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Production of Dense Red Band Around Growth of *Staphylococcus aureus* on Blood Agar Plates. (27309)

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When certain strains of *Staphylococcus aureus* are grown on blood agar plates, a dense red band develops around the growth. The band appears around the hemolytic zones produced by strains that are hemolytic for human erythrocytes and directly around the bacterial growth of strains that are not hemolytic. Investigations have been carried out to determine the conditions under which this band develops and grows, and to define some of the factors necessary for its appearance.

Materials and methods. Most of the work was carried out with 2 coagulase-positive laboratory strains of *S. aureus* previously isolated from human beings—Strain Guy which hemolyzed and Strain Oxford which did not hemolyze human erythrocytes. The culture medium consisted of BBL trypticase soy agar to which 10% human blood (ACD anticoagulant) was added. Approximately 16 ml of this blood agar was poured into plastic petri dishes 9 cm in diameter. For inoculation, one loop of undiluted 18-hour meat infusion broth culture was spread on the center of the plate. After inoculation, the plates were incubated at 37°C for 4 to 6 days, and then kept at room temperature.

Experimental procedures and results. S. aureus (*Guy Strain*). On blood agar after 3 days' incubation at 37°C definite hemolysis appeared around the growth of *S. aureus*. In addition, a dense red band 0.2 to 0.4 cm wide, with indistinct outer margin, developed around this hemolytic zone. On further incubation, 5 days in all, the hemolytic zone around the growth measured 0.2 to 0.3 cm. (There was always an opaque ring in the approximate middle of this hemolytic zone. Since this observation is not germane to this study, it will not be discussed here.) At this time, a conspicuous well demarcated red band 0.8 to 1.0 cm in width encircled the hemolytic zone. The medium immediately around the red band was a paler red than the rest of the medium, which was bright red and partially hemolyzed (Fig. 1). After the plate had been kept at room temperature for several days, the red band gradually decreased in width, apparently due to extension of hemolysis encroaching on the inside of the band. When sealed by tape and kept at room temperature, these plates retained conspicuous though narrowing dense red bands, 0.2 to 0.3 cm wide, for at least 4 weeks.

Cutting of agar during growth of S. aureus (*Guy Strain*). Blood plates were inoculated centrally and then the agar was cut with a thin scalpel immediately after inoculation, and after 1, 2 or 3 days of incubation at 37°C. The cuts were made 0.5 cm from the growth or hemolytic area and at right angles to the growth and measured 1.5 cm in length.

When the agar was cut immediately after inoculation, the hemolytic zone reached to the cut but no hemolysis with red band developed beyond the cut even after long incubation at 37°C. However, a typical dense band developed around the growth away from the cut. Moreover, no clearing of the agar

occurred outside the cut, whereas the typical pale area developed surrounding the red band. When the plates were incubated for 2 days before the cuts were made, a typical red band developed surrounding the whole growth including the area beyond the cut (Fig. 2). These results are interpreted as evidence that within 2 days a factor had diffused through the agar into the band area, eventually producing the band effect.

Effects of placing sterile sections of agar, taken from plates previously inoculated with staphylococcus, onto the surface of blood agar plates (Guy Strain). Segments measuring 2 × 1 cm were taken from (a) the hemolytic area around growth, (b) the dense red band, (c) the pale area around the dense band and (d) the medium 2.5 cm out from the band, and transplanted onto other blood plates. After 5 days at 37°C and 3 days at room temperature, the following observations were made:

(a) A segment from the hemolytic area produced a hemolytic area around the section, then a dense red band; (b) A segment from the band area produced a dense red band directly around the section; (c) A segment from the pale area produced a narrow red band; (d) A segment from the medium 2.5 cm out from the band produced no band. The red band required 4 to 6 days at 37°C to develop, and remained unchanged in color and density for several weeks (Fig. 3).

In another set of experiments, sections taken at a distance of 0.5 cm from growth on *plain* trypticase agar and transplanted onto blood agar produced red bands indistinguishable from those produced by the transplantation of red bands. These latter experiments showed that blood is not required for production of the diffusible factor. In these experiments in any one plate in the same approximate area, the width of the red band depended directly upon the size of the sections transplanted; the larger the area transplanted the larger the red band that developed.

