THE ROLES OF BIOTIN AND CARBON DIOXIDE IN THE CULTIVATION OF MYCOBACTERIUM TUBERCULOSIS[1, 2]

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During a study of the growth of tubercle bacilli on primary isolation on oleic acid-albumin agar and on the egg medium of the American Trudeau Society (ATS), the observation was made that a number of strains, which gave growth on ATS medium, did not grow, or grew very poorly, on oleic acid-albumin agar medium (Middlebrook, Cohn, and Schaefer, 1954).

In an attempt to improve this latter medium various growth factors were added, and it was found that biotin was either markedly stimulating or even absolutely required for the artificial cultivation of several strains (Middlebrook, Cohn, and Schaefer, 1954). Similarly, incubation under an atmosphere with increased CO_2 content also stimulated the growth of these strains markedly. One strain has been observed for which biotin would not support growth at 37 C, and which was permitted to grow only by incubation under an atmosphere of air containing added CO_2.

The purpose of this paper is to report our observations on the roles of biotin and carbon dioxide as growth factors for tubercle bacilli.

MATERIALS AND METHODS

The basic oleic acid-albumin agar medium[3] used in these experiments had the following composition: KH_2PO_4, 1 g; Na_2HPO_4 (anhydrous), 2.5 g; Na_3 citrate $2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.0005 g; $ZnSO_4 \cdot 7H_2O$, 0.0005 g; $CuSO_4 \cdot 5H_2O$, 0.0005 g; ferric ammon. citrate, 0.05 g; L-glutamate (sodium salt), 0.5 g; vitamin-free casein hydrolyzate, 0.1 g; $(NH_4)_2SO_4$, 0.5 g; glucose, 2 g; glycerol (reagent grade), 2 ml; oleic acid-albumin complex, 50 ml; distilled water, ad 1,000 ml; agar, 15 g.

The pH was adjusted before autoclaving to 6.8 (unless otherwise indicated). After autoclaving, its value was found to be 0.15 to 0.2 pH units lower. Before autoclaving, malachite green (Coleman and Bell), in a final concentration of 0.5 to 1.0 μg/ml, was added in order to reduce the incidence of contamination. This concentration was found to have no significant effect on the growth of tubercle bacilli in this medium. Glucose and oleic acid-albumin complex, prepared as described by Dubos and Middlebrook (1947), were added after autoclaving. The medium was poured into petri dishes. For the inoculation of the plates, the following procedures were used.

Clinical specimens were treated before inoculation by the concentration and decontamination procedures previously described (Middlebrook *et al.*, 1954). When cultures in "Tween-albumin" liquid medium were to be used as inocula, they were ground in a Teflon grinder (Pierce, Dubos, and Schaefer, 1953) and filtered through an M-type sintered glass filter in order to obtain a suspension consisting predominantly of isolated bacterial cells (Cohn *et al.*, 1954). The suspension was diluted to 10^{-5} or 10^{-6} in sterile solution of 0.2 per cent bovine serum albumin in distilled water, and 0.1 ml of each dilution was spread over the agar surface with a glass spreader while the plate was revolving on a turntable. The plates were placed in polyethylene plastic bags in order to protect them from desiccation and incubated at 37 C. For the study of the effect of incubation under an atmosphere of air with higher CO_2 content, the plates were placed in glass desiccators which were evacuated to 50 mm Hg pressure and then filled to atmospheric pressure with a gas mixture of air containing 5 per cent CO_2 obtained from a commercially available cylinder. For the routine use of incuba-

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[2] Presented in part at the General Meeting of the Society of American Bacteriologists in Pittsburgh on May 7, 1954.

[3] This medium was proved to contain less than 0.001 μg biotin/ml by microbiological assay with a biotin requiring strain of *Lactobacillus arabinosus*.

tion under increased CO_2 tension in the diagnostic laboratory, a large metal chamber with hermetic closure was employed, into which a gas mixture of 10 per cent CO_2 in air was appropriately introduced to give a final CO_2 concentration of approximately 2 to 5 per cent. The chamber was refilled with the gas mixture each time after opening.

