricted cocci. The nearly 12-fold differences in catalase activity of the high-iron cocci over the low-iron cocci is impressive evidence in itself of the effectiveness of the plasma siderophilin to restrict iron availability to the growing cocci.

Diffusible products of Staphylococcus aureus grown in serum at various iron saturation levels

Investigations were made of the production by our strain of *Staphylococcus aureus* of diffusible factors commonly associated with the pathogenicity of this organism, namely: coagulase, hyaluronidase, haemolysins, and staphylokinase. Both qualitative and quantitative estimations were made, where possible, of these products in human sera which served as test-tube growth media at different levels of iron saturation of their normally occurring plasma siderophilin. The serum iron levels chosen were

Table 2. *Coagulase activity of supernatants of Staphylococcus aureus grown in serum at different serum iron levels*

Fe sat. (%)	Fe conc. ($\mu\text{g./ml.}$)	Growth period (hr)		Growth ($d_{\lambda_{880}}^{1.00}$)		Coagulation time (Rogers, 1954)	
		<i>a</i> *	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
16	0.56	30	41	0.240	0.336	> 15 min. (undil.)	> 15 min. (undil.)
32	1.12	24	—	0.336	—	> 15 min. (undil.)	—
48	1.68	20	—	0.360	—	> 15 min. (undil.)	—
64	2.24	19	19	0.380	0.424	> 15 min. (undil.), clot-2 hr.	9.45 min. (undil.)
80	2.80	16	—	0.328	—	> 15 min. (undil.), clot-2 hr.	—
110	3.85	16	17	0.548	0.460	3.06 min. (1/1 dil.)	2.43 min. (1/1 dil.)

* *a* and *b* signify two experiments.

those obtaining in iron-deficiency states, in normal subjects, and on conditions of haemochromatosis and transfusion haemosiderosis, i.e. 5–15 %, 20–60 %, and 80–100 % siderophilin iron-saturations, respectively. Serum at 110 % iron-saturation was included to provide a comparable medium in which free ionic iron was available to the growing organism. Appropriate controls were obtained from cultures grown in trypticase or brain-heart infusion media. All media were inoculated with 200–1000 colony-forming units and kept under 5 % CO_2 + air at 37°.

Coagulase. Through use of both the qualitative method of Fisk (1940) and the quantitative method of Rogers (1954), we determined that the soluble coagulase activity of culture supernatants from cultures of comparably sized populations

increased with increasing iron-saturation of the serum medium when its siderophilin was at least half saturated with iron. In all experiments the coagulase activity of supernatants from cultures in serum 110 % iron-saturated was readily demonstrable by both methods and at a relatively high level. Data from two illustrative experiments are summarized in Table 2. Comparable results were obtained with unheated or heated (56° for 30 min.) sera as media. In general, the data indicate that human sera at normal serum iron levels and below and at normal percentages of plasma siderophilin iron-saturation and below, fail to provide media productive of detectable levels of coagulase. Whether this fact is a consequence of the slower growth rate and altered metabolism of *Staphylococcus aureus* growing in a nutrient iron-restricted medium or reflects some destruction or inhibition of coagulase normally produced is not known.

Hyaluronidase. Applying the quantitative viscosimetric method of Swyer & Emmens (1947) for estimation of hyaluronidase in culture supernatants, we found none to be active, whether from unheated or heat-treated serum media at any percentage iron-saturation. Addition of potassium hyaluronate to the serum media (0.5 mg./ml.) did not effect hyaluronidase production by the growing bacteria. Supernatants of cultures of similar cell populations produced in brain-heart infusion broth or in trypticase with 1.5 % (v/v) egg white supplement at 5 %, 50 % and 110 % iron saturation of its conalbumin component, however, possessed hyaluronidase activity in rough proportion to the population and indifferently to the availability of iron in the medium.

