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Cultivation of *Bacterium tularense* in Peptone Media.*

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(Introduced by K. H. Lewis.)
From Camp Detrick, Md.

Until recently *Bacterium tularense* has resisted attempts at cultivation in any fluid medium or on any but complex solid media[1] and until recently no dilution plate counting method was described for this organism. Since 1942 there have been described several semi-synthetic liquid media,[2,3] and a more easily prepared fluid medium containing heart infusion broth, hemoglobin, glucose and cystine.[4] Larson[5] has further modified this last medium by replacing the hemoglobin with a completely soluble erythrocyte extract, and has quantitatively confirmed earlier suggestions[2,4] that large inocula are required for propagation in liquid media.

Through a process of elimination and substitution, starting from Rhamy's hemoglobin cystine heart agar[6] we found it possible to derive more simple fluid and solid media quite satisfactory for routine use. These consist of 2% Difco Bacto Peptone, 1% sodium chloride and 0.1% glucose, with or without 2% Difco Bacto Agar. The hydrogen-ion concentration is not adjusted before sterilization. The only precaution that need be carefully observed is the use of relatively large inocula.

The 28 strains which were investigated are listed in Table I in order of decreasing virulence. All strains were originally isolated from human sources and presumably were highly virulent at the time of isolation. Most of the strains, together with information concerning their histories, were supplied us by Professor Lee Foshay. The exceptions are Strain Ince (Professor Cora M. Downs) and Strain 38 (National Institute of Health). The donors' designations are used throughout. Virulence titrations were carried out by injecting groups of 6 mice intraperitoneally with appropriate 10-fold serial dilutions of suspensions of equivalent turbidity (T-500 Fullers earth standard, National Institute of Health). The 50% lethal doses were calculated by the method of Reed and Muench[7] from the percentage mortality within 10 to 14 days, and are recorded as dilutions of the standard suspension. We are indebted to Capt. L. L. Coriell for many of these titres, which are taken from his unpublished data.

Peptone broth was tested for its ability to permit the growth of the 28 strains, using 50 or 100 ml of medium in 250 ml Erlenmeyer flasks. The primary cultures were inoculated into this medium sufficiently heavily from stock blood cysteine agar slants to produce a faint turbidity, and the remaining transfers were made with sterile pipettes. All strains were tested for at least 9 consecutive transfers before terminating the experiment. Twenty-one strains could be maintained by the use of 1% inocula (*i.e.*, one ml of 24-hour broth culture as inoculum for 100 ml sterile broth). A few representative strains of this group (Dieck, Ince and Camp) have been carried through more than 20 transfers, and Strain Schu through 98 transfers, without difficulty. The remaining 7 strains required a larger inoculum for serial cultivation, 10% being successful in all cases. Strain 38, representing this group has been maintained through about 20 transfers under these conditions. With one exception (Strain LR),

* Studies conducted at Camp Detrick, Frederick, Md., between February, 1944, and June, 1945.

[1] Francis, E., *J. Bact.*, 1942, **43**, 434.

[2] Berkman, Sam, *J. Infect. Dis.*, 1942, **71**, 201.

[3] Tamura, J. T., and Gibby, I. W., *J. Bact.*, 1943, **45**, 361.

[4] Steinhaus, E. A., Parker, R. R., and McKee, M. T., *U. S. Pub. Health Rep.*, 1944, **59**, 78.

[5] Larson, C. L., *U. S. Pub. Health Rep.*, 1945, **60**, 863.

[6] Rhamy, *Am. J. Clin. Path.*, 1933, **3**, 121 (cited by *Manual of Dehydrated Culture Media and Reagents*, 7th Edition, Difco Laboratories, Inc., Detroit, Mich., 1943).

[7] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Characteristics of Strains Investigated.