RESULTS

(1) *A strain requiring biotin or CO_2.* The effect of various concentrations of biotin and of incubation under CO_2 enriched air on a strain requiring either biotin or carbon dioxide is represented in table 1. It can be seen from this table that on the agar plates, which had been incubated in atmospheric air, no growth was obtained in the absence, partial growth in the presence of 0.02 μg/ml, and full growth in the presence of 0.1 μg/ml or higher concentrations of biotin. On the plates which were incubated in an atmosphere of 5 per cent CO_2 in air, full growth occurred in the absence as well as in the presence of biotin. In liquid Tween-albumin medium incubated in atmospheric air, the rate and total amount of growth increased with increasing biotin concentration in the medium. Desthiobiotin had the same growth stimulating effect as biotin. Pimelic acid, diamino-pimelic acid,[4] asparagine, aspartic acid, oxalacetic acid, acetic acid, and arginine had no effect. Oleic acid and pyruvic acid also did not replace biotin but stimulated growth in the presence of biotin. Variations of the pH or a decrease of the incubation temperature from 37 to 34 C had no effect on the biotin requirement of this strain. Experiments of incubation in air with different concentrations of CO_2 indicated that a concentration of 1 per cent CO_2 in air was sufficient to support growth in the absence of biotin. The described strain was resistant to 500 μg/ml of streptomycin and to 30 μg/ml of isoniazid. It maintained its requirements for biotin or CO_2 after many transfers in biotin containing Tween-albumin medium. Occasionally, however, rare colonies were recovered from biotin-free agar media incubated under atmospheric air, and these colonies proved to be biotin-independent mutants.

[4] Kindly supplied by Dr. L. D. Wright, Director of Research in Microbiological Chemistry, Research Division, Sharp & Dohme, Inc., West Point, Pa.

TABLE 1

Effect of biotin and CO_2 on growth of a biotin requiring strain of Mycobacterium tuberculosis

Biotin	Oleic Acid-Albumin Solid Agar Medium* (Growth after 17 days)		Biotin	Tween-Albumin† Liquid Medium (Optical density after 12 days)
	Air	5% CO_2 in air		
μg/ml			μg/ml	
0.5	+++	+++	0.05	0.300
0.1	+++	+++	0.025	0.328
0.02	+	+++	0.012	0.244
0.004	0	+++	0.006	0.199
0	0	+++	0.003	0.164
			0	0.089

* Inoculum: 0.1 ml of 10^{-6} dilution of culture in Tween-albumin medium.

† Inoculum: 1/100 dilution of culture in Tween-albumin medium, at pH 6.8.

+++ = full growth; + = sparse growth.

This strain was reisolated from the patient at intervals of several months without showing any change of its characteristic properties.

(2) *Strains requiring biotin or CO_2 only on oleic acid-albumin medium of acid reaction.* Several strains of tubercle bacilli have been encountered which showed a requirement for biotin or carbon dioxide on oleic acid-albumin

TABLE 2

Effects of pH and of oleic acid on the biotin requirement of a strain of Mycobacterium tuberculosis which requires biotin only at acid reactions

(Incubation at 37 C for 3 weeks under atmospheric air.)

Medium	Biotin	pH		
		6.5	6.9	7.3
	μg/ml			
Albumin agar	0.5	400*	500	450
		+	+	±
	0	400	500	450
		+	+	±
Albumin agar plus 0.005% oleic acid	0.5	387	473	412
		+++	++	+
	0	140	466	412
		++	++	+

* Numbers of colonies. The crosses indicate the relative sizes of the colonies: +++ = 2-3 mm diameter; ± = just visible, grossly.

TABLE 3

Reversal of the inhibiting effect of 4-(imidazolidone-2) caproic acid by biotin and by increased CO_2 tension

Biotin (μg/ml)	4-(Imidazolidone-2) Caproic Acid (μg/ml)						
	0	0.08	0.4	2.0	10.0	50.0	100.0
Incubation under atmospheric air (0.03% CO_2)							
0	+++*	+	0	0	0	0	0
0.01	+++	+++	+	0	0	0	0
0.05	+++	+++	+++	+++	+++	+++	+++
Incubation under air with 1% CO_2							
0	+++	+++	+++	+++	0	0	0
0.01	+++	+++	+++	+++	+++	+	0
0.05	+++	+++	+++	+++	+++	+++	+++
Incubation under air with 10% CO_2							
0	+++	+++	+++	+++	0	0	0
0.01	+++	+++	+++	+++	+++	+	0
0.05	+++	+++	+++	+++	+++	+++	+++

