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Chemical Studies of the Red Band Produced by *S. aureus* on Blood Agar. (27601)

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The development of an opaque red band surrounding the growth of certain strains of *Staphylococcus aureus* cultured on human blood agar has recently been reported(1). This band first appears after 4 to 5 days incubation at 37°C, takes final form after 18 additional hours either at 37°C or at room temperature, and when fully developed has a sharply demarcated edge surrounded by a narrower area of pale red. A similar red band was observed when sterile pieces of plain or blood agar near which *S. aureus* had been grown were placed on blood agar plates and incubated at 37°C. Production of the red bands appeared to depend upon a substance that diffused through the culture medium.

Direct observations of the red band indicated that it consisted of an accumulation of erythrocytes (red cells). The concept that red cells could aggregate in this way was novel and hence proof was sought that red cells actually moved in the agar. Preliminary efforts at sectioning the agar were unsuccessful. We therefore resorted to chemical means to provide proof of the phenomenon; the results are reported here. Since completion of these experiments we succeeded in sectioning the agar; these observations will be reported separately.

Methods and results. 1. *Hemin and nitrogen content in the red band and outside it. Staphylococcus aureus* strain Guy (hemolytic), was inoculated in the center of 10% human blood agar in plastic plates(1). After incubation, pieces of agar weighing between 0.05 and 0.50 g were cut out both from the red band which had already appeared and from areas outside of it. The pieces were immediately weighed on a Roller-Smith spring balance (accuracy of 0.1 mg), dissolved in 5 ml 0.5N HCl, capped with tinfoil, and placed in boiling water for one hour. Acid hematin was measured at λ 520 in the Coleman Junior Spectrophotometer, using standards established with CP hemin and gelatin solutions (2). The material was then washed into Microkjeldahl tubes and total nitrogen determined(3).

Table I summarizes 6 of the studies dealing with hemin and nitrogen content. In column 1 is shown the number of days that the plates had been incubated at 37°C, and then at room temperature before each assay was made. The red band had 18-61% more hemin and 8-25% more nitrogen than the area outside of it (see columns 5 and 9).

Chromium 51 studies. Although the above experiments provided some evidence that red blood cells are moved into the band, they were not conclusive. They did not exclude the possibility that the band consisted of red cells prevented from hemolyzing by the diffusible factor, plus hemoglobin from hemolyzed red blood cells outside the band which had diffused throughout the plate thus raising the total hemoglobin in the band. There-

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TABLE I. Hemin and Nitrogen in Agar in Red Band and Outside Red Band around *S. aureus* Growth.

	1	2	3	4	5	6	7	8	9
		mg hemin/g agar				mg N/g agar			
Exp. No.	Days after inoc. (37°C/RT*)	Band	Outside band	Diff.	% avg hemin change	Band	Outside band	Diff.	% avg N change
1	6/ 5	2.6(3)	1.5	+1.1	+52	10.6(3)	8.9	+1.7	+17
2	6/ 2	1.5	.8	+ .7	+61	7.7	6.0	+1.7	+25
3	4/10	2.2	1.5	+ .7	+38	10.5	8.8	+1.7	+18
4	4/ 4	1.3	1.0	+ .3	+26				
5	5/ 0	1.3	1.0	+ .3	+26				
6	5/ 1	1.2	1.0	+ .2	+18	8.0	7.4	+ .6	+ 8

All determinations in duplicate except where parentheses show number averaged.
* Room temperature.

fore experiments were repeated using red blood cells tagged with radiochromium, and radioactivity as well as hemin and total nitrogen were measured daily during the development of the bands.

To the packed red blood cells from 10 ml citrated blood was added 10-50 μc $Na_2Cr^{51}O_4$† in 5 ml of saline(4). The plasma was reserved. The red cells and isotope were mixed, incubated at room temperature for 20 minutes, centrifuged at 1000 rpm for 20 minutes, washed twice with 8 ml saline, then mixed with the reserved plasma. This tagged blood was added to 90 ml agar at 48°C and poured into 5 glass petri plates. Under the conditions of these experiments we were able to show that after an initial 15% elution of Cr^{51} from red cells due to the heating required for making the medium, further elution after the plates were prepared was negligible during the remainder of the study period.