Strain	Area and year of isolation	50% lethal dose (ml standard suspension)	Growth with 1% transfers
Chr*	Ohio, 1937	$<10^{-10}$	+
Gib*	Ohio, 1941	$<10^{-10}$	+
Ri*	Virginia, 1932	$<10^{-10}$	+
Holt*	Kentucky, 1944	$10^{-10.6}$	+
Fox	Ohio, 1940	$10^{-9.8}$	+
Dieck*	U. S. A., 1944	$10^{-9.6}$	+
Camp*	Kentucky, 1944	$10^{-9.5}$	+
Carr*	U. S. A., 1944	$10^{-9.5}$	+
Ince*	Kansas, 1944	$10^{-9.5}$	+
Schu*	Ohio, 1941	$10^{-9.5}$	+
Scherm*	U. S. A., 1944	$10^{-9.2}$	+
Coll*	U. S. A., 1945	$10^{-8.8}$	+
Chur	Ohio, 1941	$10^{-8.6}$	+
LR	Arkansas, 1927	$10^{-8.5}$	—
Hugh	Ohio, 1940	$10^{-6.3}$	+
Jap	Japan, 1926	$10^{-4.4}$	—
HD	Austria, 1935	$10^{-4.0}$	+
Sto*	Ohio, 1937	$10^{-3.0}$	+
PF*	Ohio, 1936	$10^{-2.5}$	+
Russ	Russia, 1928	$10^{-2.0}$	+
De P	Ohio, 1938	$>10^{-2.0}$	+
Die	California, 1938	$>10^{-2.0}$	—
Max	Russia, 1928	$10^{-1.7}$	—
Pi	Ohio, 1938	$10^{-1.0}$	+
Ohara	Japan, 1931	$10^{-1.0}$	—
26	Utah, 1921	$10^{-1.0}$	—
Li*	Canada, 1934	$>10^{-1.0}$	+
38	Utah, 1920	avirulent	—

* Yield in peptone broth determined by dilution plate count.

strains requiring an inoculum in excess of 1% were found among those of lesser virulence.

All strains appeared to grow quite as luxuriantly in the 1st and 2nd passages in peptone broth as in later ones, indicating that no process of adaptation was required. The yields of 14 strains (indicated in Table I by asterisks) could be determined by means of a dilution plate counting method described elsewhere.[8] All produced one to 5 billion viable organisms per ml in 24 to 48 hours at 37°C in 50 ml cultures. The effect of aeration on yield was demonstrated with Strains Schu, Camp, Dieck and Ince. An increase in the volume of culture from 50 to 100 ml in 250 ml Erlenmeyer flasks decreased the yield to between 200 millions per ml and 800 millions per ml, while active aeration by means of spargers or by constant shaking increased it to between 3 and 10 billions per ml in 24 hours. Incubation at 23 to 25°C decreased the growth rate, but similar or slightly higher final yields were obtained after 48 to 72 hours. The remaining 14 strains produced roughly equivalent turbidity, but the yields could not be determined by the plate-counting method.

The effect of alterations in the composition of the fluid medium was investigated, using Strain Schu. The average yield was halved by decreasing the peptone concentration to 1%, but some growth occurred with as little as 0.25%. Bacto Peptone could be replaced by Proteose Peptone, Proteose Peptone No. 3 or Bacto Tryptose, but not by Neopeptone or Bacto Tryptone. No difference in yield was observed with 10 different lots of Bacto Peptone. Two and 4% corn steep liquor supported somewhat less luxuriant growth than 2% peptone. Sodium chloride was found to be approximately optimum over the range 0.4 to 2.0% in the presence of one and 2% peptone, but 0.2% gave a poor yield. Glucose was included because there is evidence that

[8] Snyder, T. L., Engley, F. B., Jr., Penfield, R. A., and Creasy, J. C., *J. Bact.*, 1946, **52**, 241.

TABLE II.
Effect of Inoculum Size and Reducing Agents on Growth of Strain Schu in Peptone Broth.

Reducing agent	Inoculum (No. of cells per 100 ml flask)	No. of flasks inoculated	No. of flasks turbid after incubation (hrs)						
			16	24	40	48	88	96	336
0	380,000,000	2	2	2	2	2	2	2	2
	38,000,000	3	3	3	3	3	3	3	3
	3,800,000	3	0	0	0	0	2	3	3
	380,000	2	0	0	0	0	0	0	0
0.1% cysteine-HCl	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	3	3	3	3	3
	0.38	3	0	0	2	2	2	2	2
	0.038	3	0	0	0	0	0	0	0
0.01% thioglycollate	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	0	3	3	3	3
	0.38	2	0	0	0	0	0	0	0
	0.038	3	0	0	0	0	0	0	0

it is utilized,[1,9] but our results did not indicate any stimulation by either 0.1 or 1.0%. Variation of the hydrogen-ion concentration over the range pH 6.5 to 7.2 appeared to have no effect.