Production of circular dense red bands (Guy Strain). Blood agar was separated from plain agar by first pouring plates with trypticase soy agar. After the agar had solidified approximately one half the agar was removed in a straight line and discarded; then blood agar was poured into the empty part of each plate. Although care was taken to cover the entire empty part of the plate, microscopic examination showed that the blood agar and plain agar had not mixed; there was a definite line between the 2 types. These plates were inoculated on the surface of the *plain* agar at various distances from the dividing line between the 2 kinds of agar.

One experiment was carried out as follows: Each of 4 plates was inoculated on *plain* agar in 2 areas, 0.2 cm to 0.8 cm from the dividing

FIG. 1. *Guy Strain*—central inoculation on blood agar plate. Incubated at 37°C for 5 days shows zones from within out: bacterial growth, hemolytic area, dense red band, narrow clear area around band, partial hemolysis of outside medium.

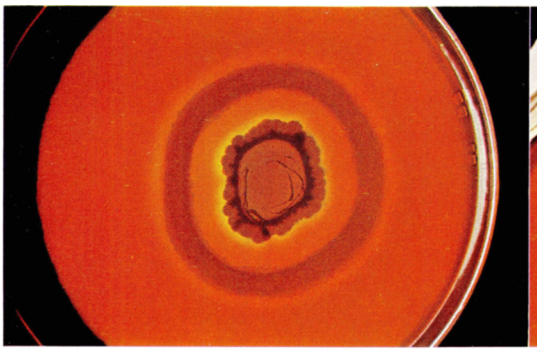
FIG. 2. *Guy Strain*—cut 2 days after inoculation. After 5 days at 37°C, 3 days room temperature, shows typical red band surrounding the whole growth including the area beyond the cut.

FIG. 3. Agar sections from blood agar plate on which Guy Strain had grown at 37°C for 5 days, room temperature for 6 days, were transplanted onto a second blood plate. The second blood plate was incubated at 37°C 5 days, room temperature 5 days. Location of sections surrounding bacterial growth below: *Right upper—Agar section from hemolytic area around growth* shows slight hemolysis surrounded by red band. *Right lower—Agar section from red band area*—shows red band directly around section. *Left lower—Agar section from narrow clear area immediately surrounding band*—shows narrow red band around section. *Left upper—Section from hemolyzing area 1.5 cm out from band*—shows no red band.

FIG. 4. *Guy and Oxford Strains*—inoculated separately on plain agar, on medium ½ plain agar, ½ blood agar. Incubated at 37°C 5 days, room temperature 5 days, shows: *Opposite growth of Guy Strain*—hemolysis invaginating the blood medium and circular red band beyond the hemolysis. *Opposite growth of Oxford Strain*—rounded red band (half-moon) in blood agar.

FIG. 5. Agar section transplanted from plain agar on which Guy Strain had grown at 37°C 5 days, room temperature 17 days was placed on plain agar of plate which was half plain agar, half blood agar. After incubation at 37°C 5 days, room temperature 3 days, shows red band in blood agar opposite section in plain agar.

FIG. 6. *Oxford Strain*—central inoculation—blood plate. Incubated at 37°C 5 days, room temperature 3 days, zones from within out: bacterial growth, red band, clear area surrounding band, partial hemolysis of outside medium.



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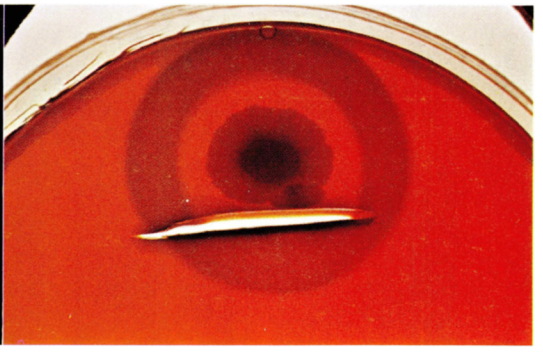

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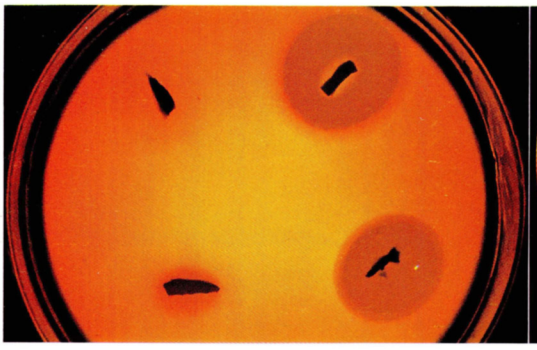

