AEROBIC RESPIRATORY METABOLISM OF *STAPHYLOCOCCUS AUREUS* FROM AN INFECTED ANIMAL

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ABSTRACT

GELLENBECK, S. MERCY (Catholic University, Washington, D. C.). Aerobic respiratory metabolism of *Staphylococcus aureus* from an infected animal. J. Bacteriol. **83**:450–455. 1962.—The separation of *Staphylococcus aureus* from the tissue fluids of an infected guinea pig has allowed a comparison of cells (GPR) separated in this manner with the same strain (PR847) grown on an artificial medium.

In general, the respiratory response of organisms separated from tissue fluid was higher than that of staphylococci grown in vitro, though endogenous respiration of the in vitro strain consistently averaged 50% higher than the GPR cells. The guinea pig serum, defined medium, Trypticase soy broth, and the guinea pig filtrate were the complex substrates, listed in order of the least to the most stimulatory. Omission of nicotinamide and thiamine from the defined medium resulted in a decrease of oxygen consumption for the PR847 strain, but not for the GPR cells; however, when several amino acids were eliminated from the defined medium, the oxygen uptake of the GPR cells was depressed more than that of the staphylococci grown in vitro. In glucose alone, the organisms separated from the tissue fluid respired over 30% more effectively than the in vitro staphylococci. The filtrate, whether whole or boiled, provided a substrate in which organisms from both sources approximately doubled their oxygen uptake over that of either whole or boiled serum. Soluble components of the 50% ammonium sulfate fractionation of the filtrate stimulated GPR and PR847 cells more than did the 100% ammonium sulfate fraction under the conditions employed.

The fact that the virulence of *Staphylococcus aureus* frequently can be increased by animal passage (Van de Velde, 1894) suggests that substances and processes connected with virulence are produced in vivo. Since many aspects of research require bacteria in large quantities and free of extraneous material, there has been, until recently, little research on the chemistry or on the metabolism of bacteria grown within the tissues of an infected mammalian host. Smith, Keppie, and Stanley (1953) established a method for collecting and separating large amounts of bacteria from the tissue fluid of experimentally infected guinea pigs, but physiological studies on large quantities of *S. aureus* taken directly from the infected host have not been reported. The purpose of this investigation is to compare *S. aureus* grown in vitro with cocci separated from the tissue fluid of an infected animal, using oxygen consumption in various substrates as the principal physiological test. Various substrates, some containing known and others unknown factors, were used in an attempt to provide a wide basis of comparison and to indicate, in a general manner at least, wherein the biochemical differences could be found.

MATERIALS AND METHODS

Source and preparation of suspensions of S. aureus grown in vitro. The *S. aureus* culture (PR847) was obtained from a local hospital and represented a recent isolate from a furuncle. It was bacteriophage typed as a type 52/80/81. The staphylococci were cultured on Trypticase soy agar, and after a 15-hr incubation period at 37 C, were harvested into buffer. All buffer used in the present work was Søresnon's 0.067 M phosphate solution at pH 6.8. After two washings, the sedimented bacteria were mixed with the aid of a glass rod in sufficient buffer to yield an even suspension with an absorbancy of 1.0 at a wave length of 525 mμ in a Spectronic 20 spectrophotometer.

Recovery of S. aureus from guinea pigs. The inocula for the injection of guinea pigs were pre-

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pared by harvesting 24-hr cultures of strain PR847 from five Trypticase soy agar slants and suspending the cells in 10 ml of Locke's solution. This represented approximately 2.9×10^9 viable cells. A male guinea pig weighing approximately 700 g was given an injection intrapulmonarily (2 ml) and intraperitoneally (8 ml); 15 hr later (7 to 9 hr after death), the animal was secured to a sloping board to allow the body fluid to collect at the bottom of the cavities. The chest and abdominal cavities were opened and the exudate from each collected in a syringe and transferred to chilled 25-ml flasks. Each cavity was then washed out with approximately 10 ml of buffer and the washings added to the exudates. The preparation was processed thereafter at 1 to 3 C. The organisms recovered from the animal's body fluids were designated as the GPR cells.