Only Strains 38, Schu and Chur were tested on peptone agar slants. These strains were twice carried through 20 successive passages without difficulty before terminating the experiment. Transfers were usually made at 24-hour intervals by spreading a 2 mm loopful of growth over the surface of the sterile slant. Growth appeared to originate within streaks of the inoculum, thence spreading over the entire surface. The yield appeared equivalent to that on Rhamy's medium,[6] but was somewhat less than that obtained with blood cystine agar.[10]

The effect of continued cultivation on retention of virulence was investigated only with Strains Schu and Chur. Titrations were carried out as described above, but in this case plate counts made on the same suspensions permit us to report the 50% lethal doses in terms of numbers of organisms. Repeated titration of stock cultures of these strains gave endpoints falling within the range 0.2 to 3.0 organisms. No decrease in virulence was detectable with either strain after 34 passages on peptone agar at 37°C, or with Strain Chur after 30 passages in peptone broth at 37°C. Broth cultures of Strain Schu were studied more extensively. Of 5 separate series, each carried through 50 passages at 25°C, none showed any decrease in virulence, whereas of 5 series carried through 50 passages at 37°C, 3 showed no loss and 2 had decreased to such an extent that the 50% lethal doses were 130 thousand and 11 million organisms respectively.

The minimum effective inoculum for consecutive cultivation in peptone broth was determined more precisely in the case of Strains 38 and Schu, which were selected as representatives of the 2 groups previously described. In this study, the incubation periods were held constant at 24 hours and the inocula varied from 0.1 to 10.0%. Strain 38 could be carried only through one or 2 transfers with 5% or smaller inocula, whereas the control series was maintained through 20 passages with 10% inocula before being discarded. Strain Schu could be maintained indefinitely with 1% inocula, but the 0.3% inoculum series failed to become turbid within 24 hours in the 3rd passage, and the 0.1% series in the second. On the basis of plate counts of 24-hour cultures it was possible to estimate that the minimum inoculum for Strain Schu under these conditions was about one to 4 million organisms per ml of fresh medium.

The constancy of the minimum inoculum size as a characteristic of the strain is open to question. Strain 38 was re-examined after

[9] Downs, C. M., and Bond, G. C., *J. Bact.*, 1935, **30**, 485.

[10] Francis, E., *J. Am. Med. Assn.*, 1928, **91**, 1155.

2 years cultivation on blood cysteine agar and still required an inoculum in excess of 1%, but one series of Strain Schu has shown a decrease in the minimum inoculum size to less than 30 organisms per ml after 43 or less passages in peptone broth. This cannot be referred to the effect of a different lot of medium since a control series in the same lot demonstrated the usual requirement for a large inoculum.

Three independent lines of investigation appear to relate the dependence of *Bacterium tularense* on large inocula to an inhibitory action by oxygen or by elevated oxidation-reduction potential of the medium. This is indicated by the effect of reducing agents and decreased oxygen tension on the size of inoculum required, and by the form of growth which occurs in agar shake cultures.

Table II shows the effect of reducing agents. Both cysteine and thioglycollate decreased the minimum effective inoculum of Strain Schu from between 4,000 and 40,000 organisms per ml to approximately one organism per flask. These compounds have also been shown to permit the growth of "strict" anaerobes in fluid cultures exposed to air,[11,12] and to decrease the minimum effective inoculum of certain facultative species.[13]

The effect of oxygen tension was investigated by inoculating peptone agar slants with serial 10-fold dilutions of Strains 38 and Schu and incubating 5 days in mixtures of air and nitrogen. Undiluted commercial nitrogen (assumed to contain 0.5% oxygen) reduced the minimum effective inoculum of Strain 38 to approximately 40,000 per slant, as compared with 4,000,000 per slant in air (21% oxygen). Strain Schu was less affected, the minimum inoculum being reduced from 400 per slant to about 4 per slant, with the optimum at an estimated 1.25% oxygen. These oxygen tensions are within the range which permits the growth of several "obligatory" anaerobes on ordinary media.[14]

In peptone agar shake cultures (0.5 to 1.0% agar) growth of *Bacterium tularense* tended to occur in a narrow zone parallel to, but separated from the surface. This was a constant observation with Strain 38, in which case the zone was always at least 10 mm below the surface and increased in depth as the size of the inoculum was decreased. The zone was about one mm thick after 24 to 48 hours, but increased by extension toward the surface and was several mm thick after a week. Strain Schu demonstrated similar growth stratification with dilute inocula (5000 organisms per ml or less), but larger inocula produced zones which approached the surface as the inoculum increased, until 500,000 to 5,000,000 organisms per ml showed only a one mm thick zone contiguous with the surface. The presence of cysteine caused the growth zone to originate nearer the surface with all inocula, and to extend thereto within 72 hours.

