

THE CULTIVATION OF *TRYPANOSOMA BRUCEI*.\*

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The first successful cultivation of *Trypanosoma Brucei* was announced by Novy and MacNeal in 1903.<sup>1</sup> The medium which gave the best results was prepared by adding to a 1 to 8 meat extract 2 per cent peptone; 0.5 per cent sodium chloride; 1 per cent normal sodium carbonate; and 2 per cent agar. Two parts of defibrinated rabbit blood was added and the mixture was allowed to solidify in an inclined position. The medium was then inoculated with two drops of white mouse, rat, or guinea-pig blood, very rich in trypanosomes. An invisible growth occurred in the water of condensation at 25°C. Novy and MacNeal after testing fifty animals (mice, rats, and guinea-pigs) found that only four gave positive results, or 8 per cent. In 1905 Smedley<sup>2</sup> reported that three out of ten attempts or 30 per cent were positive.

Because of the inconsistent results a number of experiments were made using media of quite a different nature. The first attempt along these lines was to use a medium in which the meat constituent was replaced by an extract made of beans and peas. This was obtained by macerating and boiling 1 per cent of each with distilled water. The usual amounts of sodium chloride, normal sodium carbonate and agar were added to this extract. One part of bean and pea agar was mixed with two parts of defibrinated rabbit blood, well agitated and solidified in an inclined position. On inoculating the medium with two drops of defibrinated trypanosomal rat blood and subsequent incubation at 25°C more constant results were obtained than with the original Novy-MacNeal medium.

A second medium was made similar to that employed by Nicolle<sup>3</sup> in the cultivation of Leishman-Donovan bodies. In this medium no meat extract or alkali was added. It was prepared by dissolving 2 per cent of agar, 2 per cent of peptone, and 0.5 per cent of sodium chloride in distilled water. To this modified Nicolle medium two parts of defibrinated rabbit blood was added, the same as in the case of the other media.

A third medium was employed and was made as follows: 125 gms. finely chopped lean beef, and 250 cc. of water were allowed to digest over night in the cold, or for one hour at 55°C, boiled and filtered. The filtrate was then dialyzed in a large thin collodium sac against running distilled

\*The strain of *Trypanosoma Brucei* used in these experiments was supplied thru the kindness of Dr. F. G. Novy, Ann Arbor, Michigan.

1. Jour. Am. Med. Assn. 1903, p. 1266, "Contributions to Medical Research" dedicated to V. C. Vaughan, Ann Arbor, 1903, p. 549. Jour. Infect. Dis., 1904, 1 p. 1.