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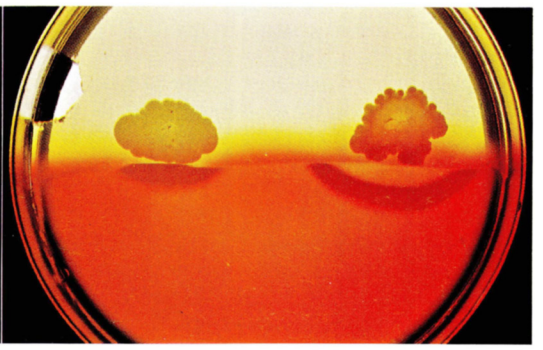

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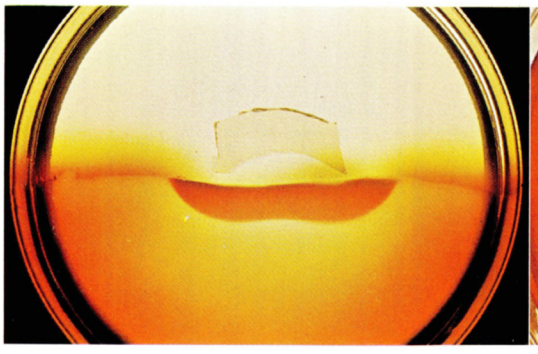

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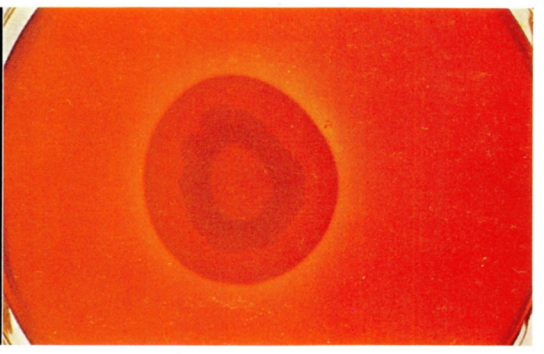

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line. After 5 days at 37°C all the plates showed virtually the same picture, a concentration of red material 1 to 1.2 cm in width, running along the *entire* dividing line on the blood agar side. There was no distinct concentration of this red material in the blood agar opposite the growth of *S. aureus* at this time. The rest of the blood medium was partially hemolyzed. After an additional 24 hours at 37°C, however, when *S. aureus* had been inoculated 0.2 to 0.5 cm from the dividing line, a 0.1 to 0.3 cm semicircle of hemolysis appeared in the blood agar opposite the growth of the organism. Directly surrounding this area, a 0.5 cm dense red semicircular band had developed reaching to the dividing line on each side (Fig. 4). Similar semicircular red bands could also be obtained in the absence of living *S. aureus* when sterile sections of red bands from blood plates or when sections of plain agar taken near the central growth of *S. aureus* were similarly placed (Fig. 5).

S. aureus (*Oxford Strain*). The principal differences between the reactions of the Oxford and the Guy Strain depended upon the presence of hemolysin (Guy Strain). Thus: (a) When the Oxford Strain was inoculated centrally upon blood plates, the red band developed directly around its growth (Fig. 6), whereas with the Guy Strain the red band developed around the area of hemolysis; (b) Certain sterile agar sections taken from previously inoculated plates with the Oxford Strain and transplanted onto other blood plates developed red bands directly around the transplanted sections, whereas comparable sections from the Guy Strain were surrounded by hemolytic areas, then the red bands; (c) On plates of one-half plain and one-half blood agar on which the Oxford Strain was inoculated on the plain agar, solid dense red bands (half-moons) developed (Fig. 4) instead of the hemo-circular red bands produced by the Guy Strain.

The red bands were produced more slowly by the Oxford than by the Guy Strain. This could be due to some difference in the postulated diffusible substance of the 2 strains or that the Oxford Strain produced less of the diffusible substance.

Discussion. A blood agar plate *macroscopically* is smooth, homogeneous, and bright red. However, *microscopically* an inverted 10% blood agar plate shows bright red clumps of erythrocytes scattered closely through the agar, whereas a dense red band produced by *S. aureus* is composed of packed clumps of red cells.

