and probably other factors of the vitamin B complex for normal growth under the conditions of these studies. Possible factors altering the requirement of choline for dogs on synthetic rations are discussed.

13142

Isolation and Pure Cultivation of the Smaller Mouth Spirochetes by an Improved Method.

THEODOR ROSEBURY AND GENEVIEVE FOLEY. (Introduced by A. R. Dochez.)

From the Department of Bacteriology, Columbia University, New York.

The characteristics and properties of the so-called commensal spirochetes of mucous membranes, and their individual or contributing rôles in fuso-spirochetal infections, remain inadequately understood largely because of a lack of effective means for their isolation and maintenance in pure culture. *Treponema vincenti,* the large delicate loosely wound spirochete of the mouth, seems never to have been obtained in pure culture; *T. buccale,* the large, thick, loosely wound form, has been reported in culture rarely,[1, 2, 3] apparently under conditions which could not be duplicated. *T. microdentium,* the small mouth spirochete which resembles *T. pallidum* in morphology and is cultivable with the least difficulty,[4–8] seems for that reason to be the only member of the group which can be accepted with little doubt as a distinct species. Studies of pure cultures of *T. microdentium,* moreover, have yielded interesting information on the pathogenesis of fuso-spirochetal infections.[5, 6, 9, 2] On the other hand, none of the other varieties which have been described and named, such as Noguchi's *T. macrodentium*[4] or the several species named by Seguin and Vinzent,[10] is so clearly defined.

1 Smith, D. T., *Oral Spirochetes and Related Organisms in Fuso-Spirochetal Disease,* Baltimore, Williams and Wilkins Co., 1932, p. 11.

2 Proske, H. O., and Sayers, R. R., *U. S. Pub. Health Rep.,* 1934, **49**, 839, 1212.

3 Vinzent, R., and Daufresne, M., *Compt. rend. Soc. Biol.,* 1934, **116**, 490.

4 Noguchi, H., *J. Exp. Ped.,* 1912, **15**, 81.

5 Kritchewski, B., and Seguin, P., *Rev. stomatol.,* 1920, **22**, 613.

6 Smith, D. T., *Am. Rev. Tuberculosis,* 1927, **16**, 584.

7 Smith, D. T., *loc. cit.,* 1932, p. 8.

8 Seguin, P., and Vinzent, R., *Ann. de l'Inst. Pasteur,* 1938, **61**, 255.

9 Smith, D. T., *loc. cit.,* 1932, p. 124.

10 Seguin, P., and Vinzent, R., *Compt. rend. Soc. Biol.,* 1936, **121**, 408.

Most successful attempts to cultivate the smaller commensal spirochetes[4-8, 3, 10, 11] have utilized methods based on those developed by Noguchi.[4, 12] These methods are laborious and intricate, particularly in that the steps necessary to obtain pure cultures from the invariably mixed source material are often discouragingly difficult, as exemplified in a recent report by Kast and Kolmer.[13] Experiences with the older methods in this laboratory[14] have led to the development of a modified technic which has greatly simplified what had been the most difficult phase of this work, namely, the isolation of spirochetes from a primary mixed culture.

The distinguishing feature of the method for primary isolation lies in the use, in place of Noguchi's deep tubes, of small Petri plates containing a thick layer of solid medium, which are inoculated by stabbing into the side of a well previously cut in the center of the plate, and incubated in anaerobic jars. Two sizes of plates have been used, 50 x 14 mm (overall) and 60 x 18 mm, either of which is satisfactory if uniform in depth. About 10 cc of medium is placed in the smaller plates and 20 cc in the larger, so that the depth of medium in the center of the plate is at least 7 mm. Noguchi's solid media[12] may be used, enriched with 1 part of sterile serum or ascitic fluid to 2 parts of agar base and containing fresh sterile tissue, the tissue, however, being minced fine and distributed on the floor of the plate instead of being added in a single piece as in tubes. In place of the usual enriching fluids, serum ultrafiltrate, as used by Simms and Stillman[15,*] for tissue culture, and by Sanders[16] and Molloy[17] for the cultivation of several viruses, has been found more satisfactory, in that repeated batches have yielded consistently effective media. Occasional batches of ascitic fluid have yielded somewhat wider

11 Vinzent, R., Seguin, P., and Daufresne, M., *ibid.*, 1936, **121**, 406; Seguin, P., and Vinzent, R., *ibid.*, 1936, **121**, 488; Vinzent, R., and Seguin, P., *ibid.*, 1939, **130**, 12; *Bull. de l'Acad. Med.*, 1939, **121**, 407.