2. Jour. Hyg., 1905, 5 p. 38.

3. Arch. de L'Inst. Pasteur de Tunis, 1908, p. 55. Ann. de L'Inst. Pasteur, 1909, 23, p. 361.

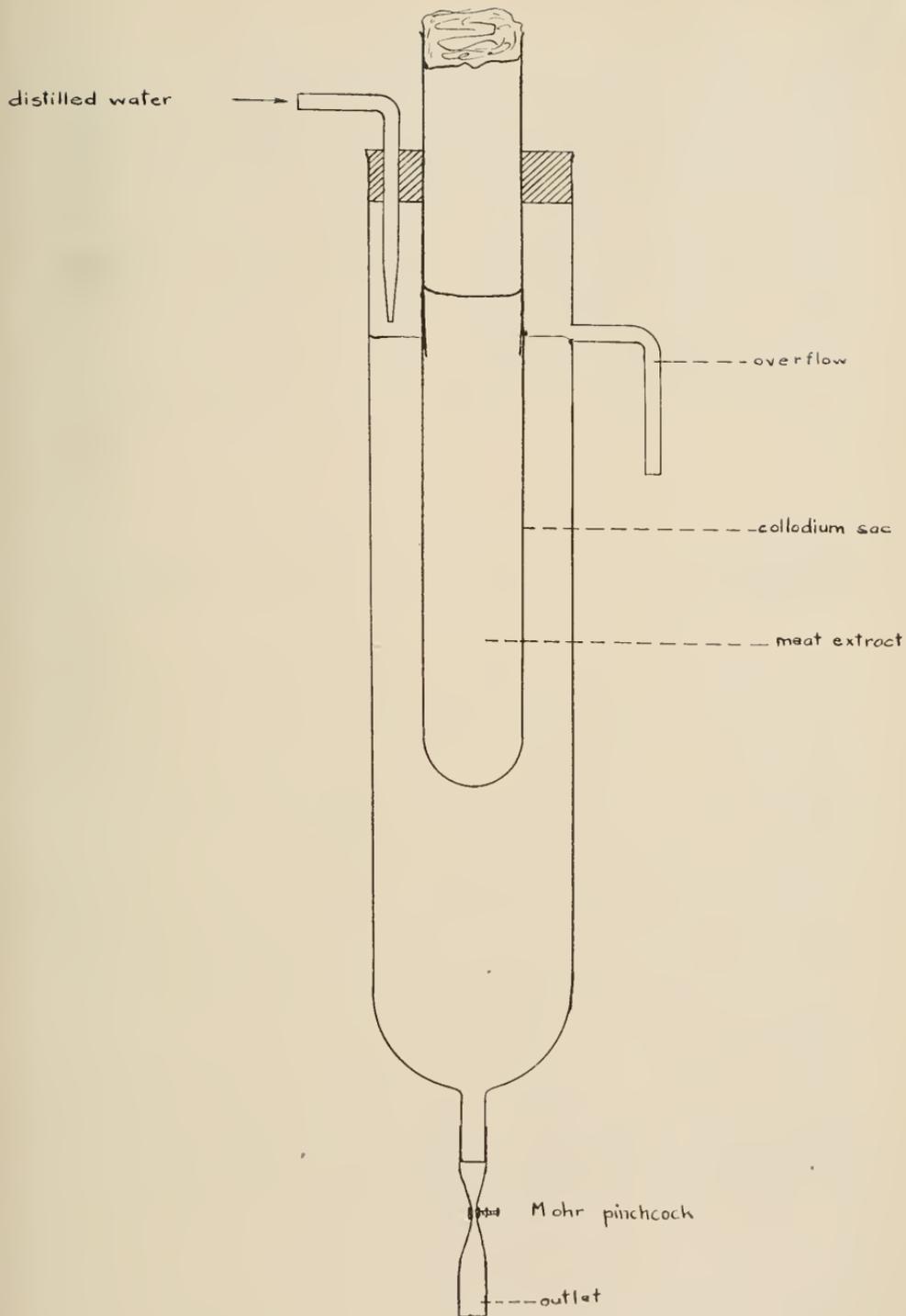


Figure I—Dialyzing Apparatus. One-fourth Size.

water (see figure I) until it gave no test for chlorides, this required twenty-four to forty-eight hours. The same results may be obtained in a comparatively short time by dialyzing in warm (50°C.) running distilled water, thus not only cutting down the amount of distilled water used and time required but also the degree of bacterial development. The dialyzed filtrate was diluted to one liter with distilled water, plus 2 per cent peptone; 0.5 per cent sodium chloride; .01 per cent calcium chloride; 1 per cent normal sodium carbonate and 2 per cent agar. One or 1½ cc. of this agar was placed in each tube and sterilized in an autoclave by heating to 105°C. for fifteen minutes. This dialyzed nutrient medium was also diluted with two volumes of defibrinated rabbit blood.

In order to ascertain the relative value of these media a series of cultures were carried out. For this purpose the blood of an infected rat was transferred by means of a Pasteur capillary pipette, bent at right angles (figure II) to usually

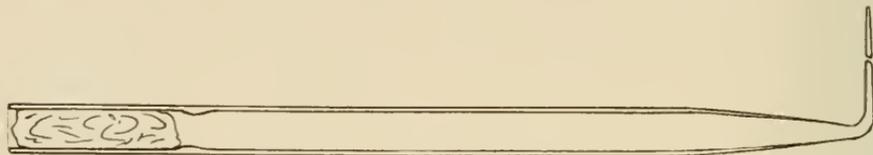


Figure II—Pasteur Capillary pipette. Full Size.

twelve tubes of each medium and incubated at 25°C. in an almost horizontal position. Six comparative trials were thus made. The results of these experiments were decidedly favorable to the dialyzed medium, since 80 per cent of the tubes gave a positive growth. In case of the original Novy-MacNeal medium which was the least favorable, only 25 per cent of the tubes gave positive results. On the modified Nicolle medium 48 per cent of the tubes were positive. The bean and pea medium revealed the presence of cultural forms in 53 per cent of the tubes (chart I).

No advantage was found by increasing the blood constituent to three parts to one of agar whereas, if it were decreased to one part no growth occurred. Also no benefit resulted by altering the amount and kind of alkali. Inoculated tubes of the various media were placed in atmospheres of different gases, such as hydrogen, nitrogen, and carbon dioxide, but all such attempts proved to be total failures, therefore, it seems that the ordinary aerobic conditions are best.

