

Survival of Tubercle Bacilli in Solutions Containing Glycerol or Its Oxidation Products.

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It has been shown by Webb, Boissevain, and Ryder¹ that tubercle bacilli die within a few weeks when their cultures are deprived of oxygen, an observation which was confirmed by Novy and Soule² and by Potter.³

A suspension of tubercle bacilli in distilled water kept in an anaerobic jar in the incubator becomes non-viable in 3 weeks. The same suspension may remain viable for a year when kept in contact with air. If, however, the tubercle bacilli are suspended in 6% glycerol-water, they die within 2 to 4 weeks in contact with air as well as in the absence of oxygen. Long's medium which is used extensively for the growth of the tubercle bacillus in addition to glycerol contains asparagine, ammonium citrate, phosphate, carbonate, potassium, magnesium, and iron. Addition of these components to the glycerol water does not prevent death of the bacillary suspension unless at least phosphate, potassium, magnesium, and a source of nitrogen have been added; the bacilli then are able to multiply. In the presence of glycerol the bacilli must either multiply or die, while in the absence of glycerol they can be maintained indefinitely in a state of suspended animation.

Experimental. A recently isolated virulent human strain "Voj" was used in the experiments reported here. Most of them have been repeated with 3 other human strains (including H₃₇) giving essentially the same results. A thin veil of a 3-weeks-old culture on Long's medium was washed several times with distilled water and ground to a suspension containing 1 mg bacilli per cc. One-half cc of this suspension was added to test tubes

containing 4.5 cc of the solution to be tested, which were then placed in the incubator, and the viability of the tubercle bacilli tested at intervals by adding one drop of the suspension to a flask containing Long's medium. All flasks were carefully cleaned according to the method of Drea,⁴ which avoids interference by substances inhibiting growth frequently present on laboratory glassware. The addition of a suspension of tubercle bacilli to Long's medium gives rise to numerous small colonies on the bottom of the flask which subsequently spread to the surface, forming a veil in the usual manner. The use of this technic permits a rough estimate of the number of viable bacteria from the number of colonies appearing on the bottom of the flask. The presence of as little as 10⁻⁵ mg viable tubercle bacilli can be regularly demonstrated in this manner. When no viable bacilli could be demonstrated, their absence was confirmed by guinea pig inoculation.

A suspension of tubercle bacilli in 6% glycerol water, in distilled water, and in Long's medium minus glycerol were kept in the incubator both in air and anaerobically in a McIntosh jar. Table I shows that all tubercle bacilli suspended in glycerol water had died after 4 weeks in a hydrogen atmosphere while only a few of those in air survived. In water or in Long's medium without glycerol about half the bacilli died in the anaerobic jar while in air there was no appreciable decrease in numbers. After 8 weeks all bacilli in glycerol water had died while a further decrease took place in the number of bacilli in the other mediums kept in the anaerobic jar. The number of bacilli kept under atmospheric pressure had not diminished appreciably.

Bakers c.p. glycerol was used in these

¹ Webb, G. B., Boissevain, C. H., and Ryder, C. T., *Am. Rev. Tuberc.*, 1924, **9**, 534.

² Novy, F. G., and Soule, M. H., *J. Inf. Dis.*, 1925, **36**, 228.

³ Potter, T. S., *J. Inf. Dis.*, 1939, **64**, 261.

⁴ Drea, W. F., *J. Bact.*, 1942, **44**, 149.

TABLE I.
Length of Survival of Tubercle Bacilli in Water and in Glycerol Solutions.

Suspension of washed tubercle bacilli in		No. of colonies in subcultures made after		
		2 wks	4 wks	8 wks
6% glycerol water	(air)	2	1	0
" " "	(anaerobic)	2	0	0
Long's medium minus glycerol	(air)	4	4	4
" " "	(anaerobic)	3	3	2
Distilled water	(air)	4	4	4
" " "	(anaerobic)	3	3	2

4—more than 100 colonies.

3—20-100 colonies.

2—5-20 colonies.

1—less than 5 colonies.

TABLE II.
Comparison of Synthetic and Natural Glycerol.

Suspension of washed tubercle bacilli in		No. of colonies in subcultures made after		
		2 wks	4 wks	8 wks
6% glycerol water, Bakers C.P.	(air)	2	1	0
" " " "	(anaerobic)	2	1	0
" " " (redistilled)	(air)	2	1	0
" " " "	(anaerobic)	2	1	0
" " " (synthetic)	(air)	2	0	0
" " " "	(anaerobic)	2	0	0
H ₂ O	(air)	4	4	4
" "	(anaerobic)	4	3	3

4—more than 100 colonies.