Haemolysins. Using the plate method of Elek & Levy (1950) with rabbit, sheep, and human red blood cells in conjunction with an anti- α and minimal anti- β -hemolysin antiserum of Connaught Laboratories (Toronto, Canada), we determined that our *Staphylococcus aureus* strain produced α , β and δ hemolysins. With the Jackson & Little (1957) quantitative haemolysin method, it was found that no haemolytic activity was present in serum supernatants of cultures at any iron-saturation level. The determined anti- α -haemolytic activity of the serum, naturally and initially present (1 ml. heated serum neutralized 2 units of α -haemolysin), was in no case decreased by the bacterial growth. Mere presence of anti-haemolysins in a medium is not responsible for failure of the organism to produce haemolysin, since trypticase plus egg-white medium to which antiserum was added had considerable haemolytic activity following growth of *S. aureus* at all levels of iron-saturation. Addition of 0.1 % iron-free agar to the serum media to provide some suspended solids did not effect a change in the failure of the bacteria to produce haemolysins in these cultures.

Staphylokinase. Through use of rabbit plasma plus nutrient agar media and the qualitative method of Christie & Wilson (1941), it was determined that our *Staphylococcus aureus* strain produced staphylokinase. However, no serum or nutrient-broth supernatants of the bacterial cultures applied to such plates gave positive reactions for kinase. Additionally, the tube method of Tillet & Garner (1933) for the quantitative estimation of kinase gave negative results for all supernatants; even overnight incubation at 37° did not give evidence of clot lysis. The positive results with the plate method involving large growing colonies probably reflect a higher concentration of kinase present in the agar at the colony site. It is also possible that this strain produces little or no kinase in the liquid media employed.

In summary, the strain of *Staphylococcus aureus* employed in these studies, although characterized by conventional methods as a producer of coagulase, hyaluronidase, haemolysins, and staphylokinase, did not elaborate any of these pathogenesis-linked

factors at detectable levels when grown in normal human blood serum. When iron was added to such serum at levels approaching saturation and beyond, then coagulase was found in increasing concentration in the culture supernatants. None of the other factors, however, were produced in response to changes in the serum iron values of normal serum. The fact of the absence of such products from growth of this pathogen in an approximation of one of its natural environments, serum, should be weighed in any assessments of their importance for its pathogenicity.

Antibiotic activities and percentage iron-saturation of serum

Preliminary tests with antibiotic sensitivity discs on brain-heart infusion agar using penicillin G, colistin, streptomycin, oxytetracycline, sulphamethizole, dimethoxyphenyl penicillin, kanamycin, and chloramphenicol indicated that our *Staphylococcus aureus* strain was resistant at high concentrations to all but the last three agents. Dimethoxyphenyl penicillin was effective at the 20 µg./disc. level, while both kanamycin and chloramphenicol were active at the 5 µg./disc level.

To determine the growth-inhibitory effectiveness of dimethoxyphenyl penicillin, kanamycin, and chloramphenicol against *Staphylococcus aureus* when grown in media of normal human serum at different levels of serum iron, we inoculated such sera with 500–1000 colony-forming units/ml. medium at measured antibiotic levels. The results established that with dimethoxyphenyl penicillin the amount of antibiotic required to inhibit growth completely after 40 hr incubation at 37° increased with increasing percentages of iron-saturation of the serum's siderophilin. For example, at 15 % iron-saturation, 0.4 µg. dimethoxyphenyl penicillin/ml. serum was sufficient to inhibit completely, while at 30 %, 60 % and 90 % saturation, 0.8, 1.6 and 3.2 µg., respectively, were required. With both kanamycin and chloramphenicol, on the other hand, the amount of antibiotic needed to suppress growth under similar conditions was independent of percentage iron-saturation of the siderophilin. Thus, 10 µg. kanamycin/ml. serum was sufficient at 18 %, 50 % and 110 % iron-saturation levels to effect complete growth inhibition; 7.5 µg. chloramphenicol/ml. serum was equally effective at 16 % and 110 % iron-saturation. From these results, it is evident that the *in vitro* effectiveness of an antibiotic against *S. aureus* grown in human serum may be determined by the iron saturation of the serum. The applicability of this conclusion is peculiar to the particular antibiotic under consideration.

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