Having apparently a satisfactory nutrient agar it seemed advisable to improve the blood constituent of the medium. Various attempts were made in this direction. Defibrinated rabbit blood was transferred to sterile centrifuge tubes and centrifugated for ten minutes at about 8,000 revolutions per minute. The serum was then drawn off by means of a Pasteur bulb

pipette (fig. III) and diluted to the original blood volume with 0.5 per cent sodium chloride solution. This clear diluted serum was then mixed with the dialyzed agar medium, in a ratio of two to one. The remaining blood cells were likewise diluted with salt solution to the original blood volume and the resulting suspension was added to the dialyzed agar in the same ratio of two to one.

Serum inactivated for one-half hour at 56°C. was also mixed with the dialyzed agar. As in the previous experiments these media were inoculated with trypanosomal rat blood and incubated at 25°C.

The results of the examination of these media at the end of 14 or 21 days showed that the diluted and inactivated serum gave practically 100 per cent positive growths, whereas, the diluted blood cell medium gave but 38 per

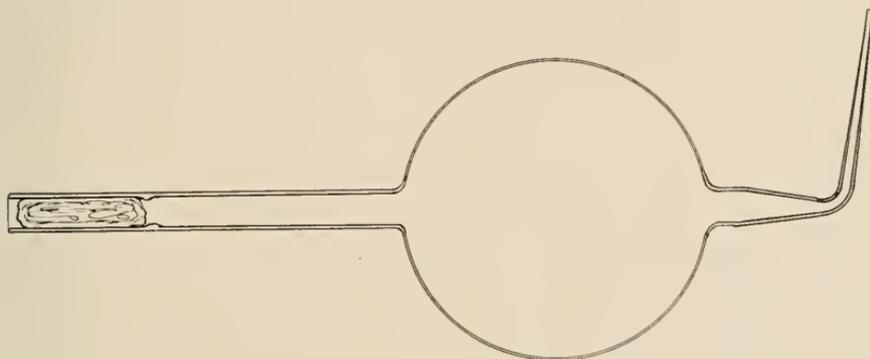


Figure III—Pasteur Bulb pipette. Full Size.

cent successful cultures (chart I). Therefore, it would seem advisable in attempting isolation of *Trypanosoma Brucei* to employ dilute or inactivated serum rather than defibrinated whole rabbit blood. Successive transplants of the cultural forms on serum agar gave at all times a very rich growth, in fact the growth in some cases became so extremely rich that a white film resulted which was easily detected by the eye. The organisms of nagana, therefore, maintain themselves without the presence of hemoglobin. This fact was also noted in regard to *Trypanosoma Lewisi*.

Isolations were attempted using ascitic fluid instead of serum and in no instance were cultures obtained. The culture medium described by Rh. Erdmann<sup>4</sup> was also used and proved to be a total failure and as a matter of fact the easily cultivated rat trypanosome of Lewis could not be grown successfully by this method.

The appearance of *Trypanosomes* of nagana *in vitro* is very characteristic. They occur either singly or in groups ranging from a few to many hundreds

4. Soc. Exp. Biol. and Med. 1914, XII, pp. 57-58.

in number. The groups are not usually arranged symmetrically like in the rosettes of *Trypanosoma Lewisi*, but the effect is that of a writhing mass with the flagella directed outward, being very suggestive of the snakes on a Medusa head. However, at times symmetrical arrangement results and because of the presence of one or more highly refractive globules and the direction of the flagella presents the picture of a jeweler's "sunburst." Therefore, *Trypanosoma Lewisi* and *Brucei* are easily and readily distinguished from each other *in-vitro*. Again, since the former only infects rats and the latter causes nagana in all the laboratory animals they may be easily separated when occurring simultaneously in cultures or in the blood of a rat.

The presence of the highly refractive globules in the cultures *in-vitro* previously referred to and their entire absence in the blood type seems to indicate an unfavorable medium. It is then probable that with an improved medium cultures may be obtained which would more nearly resemble the blood type.