At intervals from 3 to 8 days after inoculation the agar of the plates was sampled. Eighty degree truncated, pie-shaped segments were cut aseptically from the edge of the hemolytic area surrounding the growth, or from the agar section, out to near the edge of the plates. The segments were cut into pieces 1 cm wide from the center out, and each piece was cut in two. They were weighed rapidly and dissolved in 2 ml 0.5N HCl. Radioactivity was measured in a Nuclear Chicago well scintillation counter to 10,000 counts. Three ml of 0.5N HCl were then added and hemin and nitrogen determined as above.

† Squibb sodium radio-chromate (Cr^{51}) with specific activity of 134 mc/mg was used.

The results of a typical experiment are given in Table II. Fig. 1 shows the manner in which one of the plates (A) was sampled. Of the 5 plates, one was an uninoculated control (E), 2 were inoculated with *S. aureus*, strain Guy, (A and B), and 2 were seeded with a section of plain agar near which the same organism had been growing for 5 days (C and D). Since the results in plates C and D were essentially similar to those in plates A and B, the former are not presented here.

The plates were incubated at 37°C for only

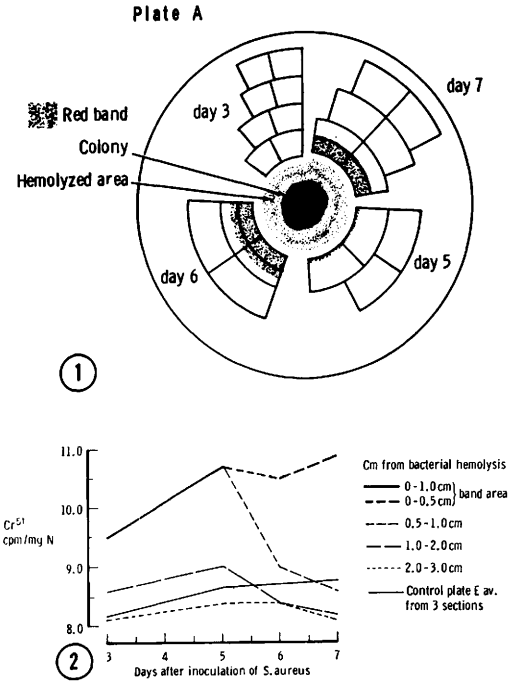

FIG. 1. Method of sampling of agar of plate A.
FIG. 2. Cr^{51} in cpm/mg nitrogen in agar during development of red band around *S. aureus* colony.

TABLE II. Hemin, Chromium[51] and Nitrogen in Agar during Development of Band around *S. aureus*.

	Days after inoc.	Distance in cm from hemolysis, plates A & B				Distance from center of control, plate E		
		0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	1.0–2.0	2.0–3.0	3.0–4.0
		0–.5 .5–1.0						
Hemin, mg per g agar	3	1.16	1.14	1.26	1.27	1.23	1.21	1.21
	5	1.34*(2)	1.28(2)	1.23		1.26	1.30	1.32
	6	1.65†(2)	1.44(2)	1.40				
		1.84 (1.46)						
	7	1.68‡(2)	1.75	2.02		1.40	1.42	1.49
		1.98(2) 1.38(2)						
Cr^{51}, cpm per g agar	3	49.0	45.4	45.6	47.2	44.6	45.5	46.1
	5	56.4 (2)	53.2 (2)	48.8		49.8	51.6	53.0
	6	61.8 (2)	51.8 (2)	48.8				
		70.9 (52.6)						
	7	(65.7)(2)	62.4	71.1		51.4	51.1	55.9
		79.9 (2) 51.6 (2)						
N, mg per g agar	3	5.15	5.27	5.64	5.93	5.37	5.53	5.78
	5	5.30 (2)	5.91(2)	5.78(2)		5.88	5.82	6.14
	6	6.28 (2)	6.19(2)	5.83				
		6.76 (5.80)						
	7	(6.68)(2)	7.61	8.81		5.83	6.02	6.21
		7.34(2) 6.01(2)						

* Beginning band on inner edge of section.
† ⅔ of the 1-cm width is band.
‡ ½ of the 1-cm width is band.
(2) Figures in which results from A and B are averaged.
( ) On day six, 0.5-cm wide and 1.0-cm wide samples were cut at the inner margin. On day seven, 2 half cm wide samples were cut at the inner margin. This permitted the calculated figures shown in parentheses for hemin, Cr^{51}, and nitrogen in the agar 0.5 to 1.0 cm from the hemolytic area on day six, and 0 to 1.0 cm from the hemolytic area on day seven, when there was insufficient agar for direct sampling. The hemolytic area encroaching on the inner rim of the band was avoided in sampling the band.

