TABLE IV. Dermal Concentration of Citrate Soluble and Insoluble Hydroxyproline (μmoles/g) in Injured Skin Distal to Site of Local Injury in Normal and Rachitic Rats.

	Normal		Rachitic	
Days after injury	.50M citrate	Insoluble	.50M citrate	Insoluble
Control	8.0	207	*25.0*	*157*
2	18.0*	148*	*22.3*	*178*
4	12.6*	90*	*28.0*	*160*
6	12.5*	120*	*23.3*	*180*
10	12.6*	149*	*28.1*	143
15	13.0*	156*	*26.8*	150
23	8.4	200	*22.0*	*142*

* Significantly different from control ($p < .05$). Italicized means are significantly different from injured normal animals ($p < .05$).

hydroxyproline (salt-soluble collagen) found in the uninjured skin of non-rachitic rats with local injury was not found in the uninjured skin from rachitic animals. Similarly no changes in concentration of isotonic saline soluble hydroxyproline were found in the skin from these animals, whereas the skin from normal animals demonstrated some significant increases in the amount of hydroxyproline soluble in this solvent.

The changes in the citrate soluble and insoluble concentrations of hydroxyproline from the uninjured skin of non-rachitic rats are contrasted in Table IV with the lack of effect of local inflammation upon these components of the skin from rachitic animals. The increased concentration of citrate soluble collagen and the loss of insoluble collagen from the uninjured skin of non-rachitic rats is typical of the previously described "distant dermal collagen response"(3,4). The rachitic rat does not demonstrate, therefore, such a response.

Summary and conclusion. Rachitic rats, which have been shown to be refractory to cortisol insofar as their connective tissue chemistry is concerned, demonstrated a chemical response to local injury which was qualitatively similar to that evidenced in non-rachitic animals. Quantitatively, the chemical alteration in inflammation in the rachitic animals was less profound than that found in the non-rachitic rats. Indeed, the wounds in the rachitic animals were chemically healed within 23 days after injury whereas the non-rachitic rats were not. Finally, the cortisol refractory rachitic animals did not demonstrate a "distant dermal collagen response" to local inflammation, confirming previous reports suggesting that this response was a hormonally potentiated response to the stress produced by local trauma.

1. Templin, V., Steenbock, H., *J. Biol. Chem.*, 1933, v100, 217.

2. Jacob, R. A., Houck, J. C., PROC. SOC. EXP. BIOL. AND MED., 1963, in press.

3. Houck, J. C., Jacob, R. A., *ibid.*, 1958, v98, 655.

4. ———, *ibid.*, 1961, v107, 280.

5. Houck, J. C., *Surgery*, 1962, v51, 770.

6. Jacob, R. A., Houck, J. C., *Surg. Gyn. & Ob.*, 1958, v109, 85.

7. Houck, J. C., Jacob, R. A., *J. Invest. Dermatol.*, 1961, v36, 451.

8. Houck, J. C., Jacob, R. A., Vickers, K., *Am. J. Path.*, 1962, v40, 531.

9. Houck, J. C., Jacob, R. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 324.

Received November 5, 1962. P.S.E.B.M., 1963, v112.

Production of Octadecenoic Acid in Plasma by *Staphylococcus aureus*. (28073)

JULIA T. WELD, B. H. KEAN AND WILLIAM M. O'LEARY
Departments of Public Health and Microbiology, Cornell University Medical College, New York City

The development of unique red bands around the growth of certain strains of *S. aureus* on blood agar plates has been reported (1). During these studies, gray plaques were observed on the surface of the blood agar. The purpose of this paper is to describe methods of producing these plaques and to report on their chemical identification.

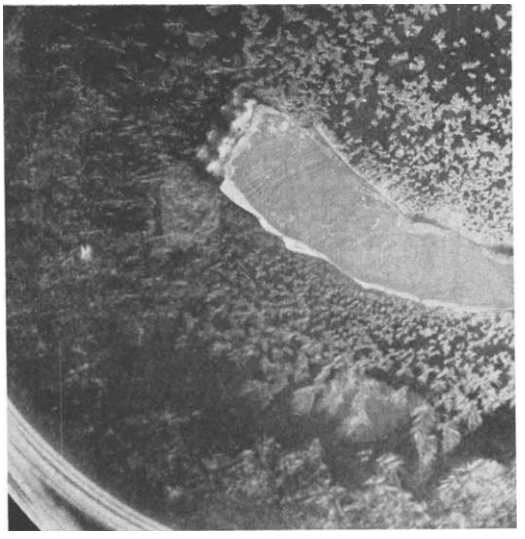

FIG. 1. Masses of plaques on the surface of an agar plate surrounding a sterile agar segment which was taken from another plate 10 days after inoculation with *S. aureus*. Photograph was taken after the plate with the agar segment had been incubated at 37°C for 5 days and at room temperature for 25 days (× 1.5).