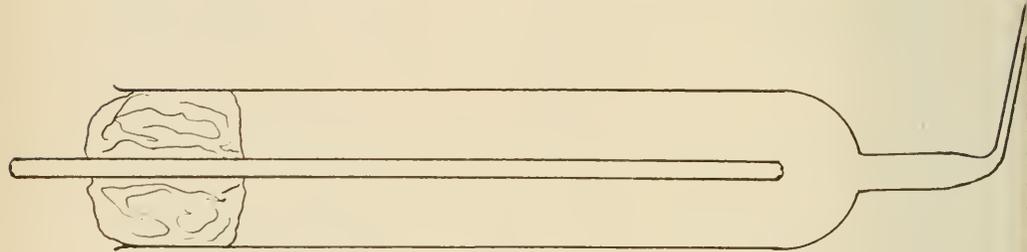


Figure IV—The Novy carotid artery pipette used in bleeding rabbits. One-half size.

The best results obtained in this direction were by employing a 1 to 8 nutrient veal extract agar. This was made as follows: 125 gms of chopped veal and 1000 cc. of distilled water were thoroughly mixed and allowed to digest over night in the ice-box. The mixture was then strained thru muslin, 2 per cent peptone; 0.5 per cent sodium chloride; 0.5 per cent normal sodium carbonate; and 2 per cent agar was added. This nutrient veal agar was then boiled and filtered thru cotton, one cc. placed in each tube and sterilized in an autoclave by heating to 105°C. for twenty minutes.

The blood used in this cultural medium was drawn from the carotid artery of a rabbit under aseptic conditions by means of a special Novy pipette (fig. IV) and immediately defibrinated. It was then drawn up into a Pasteur bulb pipette and transferred to sterile large special centrifuge tubes (fig V) and centrifugated for 5 minutes at 8000 revolutions per minute. This divides the blood into three layers serum, white, and red blood cell layers.

By means of bulb pipettes the desired constituents of the blood may be removed.

Shortly before use the desired number of agar tubes were melted in the water-bath, cooled to 60° C. and two volumes of defibrinated rabbit blood minus the white blood cell layer were added. The mixture was thoroughly

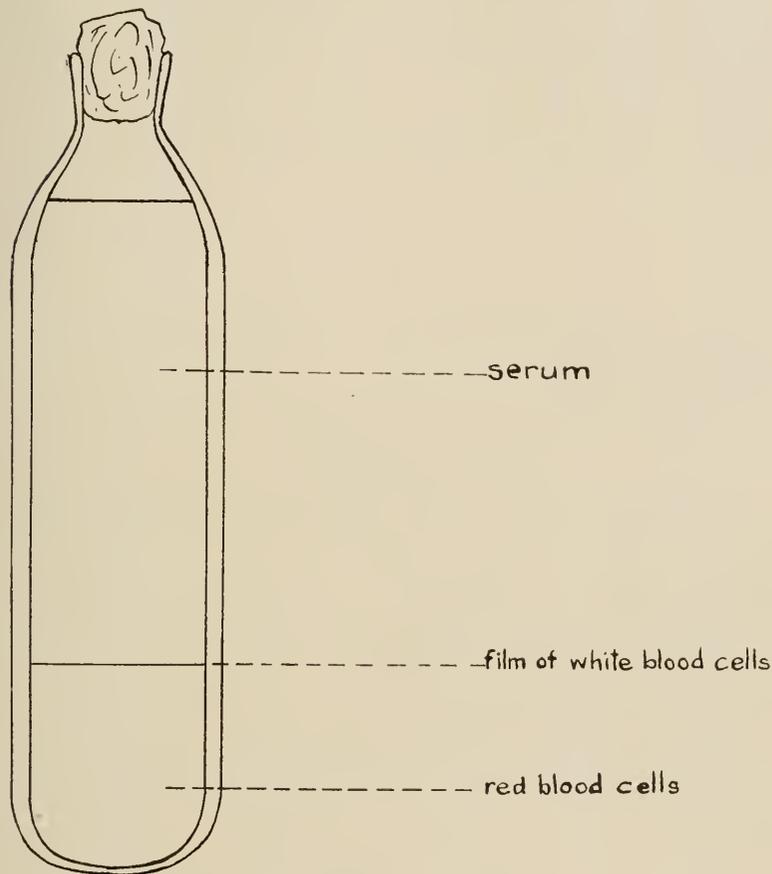


Figure V—Centrifuge tube. Full Size.

agitated by rolling in the hands in an upright position, by tapping on the tube, or by employing both methods. This well mixed medium was allowed to solidify in an incline position. It was found advisable to employ the blood

of rats which were in the early stages of the infection, having about twenty-five organisms per field (4 mm. objective). Trypanosomal blood from guinea-pigs is not suitable for isolation work. A white rat showing the desired number of organisms in its blood was etherized, after which its blood was drawn from the heart by means of a sterile special Novy heart pipette<sup>6</sup> (fig. VI). A droplet of this defibrinated nagana blood was smeared over the surface of the medium by means of a capillary pipette and incubated at the usual temperature of 25° C. after the tubes had been rubber capped to prevent dessication.

