Multiplication of the H37Rv Strain of *M. Tuberculosis* on Blood and and Its Derivatives.* (19352)

ARTHUR W. FRISCH.
From the Department of Bacteriology, University of Oregon Medical School.

In previous studies(1-3) it was demonstrated that *M. tuberculosis var. hominis* and recently isolated tubercle bacilli were capable of initiating growth from small inocula on a variety of basal media to which human blood had been added. These included Bordet-Gengou agar, blood agar base, nutrient agar, and agar-agar alone. The addition of serum, plasma, or fractions thereof to synthetic media have yielded conflicting results(4-6). Youmans and Youmans(4) observed that bovine proantithrombin (fraction III-1) when added to Prauskauer-Beck liquid medium increased the rate of multiplication of the H37Rv and freshly isolated strains to the same degree as did whole serum. Less stimulation was obtained with other fractions, among which were included two globulins, prothrombin (III-2) and fibrinolysin (III-3). In the experiments herein reported various constituents of blood have been examined for their ability to serve as a complete nutrient for the tubercle bacillus. The data indicate that the problem is complex and that growth promoting properties are present in both the plasma and cellular components of blood.

Method. The preparation of standard and blood media and the technic of wet weight inoculation are given in detail in previous publications(1,2). The agar-agar was washed three times with sterile distilled water prior to use. In Table I human bank blood collected in ACD solution 3 weeks previously was utilized. The specimens of blood in Table II were drawn with a dry syringe and needle. Half was permitted to clot and the remainder was defibrinated in a flask containing sterile glass beads. The defibrinated specimen was utilized as such. Aliquots of this material were removed, the red blood cells were washed 3 times with sterile saline, and then diluted to proper concentration based on volume of packed cells. The plasma fractions, except for the lipoproteins, were obtained in the dried state. Weighed samples were dissolved in sterile saline and put through a Seitz filter. Most of the fractions were readily soluble but a few contained slight residues which were lost on filtration. Post-filtration precipitates were observed in certain of the mixtures particularly I and II, and I and III. The same procedures were followed for the bovine plasma fractions, namely prothrombin, proantithrombin, and fibrinolysin. All solid media were prepared in a volume of 4 ml and the liquid media were tested in 2 ml quantities. The hemoglobin was obtained from Nutritional Biochemical Corporation. It was characterized as a crude bovine product containing 14.5% nitrogen and 0.17% iron. Since a considerable portion was insoluble, fractional sterilization was attempted without success. It was decided, therefore, to sterilize at 17 lb pressure for 6 minutes (123°C). The specimens of ox hemin and globin were obtained from the same source and were classed as highly purified. However, practically none of the material was soluble in physiological saline and it was sterilized either by heating to 70°C for 10 minutes on 3 successive days or else by autoclaving for 10 minutes at 17 lb pressure.

Experimental. In the initial experiment shown in Table I, it may be seen that both plasma and red blood cells in proper concentration are capable of initiating growth of the H37Rv strain from inocula consisting of as little as 0.000001 mg or between 5 and 50 organisms. The plasma appears to be somewhat less nutritive but superior to red blood cells, particularly if one considers the fact that 10% washed cells is roughly equivalent to the amount present in 20% whole blood. In other experiments serum was used either in comparison with or instead of plasma and proved just as efficacious. Data of this type were typical when whole banked blood or the constituents thereof were incorporated into agar-agar.

* Aided by a grant from the Truman Collins Fund.

TABLE I. Growth of *M. tuberculosis* on Human Bank Blood and Components Thereof Incorporated into Agar-Agar.

Component tested	%	Inoculum of H37Rv in mg						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Bank blood	10	7	14	14	14	14	21	35
	5	7	9	14	14	16	16	35
	2.5	14	14	14	21	16	35	35
	1	14	14	28	28	28	28	—
	.5	28	28	28	28	35	35	—
Washed red blood cells	10	7	7	14	14	14	14	—
	5	7	7	14	14	16	16	—
	2.5	7	7	14	14	16	16	—
	1	14	42	—	—	—	—	—
	.5	—	—	—	—	—	—	—
Plasma	10	14	14	14	14	16	16	35
	5	14	14	14	14	16	16	—
	2.5	9	14	14	16	16	21	—
	1	14	21	21	28	—	42	—
	.5	35	35	42	42	42	—	—

Numbers indicate day on which grossly visible colonies were first noted.