Separation of S. aureus from thoracic and peritoneal fluid. Buffer (20 ml) was added to approximately 8 ml of the thoracic and peritoneal fluid and centrifuged at $35 \times g$ for 30 min. The suspension of organisms was carefully removed from the sediment and the centrifugation repeated for another 20 min. The supernatant fluid was removed and fluid centrifugated at $10,000 \times g$ for 10 min and the supernatant fluid discarded. The bacterial layer was broken up sufficiently with a glass rod to provide an even suspension when the buffer was added in an amount equal to the discarded fluid. Centrifugation was repeated at $35 \times g$ for 10 min, followed by centrifugation of the supernatant fluid at $10,000 \times g$ for 10 min. After removing the buffer, the organisms were again suspended with a glass rod and adjusted to the absorbancy used in preparing the *S. aureus* grown in vitro. The in vitro organisms did not tend to clump as much as those isolated from the animal exudate, though microscopic examination showed that after resuspension the GPR cells showed no more clumping than the PR847 strain. Each bacterial preparation was stained with Gram's stain and examined microscopically as a verification of the purity of separation.

Quantitative comparison of strain PR847 and GPR cells. When suspensions of GPR and PR847 were adjusted to the same absorbancy, there were comparable numbers of viable organisms, as determined by plate counts on Trypticase soy agar. The cell concentration was 5 to 5.7×10^7 per ml. Micro-Kjeldahl analyses showed that the suspensions contained approximately 0.12 mg of bacterial nitrogen per ml. The apparent high nitrogen content may be due to adherent proteinaceous material or to the presence of dead cells. The animals used in this investigation usually yielded 5.7×10^8 cells in *purified* suspensions, though on several occasions two and three times this amount could be purified.

Preparation of substrates for respiratory studies. Endogenous respiration was determined on organisms suspended in 0.067 M phosphate buffer at pH 6.8.

1) Filtrate:—Exudates pooled from four infected guinea pigs were centrifuged at $10,000 \times g$ for 10 min to remove cellular material and bacteria. Selas filtration (size 02) of the supernatant fluid followed. The substrate was prepared immediately before use by adding one part of the filtrate to five parts buffer and then adding glucose so that the final concentration (0.0125 M) was the same as that of the defined medium.

2) Filtrate in Locke's solution:—One part of the above filtrate was added to one part of Locke's solution. The final pH was 6.8.

3) Serum:—Fresh blood was obtained aseptically from the heart of a healthy guinea pig. The serum was carefully separated from the clot, filtered, and prepared as a substrate in the same manner as the filtrate.

4) Boiled filtrate and boiled serum:—Separate samples of the filtrate and serum were placed in a pan of boiling water for 10 min, and coaguable material was centrifuged off. The boiled filtrate and boiled serum were filtered and prepared as substrates in the same manner as the filtrate.

5) Trypticase soy broth:—The Trypticase soy broth II was prepared in the manner recommended by the manufacturer, i.e., 40 mg per ml and sterilized in the autoclave. Trypticase soy broth I was prepared by adding one part of Trypticase soy broth II to five parts buffer; enough glucose was added to make the final concentration of glucose 0.0125 M.

6) Glucose:—Enough glucose was added to distilled water (pH 5.9) to bring the final concentration to 0.2 M.

7) Globulin and albumin fraction:—By adding an equal portion of saturated ammonium sulfate to another sample of the filtered exudate, a precipitate formed, which was then separated by centrifugation, dissolved in distilled water, and dialyzed against running water for 24 hr. After dialysis, the insoluble and soluble materials were

separated; glucose was added to the latter, as in the above substrates. The supernatant fluid from the 50% saturation was raised to 100% saturation to give another precipitate. This was treated in the same manner. Though more components may be present than just albumin and globulin, for convenience the results of the 50% ammonium sulfate fractionation will be designated as the globulin fraction and the results of the 100% fractionation will be designated as the albumin fraction. The pH of these fractions was not adjusted.