* The crosses indicate the relative amounts of growth after 3 weeks of incubation.

medium of more or less acid reaction, but not on media of neutral or alkaline reaction. On albumin agar medium without oleic acid, these strains were able to grow in the absence of biotin even when the reaction of the medium was acid. An example of the effect of biotin on the growth of such a strain on oleic acid-albumin agar and on albumin agar media of various pH's is represented in table 2. This group included isoniazid and streptomycin-sensitive strains as well as strains resistant to isoniazid alone or to both drugs. Their requirement for biotin or CO_2 under the above described conditions remained the same after repeated subcultures *in vitro* and on repeated isolation from the specific patient's sputa.

(3) *The inhibiting effect of the biotin analogue 4-(imidazolidone-2) caproic acid and its reversal by increased CO_2 tension.* Having shown that incubation under increased CO_2 tension substitutes for a requirement for biotin, it seemed of interest to investigate whether increased CO_2 tension actually eliminates the need for biotin as a necessary growth factor or only decreases the amount of biotin required for CO_2 assimilation. For this study the biotin analogue 4-(imidazolidone-2) caproic acid[5] was used because it is known to inhibit growth of tubercle bacilli by antagonizing endogenous biotin (Pope, 1952). The effect of increased CO_2 tension and of biotin on this inhibition was studied. The experiments were made with a nonbiotin requiring strain. Their results are recorded in table 3.

It can be seen from this table that under increased CO_2 tension about 50-fold higher concentrations of the biotin antagonist were necessary for inhibition of growth than under atmospheric air. The inhibiting effect of still larger concentrations of the inhibitor was reversed by the addition of large amounts of biotin but not by an increase of the CO_2 tension. These results suggest that minimal amounts of endogenous biotin are required for the utilization of CO_2 for growth.

(4) *A strain requiring CO_2 for growth at 37 C but not at 34 C.* A different type of CO_2 requirement, which is apparently not due to a deficiency of biotin, was found for a highly streptomycin and moderately isoniazid-resistant strain isolated from the sputum of a patient with cavitary tuberculosis under treatment with streptomycin and isoniazid. This strain was unable to grow on ATS egg medium and on agar medium even when biotin was added to the medium. Its culture could only be obtained either when such media were incubated at 37 C under an atmosphere with increased CO_2 content or under atmos-

[5] Kindly supplied by Dr. J. A. Aeschlimann, Director of Chemical Research, Hoffmann-La Roche, Inc., Nutley, New Jersey.

pheric air when incubated at 34 C. All attempts to find a medium suitable for the growth of this strain at 37 C in air failed. Variations of the pH, omission of oleic acid, the addition of various amino acids or adenylic acid in various concentrations, of yeast extract and various vitamins were tried without success. This strain was also unable to grow in liquid Tween-albumin medium when incubated at 37 C in loosely capped tubes. It gave, however, growth when it was incubated in such tubes at 34 C, and also at 37 C when the tubes were tightly closed (Thunberg tubes). In these latter tubes growth occurred, however, only after a lag phase of some 5 days. The growth curves obtained under these various conditions are shown in figure 1. Subcultures on agar media from the closed tubes incubated under atmospheric air at 37 C, as well as from those incubated at 34 C, showed that the bacteria grown under these conditions were still CO_2 requiring organisms. The delayed growth in the sealed tubes incubated at 37 C, therefore, was due, most probably, to the slow accumulation of metabolically produced CO_2 which could not escape from the closed tubes. Large inocula of this strain on agar media incubated in atmospheric air yielded rare colonies of bacilli which proved to be CO_2-independent mutants. The mutation rate of this strain from CO_2-dependence to CO_2-independence has not been determined.

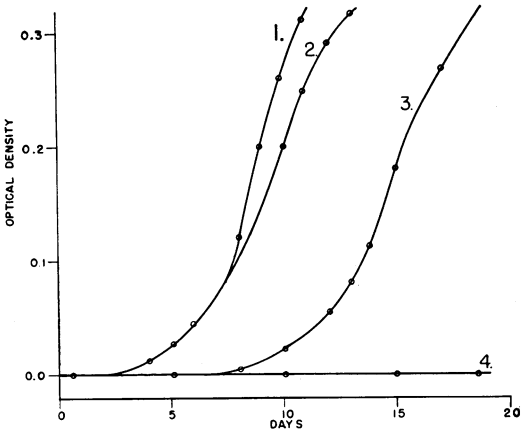

Figure 1. Effect of gaseous environment and temperature on the growth in "Tween-albumin" liquid medium of a strain of *Mycobacterium tuberculosis* which requires increased CO_2 tension at 37 C, but not at 34 C.