Cultural forms were observed as early as the fourth to the sixth day. It is advisable, however, not to disturb the tubes until after a period of 14 days

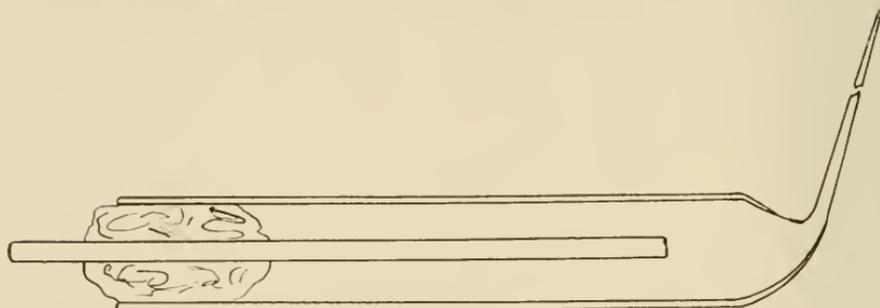


Figure VI—The Novy heart pipette used for bleeding rats. Full Size.

has elapsed when all the cultural tubes may be examined and transplants made. Transfers should be made by means of the capillary pipette instead of with a platinum loop and to perpetuate the cultural type transplants should be made weekly. This medium gave 100 per cent positive growth. These cultural forms are practically devoid of large globules, altho small ones were present, and in that respect more nearly resemble the blood type which is free from globules. They differ from the latter type in that the centrosome is near and usually situated anterior to the nucleus. Also the undulating membrane is not nearly so conspicuous as in the blood form.

Intraperitoneal and subcutaneous injections of these cultural forms after several successive growths (generation seven) *in vitro* will infect rats and guinea-pigs and the organisms which develop in the blood of the animal after an injection of the cultural form are identical with those of the blood type.

#### SUMMARY

One part of a veal nutrient agar, plus two parts of defibrinated rabbit blood devoid the white blood cell layer yields constant results and seems to indicate that the conditions are more nearly ideal for the cultivation of trypanosoma Brucei *in vitro*.

6. Jour. Infect. Dis. Vol. 20, 1917, p. 502.

The difference between the organisms grown upon the veal blood agar minus the white blood cell layer and upon the dialyzed dilute serum or inactivated serum agar is in the size of globules, being smaller on the former medium.

The organisms grown upon the improved medium differ from the blood type because of the presence of small globules; the position of the centrosome; and the less conspicuous undulating membrane.

The blood of rats in the early stages of Nagana, showing about twenty-five trypanosomes per field is best suited for isolation work. Infected guinea-pig blood is not desirable for this work.

The best results are obtained when blood is used in a ratio of 2 to 1 of agar and ordinary aerobic conditions are resorted to.

To perpetuate the growth transplants should be made weekly by means of a capillary pipette. *Trypanosoma Brucei* and *Lewisii* do not require the presence of hemoglobin for their existence.

The characteristic rosette formation of *trypanosoma Brucei* and *Lewisii* serves as a means of differentiation.

Since the cultures are pathogenic for rats and guinea-pigs the positive causal relation of the trypanosome to the disease nagana can be demonstrated by Koch's requirements.

After obtaining such excellent results with *trypanosoma Brucei* this medium seems especially promising for the cultivation of other pathogenic forms.

## CHART I

SHOWING THE PER CENT OF POSITIVE RESULTS ON VARIOUS MEDIA

No. of media used	Kind of media	No. of experiments	Av. No. of tubes in each experiment	Per cent of positive growths
1	Novy & MacNeal blood agar.	6	12	25%
2	Bean and pea blood agar. . . . .	6	12	53%
3	Nicolle blood agar. . . . .	6	12	48%
4	Dialyzed dil. blood agar. . . . .	6	12	80%
5	Dialyzed dil. serum agar. . . . .	8	10	100%
6	Dialyzed inact. serum agar. . . . .	8	8	100%
7	Dialyzed dil. bld. cell agar. . . . .	8	14	38%
8	Dialyzed Ascitic fluid agar. . . . .	3	6	0%
9	Erdmann's medium. . . . .	3	15	0%
10	Veal bld. minus white bld. cells agar. . . . .	10	10	100%