8) Defined medium:—Fildes et al. (1936) and Gladstone (1937) described the defined medium employed. The rather complex medium included 14 amino acids, salts, glucose, thiamine, and nicotinamide. Each component was prepared separately, so that the addition of a small volume of each gave the final concentration required. To maintain the same concentration, buffer was added in the same volume as a particular component when the latter was omitted. The final pH of the medium was 6.8.

Metabolic studies. Comparative response of GPR and PR847 to various substrates, as measured by oxygen consumption, was the principal metabolic study. Conventional Warburg manometric techniques were employed (Umbreit, Burris, and Stauffer, 1957). The temperature was 37 C; the gas phase was air; carbon dioxide was absorbed by 0.2 ml of 10% potassium hydroxide in the center well. The total volume of liquid in the Warburg flasks was 2.2 ml. All experiments were controlled by adjusting suspensions to the same absorbancy and by using the same volume (2 ml) centrifuged at 10,000 $\times$ g for 10 min. The bacterial layer was resuspended in 2 ml of the substrate under study and placed in the main chamber of a Warburg flask. Additional controls in all experiments consisted of simultaneous determinations of endogenous respiration, which also served as a check on the purity of separation. The time set for the first reading was from 20 to 25 min after the organisms came in contact with the substrate. Oxygen uptake values were calculated from the first and second hour of respiration and were expressed as μliters O_2 consumed per hr. Oxidation rates were not corrected for endogenous rates.

RESULTS AND DISCUSSION

The aerobic respiratory rate of organisms separated from tissue fluid (GPR) was in general higher than that of staphylococci grown in vitro (PR847). One striking deviation from this trend was the endogenous respiration of PR847, which consistently averaged 50% over that of the GPR cells. The PR847 average was calculated from respiratory activity in buffer for 2 hr, but preliminary experiments indicated that endogenous activity could be measured for a period considerably longer than that of the GPR cells.

Preliminary experiments proved that there was no difference due to centrifugation in the respiratory levels of the in vitro strain. Variations of either GPR or PR847 caused by the use of different batches prepared at different times did not appreciably alter the average oxygen uptake. When GPR and PR847 were compared with regard to mannitol fermentation, pigmentation, size, shape, and staining properties, they were indistinguishable. But the metabolic activity, as measured by oxygen consumption, revealed some broad differences.

The responses of GPR and PR847 to the defined medium and to the incomplete fractions of it are summarized in Table 1. Whether the concentration of glucose was 0.0125 or 0.2 M, the organisms separated from tissue fluid respired at least 30% more effectively than staphylococci grown in vitro.

The possibility that filtrable tissue enzymes were carried over from the separation of GPR and thus accounted for a stimulatory effect was eliminated. In the absence of cocci, the filtrate from an infected guinea pig showed no uptake of oxygen even when glucose was included in the Warburg vessels.

Boiling the filtrate from an infected animal removed components inhibiting the aerobic metabolism of GPR and PR847 (Table 2). The pH change (6.8 to 5.9 after 2 hr, recorded by a Beckman pH meter) may partially account for the marked drop in respiration in the second hour.

All of the substrates contained glucose except the Trypticase soy broth at 40 mg per ml and the buffer used for studies of endogenous respiration. The preparation of albumin and globulin fractions involved the elimination of all dialyzable components; hence, the respiration is due almost entirely to glucose and to relatively short-term proteolytic products from the protein. The globulin fraction contained factors far more conducive to the respiration of GPR than of PR847.