(1) 34 C in atmospheric air; (2) 37 C in 5 per cent CO_2 air; (3) 37 C in atmospheric air (closed system); (4) 37 C in atmospheric air (open system).

PRACTICAL APPLICATIONS

In an attempt to evaluate the practical importance of increased CO_2 tension for the culture of tubercle bacilli on primary isolation, tubercle bacilli from some 70 sputa were cultivated on biotin containing oleic acid-albumin medium by incubation under atmospheric air, on the one hand, and under air with increased CO_2 content, on the other hand. It was found that incubation under increased CO_2 tension definitely stimulated the growth in more than half of the cases and that none of the strains encountered was retarded or inhibited by increased CO_2 tension.[6]

[6] It should be mentioned that the stock laboratory strain, H37Rv, which had been repeatedly subcultured on Tween-albumin medium, was inhibited by increased CO_2 tension when plated onto oleic acid-albumin agar medium. This strain, on the contrary, was stimulated on the same medium by increased CO_2 tension when a culture, which had been recently animal-passed, was employed.

The stimulating effect of CO_2 was particularly marked on media of acid pH (6.6).

An illustration of the beneficial effect of CO_2 for the primary isolation of a tubercle bacillus strain from a sputum specimen is presented in figure 2.

DISCUSSION

A requirement of strains of tubercle bacilli for biotin has not previously been reported in the literature. The studies of Landy and Dicken (1941) and of Pope and Smith (1946) have shown, on the contrary, that the two human and one bovine strains studied synthesized biotin during growth on a synthetic medium. The experiments presented here show that strains of tubercle bacilli may manifest a requirement for exogenous biotin for their growth from small inocula on solid and in liquid synthetic media, and especially when they are cultivated on solid oleic acid-albumin media of acid reaction.

The observation that all strains could be cultivated in the absence of biotin when incubated in air with increased CO_2 content suggests that the function of biotin in the metabolism and growth of tubercle bacilli is to promote the assimilation of CO_2.

That CO_2 is essential for the growth of tubercle bacilli was indicated by the studies of Wherry