2 days to retard the development of the band, then left at room temperature. On the third day no red band was yet visible in any plate. Segments of agar were taken from plates A and E.

On the fourth day, the second at room temperature, there was no change, but on the fifth day a definite narrow red band was visible around the growth in plates A and B. Segments of agar were taken from plates A and/or B and from plate E on the 5th, 6th, and 7th days. Results of plates A and B were averaged, and are tabulated in the table with results from the control plate E.

As the footnotes of the table indicate, the 1-cm wide piece of agar taken from the area closest to the zone of hemolysis contained on day 5 an edge of increased density representing the beginning of the band. An day 6 the red band had grown to occupy ⅔ of the 1-cm width of the sample, and on day 7 it had shrunk to one-half of the 1-cm width. The narrowing of the band was due to bacterial hemolysis at its inner rim. The concentration of Cr^{51} and hemin in the remaining narrower band was unaffected by the hemolysis (Table II and Fig. 2).

The Table brings out the fact that between 3 and 5 days, hemin, Cr^{51}, and nitrogen increase rapidly in a band around the colony. The content of these substances outside the band was lower than that in the band itself and was comparable to that in the control plate.

However, all 3 substances, hemin, Cr^{51}, and nitrogen increase with time, especially in the periphery of the plates from day 6 to day 7. It was obvious by observation that the plates were drying rapidly. An attempt was made to correct for the drying by calculating hemin and chromium in terms of mg and cpm, respectively, per mg nitrogen. The data are plotted in Fig. 2 for Cr ^{51} per mg nitrogen. The high level of Cr^{51} per mg nitrogen on days 5, 6,

and 7 in the band contrasts with the low levels in the rest of the plates and in the control plate. Note in Fig. 2 that the largest increase of Cr^{51} occurred between days 3 and 5, after which there was little further increase.

The use of nitrogen as the basis for measuring changes of hemin and Cr^{51} is not entirely valid in view of the fact that the red cells being moved contain an appreciable amount of nitrogen. However, this fact would tend to decrease the difference between the hemin and chromium per mg nitrogen in the band and that outside the band. Therefore any difference found between the two would be the more significant.

Discussion. The chromium studies provide evidence to support the thesis that red blood cells are actually attracted through agar toward a substance which diffuses from the growth of *S. aureus*. There is a true increase of hemin and Cr^{51}, which presumably measure red blood cells, in the region of the red band, *over that in any other part of the* S. aureus *plates or in the control plate.* This is especially evident when the hemin and Cr^{51} are calculated per mg total nitrogen to correct for drying of the plate.

Our hypothesis is that the substance attracting red cells diffuses from the *S. aureus* growth on agar into a well-defined area of 0.6 to 1 cm width. This diffusable substance draws red cells singly and in clumps to this area from the 0.2- to 0.4-cm wide region around it. The chemical data appear to support the hypothesis. Visually it appears that the band develops quite quickly after 4 to 5 days of incubation. Data here show that the effect develops over the course of one day, and that there is little change thereafter. The red cells seem to pack into the area of the band until there is no more room for them. In some specimens, indeed, the agar in the band bulges upward as though swollen with red cells.

Summary. *S. aureus* grown on whole blood agar produced a diffusible material which caused changes in the nitrogen, hemin and red cell content of the agar. Experiments using blood cells tagged with Cr^{51} indicated that the dense red band around the growth of the organism was due to a concentration of red blood cells which had been moved into the area of the band from the area just outside it.

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Immunologic Studies on the Mechanism of Action of Erythropoietin.* (27602)

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The ability of serum obtained from rabbits previously immunized with human urinary erythropoietin to neutralize the erythropoietic activity of human urinary erythropoietin has been demonstrated(1). Injection of such sera into normal mice markedly decreased their normal 24-hour incorporation of Fe^{59} into circulating red blood cells and their peripheral reticulocyte count. This neutralization was considered to be the result of an antigen-antibody reaction directed against either the erythropoietically active molecule or the general class of molecules to which erythropoietin belongs. The present experiments are

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