Although the Trypticase soy broth I was pre-

TABLE 1. *Respiratory response of Staphylococcus aureus strains GPR and PR847 in defined media*

Substrate	Respiration, 60 min*			Respiration, 120 min*		
	GPR	PR847	Increase over PR847	GPR	PR847	Increase over PR847
	µliters O_2	*µliters* O_2	%	*µliters* O_2	*µliters* O_2	%
Glucose, 0.2 M	136.6 ± 18.6	79.2 ± 10.7	42	107.4 ± 16.3	74.5 ± 12.1	31
Complete medium†	267.1 ± 10.6	168.0 ± 5.6	37	258.8 ± 9.8	171.1 ± 10.0	34
Defined medium lacking all components except glucose	153.0 ± 15.0	101.3 ± 12.3	34	130.1 ± 16.0	88.5 ± 12.0	32
Defined medium lacking nicotinamide and thiamine ("staph factor")	292.7 ± 16.2	123.6 ± 8.3	58	285.0 ± 18.2	107.9 ± 11.0	62
Defined medium lacking alanine, valine, leucine, glycine, proline, aspartic acid, methionine	137.6 ± 13.0	148.4 ± 5.7	(7)‡	113.6 ± 14.1	141.7 ± 5.8	(20)
Defined medium lacking phenylalanine, tyrosine, arginine, histidine, lysine monohydrochloride, tryptophan, cystine	185.6 ± 11.7	165.6 ± 14.2	11	164.6 ± 16.7	153.2 ± 10.7	7
Endogenous respiration (buffer)	9.9 ± 2.8	22.8 ± 4.7	(57)	3.0 ± 2.4	5.7 ± 2.4	(47)

* Each value with its standard deviation represents the mean of three to five or more experiments. Statistical analyses were applied to replicate values according to the *t* test. A significant difference exists between each set of responses used for comparative purposes in the text. The highest values of *P* were <0.05.

† The final concentration of glucose was 0.0125 M; the final pH was 6.8.

‡ The parentheses refer to the percentage *below* the PR847 strain.

TABLE 2. *Respiratory response of Staphylococcus aureus strains GPR and PR847 in filtrate from an infected animal*

Substrate	Respiration, 60 min*			Respiration, 120 min*		
	GPR	PR847	Increase over PR847	GPR	PR847	Increase over PR847
	µliters O_2	*µliters* O_2	%	*µliters* O_2	*µliters* O_2	%
Filtrate†	405.1 ± 9.8	273.9 ± 23.7	32	305.4 ± 8.8	283.2 ± 17.0	7
Boiled filtrate†	440.6 ± 21.4	359.9 ± 13.1	18	304.7 ± 6.4	356.6 ± 26.0	(19)‡
Albumin fraction†	119.0 ± 8.0	101.2 ± 8.8	15	105.1 ± 2.8	86.7 ± 7.7	18
Globulin fraction†	194.3 ± 15.5	117.4 ± 10.0	40	156.0 ± 17.3	89.4 ± 5.5	43
Filtrate in Locke's solution (1:1)†	856.1 ± 26.0	517.0 ± 8.5	40	480.3 ± 12.0	546.9 ± 12.4	(12)
Endogenous respiration (buffer)	9.9 ± 2.8	22.8 ± 4.7	(57)	3.0 ± 2.4	5.7 ± 2.4	(47)

* Each value with its standard deviation represents the mean of three to five or more experiments. Statistical analyses were applied to replicate values according to the *t* test. A significant difference exists between each set of responses used for comparative purposes in the text. The highest values of *P* were <0.05.

† The final concentration of glucose was 0.0125 M.

‡ The parentheses refer to the percentage *below* the PR847 strain.

TABLE 3. *Respiratory response of Staphylococcus aureus strains GPR and PR847 in serum and in broth*

Substrate	Respiration, 60 min*			Respiration, 120 min*		
	GPR	PR847	Increase over PR847	GPR	PR847	Increase over PR847
	µliters O_2	*µliters* O_2	%	*µliters* O_2	*µliters* O_2	%
Serum from a normal guinea pig†	187.6 ± 2.6	145.1 ± 7.8	23	152.5 ± 4.0	139.7 ± 5.3	8
Boiled serum†	176.9 ± 8.7	161.5 ± 6.8	9	129.1 ± 2.8	145.4 ± 8.4	18
Trypticase soy broth I†	290.1 ± 6.8	198.1 ± 5.7	32	335.1 ± 15.4	202.9 ± 12.8	39
Trypticase soy broth II, 40 mg/ml	516.9 ± 10.6	345.6 ± 13.0	33	503.3 ± 2.0	385.1 ± 7.0	23
Endogenous respiration (buffer)	9.9 ± 2.8	22.8 ± 4.7	(57)‡	3.0 ± 2.4	5.7 ± 2.4	(47)