3—20-100 colonies.

2—5-20 colonies.

1—less than 5 colonies.

experiments; according to the analysis of the manufacturers it contains 0.03% fatty acids. The soaps of fatty acids and the fatty acids themselves are bacteriostatic for the tubercle bacillus in very low concentrations, and although the fatty acids present in glycerol are probably the short chain fatty acids that have no inhibiting action, the glycerol was further purified by redistillation *in vacuo* and a sample of glycerol was used that had been synthesized from petroleum products and presumably was free from contamination by fatty acids.*

Table II shows that these samples of glycerol had the same effect as Bakers c.p. glycerol.

Since the tubercle bacillus actively metabolizes glycerol the possibility exists that its

lack of survival in glycerol water is connected with this metabolism. The effect on the survival of suspensions of tubercle bacilli was studied for the following glycerol metabolites: glycerophosphoric acid, phosphoglyceric acid, glyceric acid, glyceraldehyde, dioxyacetone, lactic acid, and pyruvic acid. A concentration of 0.1% was used in all cases and the solutions were brought to pH = 7.0 with N/1 NaOH. The results are recorded in Table III. Glyceraldehyde and dioxyacetone kill the tubercle bacilli more rapidly than glycerol, while the others are less active, pyruvate being without any lethal effect. The hypothesis that the death of tubercle bacilli in glycerol water is due to the formation of glyceraldehyde is tempting, but its presence could not be demonstrated in the glycerol water suspensions.

Pyruvic acid may be substituted for glycerol in culture media if its concentration

* We wish to thank the Shell Chemical Co. through whose courtesy we obtained this sample of synthetic glycerol.

TABLE III.
Survival of Tubercle Bacilli in Solutions of Glycerol and Some of Its Derivatives.

Suspension of tubercle bacilli in	No. of colonies in subculture made after			
	2 wks	4 wks	6 wks	8 wks
6.0% glycerol	2	0	0	0
.1% glyceraldehyde	0	0	0	0
.1% dioxycetone	0	0	0	0
.1% glycerophosphate	3	3	1	0
.1% phosphoglycerate	2	1	0	0
.1% glycerate	3	3	0	0
.1% lactate	2	1	0	0
.1% pyruvate	4	4	4	4
Distilled water	4	4	4	4

4—more than 100 colonies.

3—20-100 colonies.

2—5-20 colonies.

1—less than 5 colonies.

is maintained below 0.1%. At higher concentrations it inhibits growth. Tubercle bacilli multiply in pyruvate suspensions when Na-phosphate is present. Potassium and magnesium are not required as they are for the glycerol metabolism.

The formation of pyruvic acid in Long's culture medium during the growth of tubercle bacilli was demonstrated through the di-nitro phenylhydrazone. It is apparently an intermediary metabolite in the oxidation of glycerol by the tubercle bacillus.

Glyceraldehyde may be formed from glycerol by removal of 2 hydrogen atoms and pyruvic acid by the removal of 4 hydrogen

atoms. Since pyruvate allows the tubercle bacilli to survive, it may well be that this attempted dehydrogenation is the direct cause of death for tubercle bacilli in glycerol water. A similar derangement of the oxidation mechanism may be responsible for the lack of survival of the tubercle bacillus under anaerobic conditions.

Summary. Tubercle bacilli die more or less rapidly in solutions in water of glycerol, glycerophosphate, phosphoglycerate, glycerate, lactate, glyceraldehyde, or dioxycetone. Similar suspension in distilled water in solutions of inorganic salts and in pyruvate survive for a prolonged period.

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Growth Promotion of Bovine Tubercle Bacilli by Pyruvate.

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It has been shown in the preceding paper¹ that human tubercle bacilli die when suspended in a 6% glycerol solution while they remain viable for at least 8 weeks in distilled water or in a one-tenth of one per cent pyruvate solution. If phosphate is added to the pyruvate solution the tubercle bacilli multiply

while a similar addition to the glycerol solution has no effect.

Glycerol is believed to be harmful for the growth of the bovine type of tubercle bacilli, and many strains of bovine tubercle bacilli grow very slowly or not at all on Long's medium. Addition of 0.1% pyruvate makes it suitable for the growth of the majority of the dysgonic type of bovine tubercle bacilli.

¹ Boissevain, C. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, **54**, 342.