There is no doubt that there is an *appearance* of accumulation of red material in the dense red band around the growth of *S. aureus* on human blood agar. Neither is there any doubt that there is an appearance of accumulation of red material surrounding sterile sections of agar—either blood or plain—taken from near the growth of *S. aureus* and placed on blood agar. The question is whether such accumulation of red material is actually an accumulation, that is, a movement of red cell clumps and red cells into a specific area into which a hypothetic diffusible factor had previously diffused, or whether the diffusible substance merely prevents the clumps of red cells already in the band area from being hemolyzed, thus giving a semblance of accumulation.

Studies of the hemolysis of blood agar which eventually occurs in the medium outside the red bands will be reported later. That such hemolysis does not depend upon the growth of staphylococcus is evident from the fact that hemolysis eventually occurs in uninoculated blood plates when incubated at 37°C or kept at room temperature.

Since sterile sections of plain agar from plates previously inoculated with *S. aureus* produce red bands when transplanted onto blood plates, it is obvious that blood is not required for production of the diffusible factor, but that whole blood acts as indicator for demonstrating the phenomenon.

The nature of the phenomenon reported here is currently being studied, and various other strains of staphylococcus and other micro-organisms are also being tested for this phenomenon.

Summary. 1. When certain strains of *S. aureus* are grown on human blood agar, a dense broad red band develops around the growth. This band surrounds a hemolytic area produced by strains that are hemolytic

for human erythrocytes (*e.g.*, Guy Strain) and surrounds directly the growth of strains that are not hemolytic (*e.g.*, Oxford Strain). 2. Production of the red bands appears to depend upon a substance that diffuses through agar. 3. When sterile agar sections from plates previously inoculated with *S. aureus* are transferred onto blood plates, red bands develop around the transplanted section. 4. When plain agar is inoculated with the Guy Strain at a point near blood agar, a circular dense red band develops around the hemolytic zone in the blood medium; when inoculated with the Oxford Strain, a dense solid red band (half-moon) develops without hemolytic zone.

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Creatine Phosphorylkinase in Muscles of Dystrophic Mice.* (27310)

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Anomalies of creatine metabolism occur regularly in human muscular dystrophy, showing marked creatinuria(1) and diminished concentration of muscle creatine(2). Perkoff and Tyler(3) reported that the creatinuria of the Bar Harbor 129 strain of dystrophic mice is much less severe than human dystrophy. Kandutsch and Russell(4) showed that the decrease in muscle creatine is small in dystrophic mice when based on fat-free dry weight. This implies that certain biochemical dissimilarities exist between human dystrophy and the inherited dystrophy of mice.

From data obtained on biopsy specimens taken from gastrocnemius muscles of patients suffering from progressive muscular dystrophy, Ronzoni, Berg and Landau(5) found a close correlation between the low muscle creatine content and a depressed creatine-kinase activity. These investigators suggested that the low creatine content of dystrophic muscles is due to defective phosphorylation. However, Read and Nehorayan(6) showed that, in early vit. E-deficient rabbits, at a time when urinary excretion of creatine is rising, the creatine phosphorylating enzyme is actually in excess. Thus it would appear that increased urinary excretion of creatine and decreased muscle creatine may be due to causes other than depressed creatine phosphorylation.

The present report shows that there is no difference between the creatine phosphorylkinase activity from muscles of dystrophic mice and their littermate controls and is evidence of one further biochemical dissimilarity between human muscular dystrophy and that exhibited by the muscular dystrophic strain of mice.

Materials and methods. The mice studied were dystrophics (dy, dy) and heterozygous "carriers" (Dy, dy) obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The animals were 56 to 63 days old when used. Average weight of control animals was 24 g; that of dystrophic animals was 16 g.

The animals were given an intraperitoneal injection of sodium pentobarbital (40 mg/kg), the gastrocnemius muscles removed and chilled in chopped ice. Creatine phosphorylkinase was isolated from pooled muscle samples using the first 2 steps of procedure B of Kuby, Noda and Lardy(7). The enzyme was assayed using the procedure as outlined by Read and Nehorayan(6). Phosphorus was determined by the method of Fiske and Subbarow(8). Non-collagenous nitrogen (NCN) was extracted according to Lilienthal *et al.* (9). Nitrogen was determined by the method of Lanni, Dillon and Beard(10).

Results. In comparing the enzyme activi-

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