12 Noguchi, H., *J. Exp. Med.*, 1912, **15**, 90, 466; 1912, **16**, 194; 1913, **17**, 89.

13 Kast, C. C., and Kolmer, J. A., *Am. J. Syph.*, 1940, **24**, 671.

14 Rights, F. L., unpublished data.

15 Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, **20**, 649; *Arch. Path.*, 1937, **23**, 316, 322.

* We are grateful to Dr. Simms for his interest in these experiments and for supplying us with serum ultrafiltrate in the early part of this work, and to Warner Institute for Therapeutic Research, 113 West 18th Street, New York City, for supplying the ultrafiltrate more recently. This institute is now prepared to supply serum ultrafiltrate made according to Dr. Simms' directions.

16 Sanders, M., *J. Exp. Med.*, 1940, **71**, 113; Sanders, M., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 327.

17 Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 563.

zones of spirochetal growth than comparable media containing serum ultrafiltrate, but other batches of ascitic fluid have been worthless.

The agar base used in these studies, both for isolation and for maintenance of pure cultures, is the veal heart agar of Proske and Sayers,[2] without modification. This agar base may be prepared in large batches and stored in the refrigerator for several months. Sterile serum ultrafiltrate may likewise be stored in sealed containers for at least two months.

The tissue used in most of these experiments has been guinea pig kidney; of the other tissues tried, guinea pig testicle seemed less effective, and rabbit kidney or testicle were not more effective. A guinea pig is exsanguinated and the kidneys removed aseptically and carefully freed from capsular connective tissue and fat. The two kidneys are then minced fine—so that the largest particles are about 2 mm wide—in 10 cc of serum ultrafiltrate diluted 1 part with 2 parts of Simms' salt solution.[18] Simms and Sanders[18] report that the cells in such finely minced adult tissues remain healthy for long periods in diluted serum ultrafiltrate, whereas larger fragments rapidly become necrotic at the center. About 0.1 cc of the suspension of minced kidney is used for each 10 cc of final medium. The tissue is transferred to the plates first with a sterile wide-tip pipette or glass tube. The agar base, liquefied and cooled to about 50°C, is then added to undiluted serum ultrafiltrate (warmed to about 40°C) in the proportion 2 parts of agar base to 1 part of ultrafiltrate, and the mixture poured into the plates over the minced kidney in such a way as to distribute the tissue particles uniformly. Plates are incubated aerobically at 37°C overnight, and then anaerobically at 37°C for an additional 24 hours or more to assure sterility before use. The plates may subsequently be stored in air at room temperature for at least 2 weeks without impairment of their ability to grow spirochetes.

Just before use a well is cut in the center of each plate with a pipette drawn from thin-walled glass tubing so that the diameter of the tip is about 2 mm. The well should preferably not reach the bottom of the plate, and care must be used to avoid undercutting the agar. A single stab inoculation is made with a straight platinum needle to a depth of about 2 mm obliquely into one side of the well, in such a way that neither the upper nor the lower surfaces of the agar are touched by the inoculum. In general, and particularly when a concentrated inoculum such as undiluted exudate is used, three or more plates should be stabbed serially without recharging the

[18] Simms, H. S., and Sanders, M., to be published.

needle. Disruption of the medium by gas-forming bacteria is rarely seen in these plates because the well provides a pathway of escape for gas; but such disruption—one of the common difficulties with the tube method—may occur if the inoculum is too heavy or stabbed too deeply.

The inoculated plates are incubated at 37°C inverted in jars made anaerobic with hydrogen, catalyzed by electrically heated platinized asbestos, and containing 5% of carbon dioxide. Spirochetes begin to appear on about the 4th day in primary plates, and are ready for examination and transfer on the 5th to the 7th day.