Since it was entirely possible that the agar may have contributed in some manner to the ability of tubercle bacilli to grow on blood, it was decided to utilize defibrinated blood, serum and washed cells in a saline menstruum. In these experiments freshly drawn blood was employed and to avoid hemolysis the serum was removed as soon as clotting had occurred. In order to rule out a possible stimulating effect of anticoagulants, a portion of the whole blood was defibrinated and either used as such or the red cells were removed and washed thoroughly with saline. Some of the data are presented in Table II and represent single specimens drawn from 6 normal adults of a total of 10 tested. It is apparent that both washed human red cells and serum are capable, under proper conditions, of supporting growth of inocula ranging from 0.001, 0.0001, and 0.00001 mg of tubercle bacilli. In the experiment presented, this represented starting inocula of approximately 50,000, 5,000, and 500 organisms, respectively. Growth of the H37Rv strain was approximately equal in both the defibrinated blood and washed red blood cells, the endpoint varying between 0.5 to 2.5%. It should be pointed out that the final readings represent smears made with a single drop from a 1 ml pipette. If loops were used to prepare the smears, the data were entirely irregular because of the fact that the organisms tended to grow in such tight skeins that they were not picked up.

The specimens containing serum were examined both macroscopically and microscopically at intervals and the final readings represent the degree of growth after 8 weeks. The results were unexpected and as can be seen from Table II, 2 major patterns of growth in serum were observed. The first type illustrated by J, B, and W, were specimens in which organisms grew in the greater but not in smaller amounts of serum. The least concentrations showing growth varied from 1.0 to 2.5%. The second type illustrated by Ja, G, and D was characterized by a zone in which concentrated serum proved inhibitory and with progressive dilution, an amount which supported growth was finally reached. This varied for each of the serums tested and in general, the final degree of growth was less than in the first type.

Attempts were made to identify more accurately the components in plasma capable of initiating growth of small inocula of tubercle bacilli. For this purpose various fractions were obtained and tested either in saline or incorporated into agar-agar.† In experiments of this type using physiological saline as a menstruum growth was demonstrated with combinations as follows: I,V; I,V,IV;

† The plasma fractions were supplied through the generosity of Dr. L. D. Wojick of Harvard University Laboratory of Physical Chemistry; Dr. H. D. Anderson of the Michigan Department of Health Laboratories; Dr. F. D. Johnson of the Cutter Laboratories.

TABLE II. Growth of *M. tuberculosis* on Human Blood and Components Thereof.

Sample	%	Inoculum of H37Rv in mg: Defibrinated blood 10^{-3}	10^{-4}	10^{-5}	Red blood cells 10^{-3}	10^{-4}	10^{-5}	Serum 10^{-3}	10^{-4}	10^{-5}
J	10	+	+	+	+	+	+	+++*	++	+
	5	+	+	+	+	+	+	+++	+	+
	2.5	+	+	+	+	+	+	++	+	—
	1	+	+	+	+	+	+	+++	++	++
	.5	+	+	—	+	—	—	—	—	—
	.1	—	—	—	+	—	—	—	—	—
B	10	+	+	+	+	+	+	+++	+++	+++
	5	+	+	+	+	+	+	++	++	++
	2.5	+	+	+	+	+	+	+	+	—
	1	+	+	—	+	+	—	—	—	—
	.5	—	—	—	—	—	—	—	—	—
	.1	—	—	—	—	—	—	—	—	—
W	10	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+++	+++	++
	2.5	+	+	+	+	+	+	+++	++	++
	1	+	+	—	+	+	+	+++	+	+
	.5	+	—	—	+	+	+	—	—	—
	.1	—	—	—	—	—	—	—	—	—
Ja	10	+	+	+	+	+	+	—	—	—
	5	+	+	—	+	+	+	—	—	—
	2.5	+	+	+	+	+	+	—	—	—
	1	+	+	+	+	+	+	+	—	—
	.5	+	+	+	+	+	+	+	+	—
	.1	+	—	—	+	—	—	—	—	—
G	10	+	+	+	+	+	+	++	—	—
	5	+	+	+	+	+	+	—	—	+++
	2.5	+	+	+	+	+	+	++	++	++
	1	+	+	+	+	+	+	+	—	—
	.5	+	+	+	+	+	+	—	—	—
	.1	—	—	—	—	—	—	—	—	—
D	10	+	+	+	+	+	+	—	—	—
	5	+	+	+	+	+	+	+	—	—
	2.5	+	+	+	+	+	+	—	—	—
	1	+	+	+	+	—	—	—	+	—
	.5	+	+	+	+	—	—	++	+	+
	.1	—	—	—	—	—	—	—	—	—