* Each value with its standard deviation represents the mean of 3 to 5 or more experiments. Statistical analyses were applied to replicate values according to the *t* test. A significant difference exists between each set of values used for comparative purposes in the text. The highest values of *P* were <0.05.

† The final concentration of glucose was 0.0125 M.

‡ The parentheses refer to the percentage *below* the PR847 strain.

pared in the same manner as the filtrate (Table 2), the filtrate was decidedly more stimulatory (Table 3). Trypticase soy broth II, prepared in full strength (40 mg/ml), still did not provide a substrate for a rate of oxygen uptake in the first hour comparable to that found when GPR and PR847 were respiring on filtrate diluted (1:1) with Locke's solution. The filtrate, whether whole or boiled, provided a substrate in which either GPR or PR847 approximately doubled its oxygen uptake over that of either whole or boiled serum.

The metabolic differences observed in the present work between *S. aureus* grown on Trypticase soy agar and after passage through an animal justify the latter as a metabolically altered organism. It should be possible to determine whether the organisms are changing because of an adaptive mechanism or whether selective processes, such as antistaphylococcal factors in healthy tissue and phagocytosis in the living animal, are necessary for such a metabolic change. The cavities of the guinea pigs in the present study were opened 7 to 9 hr after death. The chemical environment of the host and parasite may be significantly different before death or just as death occurs. This condition must be considered in the present conclusions and is presently undergoing investigation.

The extensive adaptability or mutability of staphylococci is well known. Gladstone's (1937) studies on the nitrogen requirement of *S. aureus* suggested that differences in nutritional requirements between strains of staphylococci are largely dependent on their previous nutritional conditions. The high endogenous respiration of PR847 compared to that of GPR may be a reflection of the greater synthetic powers of the former. The present results are also in accord with the general observation that growth in a complex medium almost invariably results in cells with a lower endogenous metabolism than does growth in a simpler medium.

If the assumption is made that the organisms separated from the peritoneal and thoracic exudates had the opportunity to possess more thiamine and nicotinamide than the organisms grown in vitro, then the striking difference in glucose metabolism is more easily understood. Kligler, Gossowicz and Bergner (1943) showed that nicotinamide and thiamine had a definite effect on the glucose metabolism of *S. aureus*. Thiamine, which catalyzes many reactions involved in the breakdown of pyruvic acid, had no effect on the anaerobic glycolytic reaction. But when both vitamins were present under aerobic conditions, 2.5 times as much glucose was utilized (compared to the glycolysis by *S. aureus* in the presence of nicotinamide alone). The defined medium lacking thiamine and nicotinamide stimulated the oxygen uptake of GPR just as much as did the complete medium. Such was not the case for the PR847 strain. That the GPR cells already had sufficient amounts of nicotinamide and thiamine stored may be a plausible explanation.

Filtrate from an infected animal, Trypticase soy broth, and boiled or whole serum, all prepared according to the same method, enhance the respiratory activity of both strains to different degrees: the filtrate proving far more stimulatory than the other complex substrates. A biochemical analysis of this filtrate to determine the various metabolites, bacterial products, and catalytic accessories may reveal factors which are not produced in vitro and which may be involved with the virulence of the organism. Antigenic and biochemical analyses of the filtrate and cells and the effect of endogenous respiration on the rate of oxygen consumption of *S. aureus* in various substrates are presently under consideration.

Though future investigation may elucidate mechanisms involved in producing the dissimilarity between the two strains, the existence of physiological differences is definite.

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