An apparently identical growth formation in agar shake cultures has been described for *Brucella abortus*, and has been ascribed to this species' requirement for carbon dioxide.[15] The preceding experiments on the effect of reducing agents and air-nitrogen mixtures on *Bacterium tularense* cannot lead to a similar conclusion, nor were we able to demonstrate any stimulation by the 2 or 3% carbon dioxide atmosphere obtained by burning a candle in a closed jar. Similar stratification of growth has been observed with a few other bacterial species in ordinary media (see Braun[16] and Prévot[17]) and ascribed to oxidation-reduction potential requirements,[18] and can be induced with numerous facultative anaerobes by the

[11] Quastel, J. H., and Stephenson, M., *Biochem. J.*, 1926, **20**, 1125.

[12] Valley, George, *J. Bact.*, 1929, **17**, 12.

[13] Dubos, René, *J. Exp. Med.*, 1929, **49**, 559; 1930, **52**, 331.

[14] McLeod, J. W., *Acta path. microbiol. Scand.*, 1930, **20** (Suppl. III), 255.

[15] Wilson, G. S., *British J. Exp. Path.*, 1931, **12**, 152.

[16] Braun, H., *Schweiz. Z. allgem. Path. Bakt.*, 1938, **1**, 201, 257, 267; 1939, **2**, 309.

[17] Prévot, A. R., *Ann. Sci. Natur.*, Ser. Bot., 1933, **15**, 23.

[18] Prévot, A. R., *C. R. Soc. biol.*, 1938, **127**, 489.

use of heavy-metal precipitants,[16,19,20] high oxygen pressures,[21] or decreased concentration of nutrients.[22]

The preceding observations appear to relegate *Bacterium tularense* to the poorly defined group of "microaerophiles," as was suggested at one time by Foshay.[23] The species is apparently unusually sensitive to mildly oxidizing intensity in the environment. Our data do not indicate whether this is referable directly to molecular oxygen, or indirectly through the reaction of oxygen with constituents of the medium. As in the case of obligatory anaerobes and of other "microaerophiles," inhibition tends to be abolished by reducing agents, decreased oxygen tension or large inocula. On the other hand, the need for oxygen is indicated by the absence of growth below characteristic levels in agar shake cultures, and stimulation of growth by oxygen is shown by the increased yields obtained in aerated broth cultures.

Summary. 1. *Bacterium tularense* grew luxuriantly and retained virulence during consecutive transfers in peptone broth and on peptone agar without blood or blood constituents. 2. Large inocula were required with the strains tested. The minimum effective inoculum was decreased by reducing agents or by a lowered oxygen tension over the culture, but growth was found to be dependent upon oxygen and stimulated by increased aeration. 3. *Bacterium tularense* appears to be an obligatory aerobe sensitive to mildly oxidizing environment.

[19] Burnet, F. M., *J. Path. Bact.*, 1927, **30**, 21.

[20] King, J. W., and Rettger, L. F., *J. Bact.*, 1942, **44**, 301.

[21] Williams, J. W., *Growth*, 1939, **3**, 21.

[22] Cahn-Bronner, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 454.

[23] Foshay, Lee, *Am. J. Clin. Path.*, 1933, **3**, 379.

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Exudative Trypanosome Pleuritis of Mice Infected Experimentally with *Trypanosoma cruzi.*

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In the course of the routine transfer of a strain of *Trypanosoma cruzi* from mouse to mouse it was found that some of the animals developed a pleuritis which seemed to be of specific origin. Since we were unable to find a description of this pathological condition in the literature, our observations are presented in this note.

The strain of *T. cruzi** was carried in mice since August 1942 and was regularly transferred by subcutaneous, occasionally by intraabdominal, injection of blood containing the parasites. The virulence of the strain at the time when the occurrence of pleuritis was first noted had been increased by frequent passages through mice of 3 weeks of age (9-13 g). All the mice injected subcutaneously with 0.05 cc blood containing approximately 50 trypanosomes per microscope field (about 300 × lin. magnification) died within 10-17 days. In general, trypanosomes appeared in the blood of infected animals 2-8 days after inoculation, according to the number of organisms present in the inoculum.

The first observation was made in a young mouse (No. 234) which was inoculated subcutaneously the 28th of May, 1945 and died the 16th of June. At the autopsy ca. 0.5 cc of a clear pleural exudate was found containing 20-30 trypanosomes per microscope field; 0.1 cc of this exudate was injected intraperitoneally into another mouse which showed prostration 13 days later. Its blood contained at this period a very large number of trypanosomes. The animal was sacrificed and

* Strain of the *T. cruzi* (Chagas 1909) was received from the American Type Culture Collection.