time of the blood of treated animals has been reported for trypan blue,[5] while on prolonged administration of this agent to rabbits we have noted grossly a shortening of coagulation time after 2 acute toxic doses are administered in divided amounts over a period of 3 weeks. Certain observations lead us to suspect central nervous system depression in trypan blue treated animals; the brain and spinal fluid are reported to be impermeable to gentian violet, which is rapidly fatal in small amounts intracisternally.[6]

Experimental therapy on leprous rats has shown gentian violet and brilliant green to be too toxic for continued administration, while trypan blue, and other dyes which seemed preferable to us[7] because of their high leprocidal activity, are better tolerated.

7338 C

An Improved Method for In Vitro Cultivation of B. Leprae.*

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Many attempts have been made by various investigators to culture Hansen's bacillus of human leprosy. Undoubtedly, in a number of these attempts the investigators have mistaken the increase in number of acid-fast rods, which occurs in removed autolysing pieces of leprous tissue independent of any specially supplied cultural conditions, for multiplication *in vitro* upon artificially prepared nutrient medium. The failure to appreciate the facts that *B. leprae* continues to multiply and that growth ceases when the leprous tissue autolysate is no longer available, explain the increasing difficulty to obtain macroscopic growth of the specific microorganism in transplants through successive generations, and accounts for the absence of growth in subculture in or upon a medium that does not contain the split protein products (amino-acids).

One of us (Duval[1]) reported that *B. leprae* seemed unable to

[5] Hugget, A. St. G., and Rowe, F. M., *J. Physiol.*, 1933, **80**, 82.

[6] Kolmer, J. A., *Principles and Practice of Chemotherapy*, Philadelphia, 1926, p. 94.

[7] Emerson, G. A., and Salle, A. J., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 428.

* Aided by a grant from David Trautman Schwartz Research Fund.

[1] Duval, C. W., *J. Exp. Med.*, 1910, **12**, 649.

utilize whole protein *in vivo*. This was substantiated by repeated observations that the Hansen bacillus would grow in transplanted leprous tissue after autolysis had commenced. The initial culture away from the influence of the host-tissue was possible if the artificial culture medium contained certain split-products of protein. In our recent studies upon the cultivation of *B. leprae*, we have modified Duval's[2] earlier method. An improved procedure as described below is recommended.

The uncontaminated subcutaneous leprous nodule, rich in acid-fast bacilli, is the material of choice. Contaminated tissue is unsatisfactory, because one is likely to injure or destroy the viability of the Hansen bacillus when killing the contaminants. The use of sodium hydroxide, or the like, therefore, is not recommended as a germicide. The skin over the selected leprous nodule is cleansed with soap and water and then rinsed with alcohol. Mercurochrome, iodine or other antiseptics should not be employed. The overlying skin is next incised and the exposed nodule is dissected free and removed under strict asepsis.

The removed nodule is cut into small pieces (circa 4 mm. diameter) with sterile scissors and placed in a 1% trypsin solution previously made sterile by passage through a Berkefeld V or Seitz filter. The trypsin immersed leprous tissue bits are incubated at 37°C. for 36 to 48 hours. This period of incubation is sufficient to digest them so that their consistence is of soft butter. When the softened bits are removed and placed on the surface of slanted agar, they are readily spread with an ordinary platinum loop.

Smear-preparations are made from the trypsinized leprous material and stained by Ziehl-Neelsen's method to observe the morphology and to determine the approximate number of acid-fast rods per microscopic field. These stained preparations afford an index to the subsequent multiplication of *B. leprae*. At this time, control cultures are also made to determine possible bacterial contamination. However, when the leprous tissue contains ordinary extraneous bacteria, they evidence themselves by clouding the trypsin fluid used as a tissue digestant. It should be mentioned here that 1% trypsin solution does not destroy or inhibit the growth of common bacteria, nor has it any detrimental effect upon *B. leprae*.

The uncontaminated trypsinized leprous pieces of tissue which now have the consistence of soft butter are carefully transferred and smeared over the surface of slanted amino-acid agar by means of a sterile platinum loop. The inoculated culture tubes are plugged

[2] Duval, C. W., *J. Exp. Med.*, 1911, **13**, 365.

with paraffined cotton to prevent undue evaporation during the long period of incubation at 37°C. To permit the entrance of fresh atmosphere into the culture tubes, the paraffined cotton plugs at intervals of 3 to 5 days are removed from the tubes and replaced before the paraffin cools.