Materials. 1. Most of this work was carried out with coagulase-positive *S. aureus* (Guy strain) which was hemolytic for human blood and which produced the red bands on blood medium. Thirty other strains of *S. aureus* were also tested for the production of plaques.

2. Two types of agar plates were used: (a) B.B.L. Trypticase Soy Agar to which 5% human blood (ACD anticoagulant) was added; (b) 10% plasma agar, 25 ml for each plate.

3. To obtain active sterile agar segments (ASA segments) plain Trypticase Soy Agar plates (25 ml for each plate) were inoculated centrally with *S. aureus* and incubated at 37°C for 5 days and at room temperature for 2-5 weeks. ASA segments were cut approximately 0.5 cm from the growth of the organism and measured approximately 0.7 cm in width and 2 cm in length.

4. To prepare 10% plasma liquid, ACD plasma was diluted to 10% with broth or saline.

Methods and results. The 5% blood agar plates and the 10% plasma agar plates were inoculated centrally with *S. aureus*, incubated at 37°C for 5 days, then left at room temperature. Usually 2 ASA segments were placed near the center of similar plates which were incubated in the same fashion. Discrete, plainly visible gray plaques developed on the surfaces of all these plates. The plaques were first seen scattered on the surface of the red bands after incubation at 37°C for 5 days. With longer incubation at room temperature these plaques progressively increased in size and number until hundreds appeared beyond the red bands and eventually were scattered irregularly over the entire plate.

When ASA segments were placed on 5% blood agar plates and on 10% plasma agar plates, plaques similar to those described with the living organism appeared. The plaques, however, developed closer to the ASA segments than they did to colonies in plates with living organisms.

Surface plaques also developed in liquid media. ASA segments were inserted into tubes containing 10% plasma diluted with Trypticase broth or saline and incubated at 37°C. After 1-5 days, thick plaques similar to those observed on the solid media covered the surface of the liquid.

The blood from some individuals was not able to act as substrate for the development of plaques. Thus of 11 different transfusion bloods obtained from the blood bank, 7 were consistently effective as substrate and 4 were not. Plaques did not develop on blood plates prepared with washed red cells.

Twenty different strains of *S. aureus* that produced red bands and 10 strains of *S. aureus* that did not were tested for their ability to produce plaques on blood media. With the exception of the Smith strain(2), all strains that produced red bands also produced plaques whereas those that did not produce red bands did not produce plaques.

Experiments with Staphylococcus antitoxin. Commercial antitoxin prevented the development of plaques on both solid and liquid media. Plasma agar plates were prepared with 22 ml agar plus 2.5 ml plasma plus 0.5 ml antitoxin (Burroughs Welcome Staphylococcus antitoxin containing 20,000 units in 18.5 ml). Control plates were prepared with 0.5 ml saline instead of antitoxin.

ASA segments produced no plaques in the

plates containing the antitoxin even after 2 weeks whereas on the control plate typical plaques appeared after 3 days.

ASA segments were inserted into tubes containing plasma 0.8 ml, saline 6.9 ml, and antitoxin 0.3 ml. Controls were set up with saline instead of antitoxin. In the tubes containing antitoxin no plaques developed, whereas in the control tube without antitoxin plaques developed after 3 days.

Chemical investigations. Recovery of the material from agar surfaces was difficult and gave low yields, but preliminary observations suggested that the plaques were composed of lipid-like material.

Higher yields were obtained by inserting several ASA segments into tubes containing 20 ml of 10% plasma diluted with trypticase-soy broth or saline. After 1-5 days at 37°C a thick lipid-like plaque material developed on the surface of the broth. This material resembled the plaques seen on the surface of the agar plates and later was proved identical.

The surface plaque material was separated from the subjacent liquid by mechanical methods, washed 3 times with distilled water, dissolved in hexane and transferred to a small flask. After drying over anhydrous sodium sulfate, the hexane was evaporated leaving a colorless, slightly turbid oil.

Gas chromatography. The material was hydrolyzed with methanolic potassium hydroxide and the free fatty acids were isolated using the conventional technics of ether extraction. These acids were methylated using the boron trifluoride technic of Metcalfe and Schmitz(3). The sample so prepared was then analyzed using a Perkin Elmer model 154D vapor fractometer fitted with a 4 meter silicone column and a thermistor detector.

Repeated analyses showed the sample to be composed of octadecenoic acid (presumably oleic), 70%; stearic acid, 8%; palmitic acid, 15%; and minor amounts of myristic and other shorter chain acids totaling 7%. Following microhydrogenation of the remaining sample, it was found that the octadecenoic had disappeared and that there was a corresponding increase in the amount of stearic acid.

Thin-layer chromatography. After finding that hydrolysis of the surface material did, indeed, yield fatty acids, efforts were made to determine the types of compounds in which these acids occurred in the original plaque material. Thin-layer chromatography was the technic chosen to elicit this information. Silica Gel G (Merck, Germany) was used as the absorbent in all analyses. Two different solvent systems were employed, petroleum ether-ethyl ether (90/10/1) and chloroform-methanol-water (7/3/.5). After development, all plates were sprayed with 2,7-dichlorofluorescein, and spots were observed under ultra-violet light.