Growth determined at 8 wk from stained smears.
* Degree of growth measured in serum only since colonies were visible.

I,V,II,III; I,V,III,IV.‡ In evaluating these data it should be borne in mind that the inocula which grew were relatively heavy and represented between 5 million and 500,000 organisms. In a number of additional experiments in which the concentration of fractions I, II, II-1,2, III-0, III-1, III-2, III-2,3, IV-1, IV-4, V, VI, and lipoproteins was varied, multiplication of tubercle bacilli never occurred with any single one when an inoculum of 50,000 or less was used. We have at times, obtained growth with certain combinations of fractions incorporated into agar-agar, but the results were not considered reliable because of the stimulating influence of the solidifying agent. In addition to the plasma fractions from human blood it was possible for us to study relatively purified components of bovine plasma in the same manner.§ Also included are samples of bovine hemoglobin, hemin, and globin. All of these were examined in a saline menstruum and growth was estimated macroscopically where possible, and finally by the smear method. It may be noted that none of the bovine plasma fractions was capable of growing an inoculum of 0.0001 mg or less of the H37Rv strain but that colony formation was evident with larger numbers of organisms. The data do not indi-

‡ Fraction V 2.5 mg/ml; all others 0.5 mg/ml.

§ Obtained through the generosity of Dr. E. C. Loomis of Parke-Davis and Co.

TABLE III. Growth of *M. tuberculosis* on Bovine Blood Derivatives.

Component	mg/ml saline	Inoculum of H37Rv in mg					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Proantithrombin	20	20	30	—	—	—	—
	10	30	—	—	—	—	—
Fibrinolysin	20	20	—	—	—	—	—
	10	30	—	—	—	—	—
Prothrombin	20	20	50	—	—	—	—
	10	20	50	—	—	—	—
Hemoglobin	15	6	12	20	30	30	50
	10	6	12	20	30	50	—
	5	6	12	20	50	50	50
	1	6	12	—	—	—	—
Globin	28.6	—	—	—	—	—	—
Hemin	12.8	—	—	—	—	—	—
Globin + hemin	—	—	—	—	—	—	—
Lowenstein	—	6	10	12	12	16	—
Petragnani	—	8	10	12	14	16	—
10% human blood + agar	—	6	6	10	12	12	17

Numbers indicate day after inoculation on which growth was first observed.

cate that one fraction was more efficacious than any other (Table III).

The result with bovine hemoglobin was not surprising since human hemoglobin solutions obtained by lysing and sedimenting the stroma of human red blood cells were also capable of initiating growth from small inocula. The data show that the limiting concentration of the hemoglobin lies somewhere between 1 and 5 mg and that an inoculum of between 5 and 50 organisms will suffice. In some tubes growth was evident in 6 days. The tubercle bacilli on microscopic examination showed marked skeining and were typical in appearance. Similar results have also been obtained with bovine hemoglobin incorporated into agar-agar. On the other hand bovine hemin, globin, or combinations thereof in a variety of concentrations have never shown evidence of supporting growth of the H37Rv strain. The data regarding these derivatives of hemoglobin are by no means final since the products studied were purified but denatured.