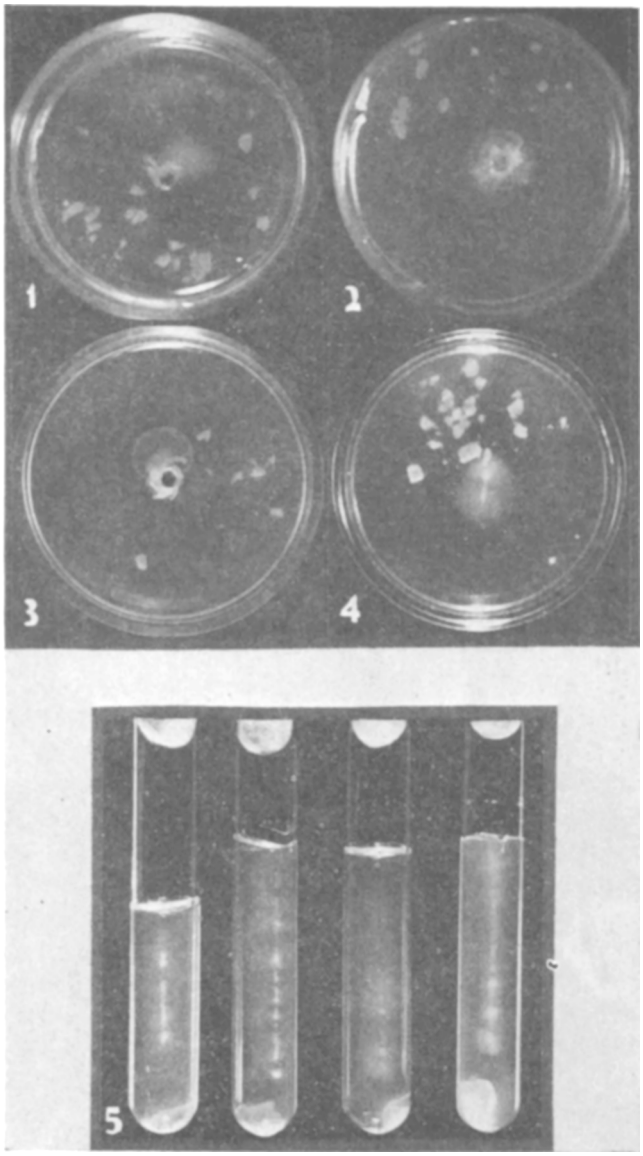
In a successful primary plate bacterial contaminants are limited chiefly to the immediate vicinity of the stab and to the sides of the well, and do not extend more than a few millimeters over the surface of the plate from the well edge. The spirochetes, on the other hand, grow in the depths of the medium as a clearly visible haze extending well beyond the surface bacterial growth under the sterile agar surface. They can, therefore, be obtained free from bacteria merely by removing a portion of the infiltrated agar through the sterile surface at a distance from the bacterial growth.

By the use of this method of primary isolation, spirochetal growth accessible without contamination has been obtained in plates from 23 of a total of 29 clinic cases, chiefly fuso-spirochetal mouth infections.† Of the 6 failures one was a case of fuso-spirochetal empyema in which exudate taken at operation showed no spirochetes under darkfield illumination but nevertheless induced a typical fuso-spirochetal response when inoculated subcutaneously into a guinea pig. The other 5 samples which yielded negative cultures were also either poor in spirochetes originally, or had been allowed to stand before inoculation until motility was lost. It is interesting that primary plates inoculated with exudates from experimental fuso-spirochetal infection in guinea pigs[19] have been successful from all of the 21 animals tested.

These findings indicate the positive nature of the method of primary isolation. On the other hand, whereas mixed bacterial and spirochetal growth can easily be subcultured repeatedly merely by transferring mixed growth from the central well with a straight needle to similarly prepared sterile plates, successful pure subculture of spirochetes is more difficult. Pure cultures have been obtained

† Heavy suspensions of gingival scrapings in 1 cc of broth were prepared for this purpose through the courtesy of Dr. D. E. Ziskin and his staff in the School of Dental and Oral Surgery, Columbia University.

19 Rosebury, T., and Foley, G., *J. Am. Dent. Assn.*, 1939, **26**, 1798.



FIGS. 1, 2, 3.

Primary plates inoculated by stab in central well with fuso-spirochetal exudates and incubated anaerobically at 37°C for 5 to 7 days. Scattered particles are fragments of minced guinea pig kidney; lighter sector in upper portion of Fig. 1 is kidney sediment. Mixed bacterial growth is present around the central well, with spirochetal growth extending beyond it in the depths of the medium. The three plates show different types of spirochetal growth.

FIG. 4.