The split protein products are prepared separately in the form of clear neutral filtrates by dissolving the powders or crystals (C.P.) in sterile distilled water to the point of saturation. These filtrates are sterilized by passage through Berkefeld V or Seitz filters.

Agar is used only as a menstrum for the special nutrients. It is prepared in 3% concentration to allow for dilution with the various filtrates. Ordinary agar-powder is melted in Ringer's solution, cleared with whole egg, titered to pH 7, filtered, tubed in the desired quantity and sterilized in the autoclave.

The placenta autolysate is obtained by mixing 500 gm. of ground human placenta in 500 cc. of Ringer's solution. The infusion is allowed to stand for 10 days at refrigerator temperature. It is first filtered through cotton to remove the solid particles, then passed through a Seitz filter. The sterile filtrate constitutes the placenta autolysate.

The leucocytic extract is secured by infusing for 10 days at refrigerator temperature 500 gm. of ground human pneumonic lung (grey stage of hepatization preferred) in 500 cc. of Ringer's solution. It is then filtered through Berkefeld V or Seitz filter and the filtrate is designated as the leucocytic extract.

Banana extract is prepared by emulsifying 500 gm. of peeled over-ripe bananas in 500 cc. Ringer's solution. The emulsion is first filtered through cotton and then through the Berkefeld or Seitz filter to obtain the sterile amber color filtrate which is designated as the banana infusion.

The procedure and proportions of ingredients employed in the preparation of the special split protein medium are as follows: to individual tubes containing 5 cc. of melted 3% sterile neutral agar,

	cc.
Tryptophane, 2.0% aq. sterile filtrate (Tryptophane solubility, 20 mg. per cc. dist. H_2O).	2
Cystine, 0.1% aq. sterile filtrate (Cystine solubility, 0.1 mg. per cc. dist. H_2O).	2
Tyrosine, 0.5% aq. sterile filtrate (Tyrosine solubility, 0.5 mg. per cc. dist. H_2O).	2
Leucine, 2.0% aq. sterile filtrate (Leucine solubility, 20 mg. per cc. dist. H_2O).	2
Placenta autolysate or leucocytic extract, sterile filtrate	1
Banana infusion, sterile filtrate	0.5
Glycerine, sterile	0.5

the various quantities of the special ingredients are added. Each ingredient is added separately under strict asepsis to the melted agar which is kept liquid in a hot water bath between the filling procedures. Upon adding the last ingredient, the tube is slanted to allow the content to solidify in this position.

Summary. The trypsinization of the leprous tissue bits, before attempting the cultivation of the contained *B. leprae,* affords at once the split-protein products which we consider the necessary nutrient factor. The trypsin apparently exerts no harmful effect upon the Hansen bacillus as evidenced by its unmistakable growth in 4 to 6 weeks. This initial *in vitro* multiplication occurs in the trypsinized leprous tissue without the aid of other nutrients and under ordinary atmospheric conditions at 37°C. Growth continues in subculture on ordinary agar medium as long as the trypsinized leprous material is transferred. The crucial point in the initial cultivation of *B. leprae* away from the influence of the host tissue is experienced at this juncture. After the complete utilization of the nutrient material of the trypsinized host tissue, the acid-fast bacillus of Hansen will only multiply in a medium that is enriched with sufficient quantities of protein cleavage products.

7339

Repeated Determinations of Pulse Wave Velocity on Normal Individuals.*

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The existing literature concerning pulse wave velocity deals with the effect of disease, age, sex and physiologic and pharmacologic stimuli on pulse wave velocity in groups of individuals. Spontaneous fluctuations in the same individual from time to time have received slight consideration.[1, 2, 3, 4]

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1 Beyerholm, O., *Acta Med. Scand.,* 1927, **67**, 203.

2 Bazett, H. C., and Dreyer, N. B., *Am. J. Physiol.,* 1922, **63**, 94.

3 Schafer, E. A., *Textbook of Physiology,* Vol. II, Edinburgh, 103.

4 Keyt, A. T., *N. Y. Med. J.,* 1878, **28**, 30.