Discussion. In the studies presented it has been observed that human blood contains all of the ingredients necessary to grow an inoculum of between 5 and 50 tubercle bacilli. For this purpose a minimal concentration of 1% defibrinated blood in saline is required. Multiplication is relatively rapid and in more concentrated specimens of blood, occurs within 2 to 3 weeks after inoculation. Variations in the ability of individual bloods to grow tubercle bacilli were observed, but were not considered to be significant. Further inquiry has revealed that both red blood cells, plasma, and serum are capable of supporting multiplication of the H37Rv strain. With regard to the red blood cell, evidence is presented that the active ingredient is hemoglobin. Although the material used was of bovine origin and relatively impure, it seems unlikely that any other constituent could be responsible if one considers that as little as 5 mg per ml was sufficient to grow a minimal inoculum of between 5 and 50 organisms. The fact that hemoglobin acts as a complete nutrient coincides with numerous observations on the essentially aerobic respiration of *M. tuberculosis*(7). It may also be recalled that cytochromes and prophyrins(8,9) have been identified as constituents of tubercle bacilli and other acid fast organisms. The factor in hemoglobin which is responsible for growth appears to resist denaturation and precipitation by autoclaving. Its relationship to the X and V substances of *Hemophilis* are under investigation.

Plasma and its constituents as growth promoting substances for the tubercle bacillus have usually been utilized as stimulants in an otherwise complete medium(4-6). In these studies it has been shown that multiplication occurs when plasma or serum is employed as a

sole source of nourishment. The fact that zones were demonstrated in certain of the specimens suggests the presence of inhibitors which are eliminated by dilution. It is also possible that the effect described may be due to concentration, physical state of the proteins, or to a change in oxidation-reduction potential under the conditions of the experiment. The data which have been obtained with the six major Cohn fractions of plasma cannot be considered as conclusive. They merely indicate that the factors necessary for the cultivation of tubercle bacilli in plasma fractions have not, as yet, been elucidated. Just why fractions I and V proved most efficacious requires further study.

The incorporation of bovine proantithrombin, fibrinolysin, and prothrombin either into agar-agar or into physiological saline did not result in growth of tubercle bacilli equal to that noted in human plasma or serum. Multiplication with large inocula occurred to the same extent in all three fractions and although our data are not comparable with those of Youmans and Youmans(4) they indicate that proantithrombin cannot, as yet, be assigned a special role as a plasma growth factor for the tubercle bacillus. In fact, the entire problem requires reinvestigation with appropriate technics. Thus even agar-agar as a solidifying agent in conjunction with blood or its products was responsible for contradictory results. It is believed, therefore, that any measurement of stimulation or inhibition due to the addition of a substance to a medium which by itself will support growth *M. tuberculosis* should be interpreted with caution. It seems even less likely that such data could be applied directly to infections in animals and man. Finally the demonstration that whole human blood and derivatives thereof are in themselves capable of furnishing a suitable environment for the multiplication of small numbers of tubercle bacilli makes it necessary to consider these substances and particularly hemoglobin, as factors in the pathogenesis of the naturally occurring disease.

Summary. It has been found that human blood, washed red cells, plasma, and serum contain all of the factors necessary for growing the H37Rv strain of *M. tuberculosis* from small inocula. The active component in red blood cells has been tentatively identified as hemoglobin. The essential ingredients in plasma or serum are more complex and require further study. A mixture of human fractions I and V was shown to be capable of initiating growth from large inocula but the data were not comparable with those obtained using whole serum. Similar results were obtained with bovine proantithrombin, fibrinolysin, and prothrombin.

The technical assistance of Vera Jackets is gratefully acknowledged.

1. Tarshis, M. S., and Frisch, A. W., *Am. J. Clin. Path.*, 1951, v21, 101.

2. Frisch, A. W., and Tarshis, M. S., *Am. Rev. Tuberc.*, 1951, v64, 551.

3. Dunphey, D. L., and Fousek, M. D., *Yale J. Biol. and Med.*, 1947, v19, 1013.

4. Youmans, A. S., and Youmans, G. P., *J. Bact.*, 1950, v60, 561, 569.

5. Davis, B. D., and Dubos, R. J., *J. Bact.*, 1948, v55, 11.

6. ———, *J. Exp. Med.*, 1947, v86, 215.

7. Edson, N. L., *Bact. Rev.*, 1951, v15, 147.

8. Crowe, M. O'L., and Walker, A., *Br. J. Exp. Path.*, 1951, v32, 1.

9. Todd, C. M., *Biochem. J.*, 1949, v45, 386.

Received January 14, 1952. P.S.E.B.M., 1952, v79.