Pure plate culture of *Treponema microdentium* incubated 7 days anaerobically at 37°C. Kidney fragments are clumped chiefly in the upper portion. The vertical white line at the center of the area of spirochete growth is the residue of the inoculum, which apparently increases in density during incubation.

FIG. 5.

Four pure tube cultures of spirochetes, incubated 7 days anaerobically at 37°C. The vertical white line in each is the inoculum, intensified during growth; the infiltrating hazes of spirochetal growth show different characteristics in the 4 tubes.

in plates, using the same medium without wells, by transferring a column of spirochete-infiltrated agar with a sterile capillary pipette. Spirochetes growing nearest to the bacteria in the well seem to be most viable; hence the inoculum should be taken from an area as close to the well as is possible without contamination. The inoculum is introduced under the surface of the fresh plate by blowing through a rubber tube attached to the pipette, with care to avoid introducing air bubbles or otherwise breaking the agar surface. Successful pure subcultures have been obtained only when such transfers were made immediately after removing the primary plates from the anaerobic jar, without taking time, for example, to examine a portion of the inoculum under the darkfield microscope until after the transfers had been made and placed under anaerobiosis. Pure subcultures in plates have not been so consistently successful as subcultures from the primary plates into tubes. For this purpose the same medium is employed, using 5 cc in Wassermann tubes, except that a single piece of guinea pig kidney is used in the bottom of the tube in place of the minced tissue. One guinea pig kidney may be cut into at least 20 pieces for this purpose. Again the transfer is made with a capillary pipette, the inoculum being drawn up with the aid of a rubber tube as an unbroken column about 2 cm or more in length, and carefully expelled without bubbles as the pipette is withdrawn in the vertical axis of the tube beginning a few millimeters above the kidney fragment. The tubes, like the plates, are incubated in anaerobic jars under hydrogen and 5% CO_2. Pure cultures grow more slowly than primary cultures, requiring 7 to 9 days at 37°C; the spirochetes are then easily removed with capillary pipettes through the upper surface of the medium. This method of pure subculture has been applied only during the past few weeks. In that time 11 strains of small mouth spirochetes, including *T. microdentium* and other as yet unidentified varieties, have been isolated and passed successfully through as many as 7 subcultures. These studies are being continued.

Summary. Details are given of a method for primary anaerobic cultivation in small plates of spirochetes from human and experimental fuso-spirochetal exudates, and for their pure subculture in

plates and tubes. Successful primary plates were obtained in 23 instances out of 29 cases of human infection, and from all of 21 experimentally infected guinea pigs. Eleven strains of spirochetes have been isolated and subcultured in a short period with little difficulty.

13143 P

Comparative Amino Acid Content of Serum Proteins in Normal Humans and in Patients with Rheumatoid Arthritis.

WALTER D. BLOCK AND WILLIAM A. MURRILL.* (Introduced by H. B. Lewis.)

From the Rackham Arthritis Research Unit,† the Medical School, University of Michigan, Ann Arbor, Mich.

The existence of a disturbance in sulfur metabolism in patients with chronic rheumatoid arthritis has been emphasized by Wheeldon.[1] Race[2] has extended this concept of disturbed sulfur metabolism to include an alteration in the cystine content of serum proteins. In order to test the validity of this finding the determination of several amino acids, as well as the elemental analyses of total serum protein, were carried out in 11 patients with chronic rheumatoid arthritis and compared with the findings in 6 normal control subjects.

Procedure. Fifty cc of venous blood were withdrawn from each subject (150 cc in the cases of control No. 6 and arthritic No. 11). The serum was separated and the total serum protein precipitated with 2 volumes of alcohol. The precipitate was dialyzed until it was sulfate and chloride free. It was then washed several times with 95% alcohol, once with absolute alcohol and several times with ether, and dried in a vacuum desiccator over sulfuric acid for several days. The analytical methods for total nitrogen, total sulfur and the various amino acids were those previously employed by Block and Lewis.[3] The values for serum protein were corrected for ash and moisture

* Research Fellow in Internal Medicine, 1940-1941.

† The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies, of the University of Michigan.

1 Wheeldon, T., *J. Bone and Joint Surg.*, 1935, **17**, 693.

2 Race, J., *Reports on Chronic Rheumatic Diseases*, H. K. Lewis & Co., Ltd., London, 1935, **1**, 55.

3 Block, W. D., and Lewis, H. B., *J. Biol. Chem.*, 1938, **125**, 561.