These studies showed that the plaque material produced in the original tubes of media consisted of free octadecenoic acid and triglycerides. The ratio of free acid to triglyceride was approximately one to three.

Discussion. Apparently many strains of *Staphylococcus aureus* produce a diffusible extracellular substance which causes the appearance of octadecenoic acid both free and in triglycerides in preparations containing plasma. The evidence indicates that this lipid material is derived from the plasma rather than being formed *de novo*. In plasma, triglycerides occur in complexes with alpha and beta-globulins, and unesterified fatty acids are combined as lipoproteins with the albumin fraction(4). Also, the chylomicrons are known to be 85-90% triglyceride. Thus the agent producing plaques may be an extracellular lipase elaborated by staphylococci.

Lipolytic effects have been detected in cultures of *S. aureus* by other workers (Elek) (5). Gillespie and Adler(6) observed as in the present study that lipolysis was inhibited by addition of Staphylococcus antitoxin.

The coalescence of chylomicrons into particles so large that they no longer remain dispersed is another, if unlikely, explanation of the formation of plaque material.

Preliminary studies now under way indicate that serum may be substituted for plasma; that only a percentage of serums can release plaque material under standard conditions; and that plaque formation varies in different animals. These host variations may

have clinical significance which is being evaluated currently. Other efforts are being directed toward isolation and identification of the diffusible substance and the plaque material which it produces and an elucidation of the specific substrates involved.

The diffusible substance that activates production of the red band differs from the substance that produces the plaque material. This is demonstrated by the fact that whereas all the bloods used for preparation of the media were able to act as substrate for the red band, a few did not act as substrate for production of plaques.

Summary. Macroscopically visible lipid plaques were produced on the surface of human blood agar plates and on 10% plasma agar plates when they were inoculated centrally with certain strains of coagulase-positive *S. aureus* or exposed to active sterile agar segments (ASA). Similar lipid plaques covered the surface of liquid media containing plasma when activated sterile agar segments were suspended in the media. Staphylococcus antitoxin prevented the appearance of the lipid plaques. The lipid plaques consist primarily of octadecenoic acid, both free and in triglycerides. Lesser amounts of stearic, palmitic and various shorter chain acids are also present. The most likely explanation for the phenomenon is that a lipase released by certain strains of staphylococci acts upon the lipids in human plasma.

1. Weld, J. T., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 693.

2. Hunt, G. A., Moses, A. J., *Science,* 1958, v128, 1574.

3. Metcalfe, L. D., Schmitz, A. A., *Anal. Chem.,* 1961, v33, 363.

4. West, E. S., Todd, W. R., *Textbook of Biochemistry,* 3rd ed., Macmillan, N. Y., 1961, p891.

5. Elek, S. D., *Staphylococcus Pyogenes,* E. S., Livingstone, Ltd., Edinburgh and London, 1959, p290.

6. Gillespie, W. A., Adler, V. G., *J. Path. Bact.,* 1952, v64, 187.

Received November 7, 1962. P.S.E.B.M., 1963, v112.

A Permanent Rat Gastric Fistula. (28074)

S. A. KOMAROV, S. P. BRALOW AND E. BOYD (Introduced by Harry Shay)
Fels Research Institute, Temple University School of Medicine, Philadelphia, Pa.

Our preliminary report(1) on basal gastric secretion in chronic fistula rats aroused much interest in our method of producing a permanent type of rumenal gastric fistula. We and others(2-4) believe that a chronic fistula in the rat may be of great value for investigation of basic problems of gastric secretion. It, therefore, seems desirable to describe in detail the technic used during the past 3 years.

Methods. A miniature stainless steel cannula of the type extensively used in Pavlov's laboratories was developed. The cannula is 10 to 12 mm in length with an inner bore of 3 mm diameter and a beveled flame at each end (Fig. 1). The inner bore at one flange is threaded to permit insertion of a removable screw which acts as a stopper between experiments. The lumen of the cannula permits a snug fit with a No. 8 French catheter.

Fasting prior to surgery was found to be unnecessary and a rumen containing food is easier to identify and to exteriorize. Under ether anesthesia, the left upper quadrant of the abdomen is shaved and the animal placed on its right side. An incision 5-10 mm in length is made about 5 mm below and parallel to the lower left costal margin between the parasternal and left mammary lines. This approach has been described(5). When the deep abdominal muscles are divided, 2 guide sutures are inserted at the margins of the muscular incision and held in place by small hemostats. The most dependent area of the rumenal wall is then drawn up through the incision. This area should be close to the greater curvature but far enough away from the glandular portion of the stomach to avoid sacrificing secretory mucosa. To permit ade-