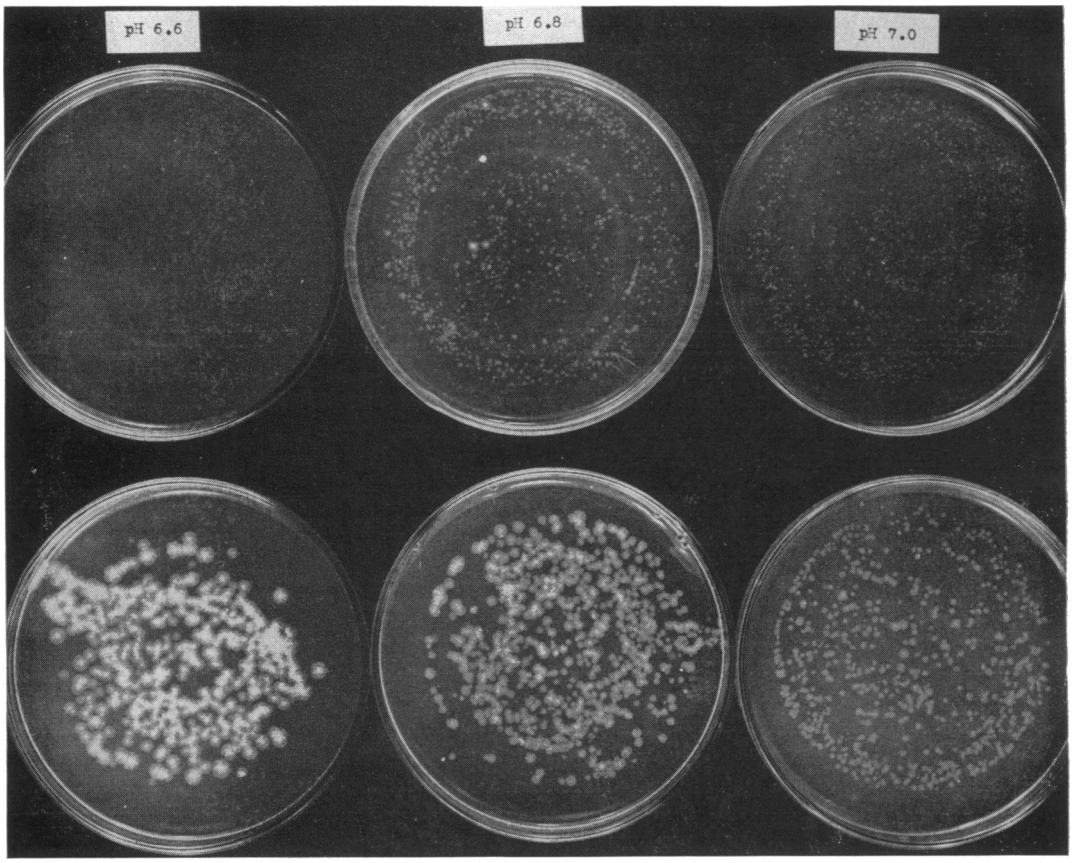

Figure 2. Growth on primary isolation from sputum of a strain of *Mycobacterium tuberculosis* on oleic acid-albumin medium at various hydrogen ion concentrations incubated under air (top row) and under air with 5 per cent CO_2 (bottom row) at 37 C for 3 weeks.

and Erwin (1918) and conclusively proven by Rockwell and Highberger (1926) and by Davies (1940). The CO_2 content of atmospheric air is apparently sufficient for the growth of most strains of tubercle bacilli on the classical egg yolk media which contain variable amounts of "free" biotin. Growth on such media is further favored by the customary enclosure of these media in sealed tubes, which permits an accumulation of metabolically produced CO_2. In contrast, in the case of agar media poured in flat petri dishes, CO_2 escapes more easily, and it is removed from the medium if the reaction of the medium is acid. The long incubation time necessary for the growth of tubercle bacilli and the slow rate of production of CO_2 by the bacteria themselves prevent the amount of CO_2 from reaching "physiologic" concentrations. Biotin, under these conditions, compensates for the low CO_2 content of the environment.

The experiments reported here have shown that the biotin or CO_2 requirement of tubercle bacilli on acid culture media is increased by the presence of oleic acid. The mechanism by which oleic acid exerts this effect is not clear. The hypothesis that the ability of the medium to retain CO_2 may be decreased in the presence of oleic acid was tested in manometric experiments with the Warburg apparatus[7] and disproved. It is possible that under those conditions where CO_2 is lost from the aqueous environment (acid reactions), oleic acid, which normally stimulates growth (Dubos and Davis, 1946; Schaefer, 1952), may be injurious to the bacterial

[7] We are indebted to Mr. Charles Coleman for performing these experiments.

cells because the stimulation of the metabolic reactions produced by oleic acid is not accompanied by an equally increased rate of CO_2 uptake. It is evident from the results of studies described here that fatty acid stimulation of growth of these organisms is most strikingly seen when the environmental concentration of CO_2 is elevated above that found in atmospheric air.

Experiments on the effect of increased CO_2 tension on the inhibition of growth produced by the biotin antagonist, 4-(imidazolidone-2) caproic acid, have lead the writers to conclude that the metabolic activity of small amounts of endogenous biotin is essential for the assimilation of CO_2, even when CO_2 is supplied in high concentration.

The hypothesis that biotin functions as a coenzyme of CO_2 transfer was first proposed by Burk and Winzler (1943) and further supported by Lardy, Potter, and Elvehjem (1947) who showed that the growth of *Lactobacillus arabinosus* was stimulated by bicarbonate in a medium with high, but not in a medium with low, biotin content. Proof of the role of biotin in the incorporation or transformation of CO_2 into an essential metabolite was given by Wessman and Werkman (1950) who showed that acetone dried preparations of *Micrococcus lysodeikticus* were able to incorporate CO_2 labeled with heavy carbon into oxalacetate and that this reaction was inhibited by avidin, an agent known to bind and render biotin unavailable to bacteria.

That factors other than biotin deficiency may be responsible for a requirement for increased CO_2 tension is indicated by our observations on a strain which requires an increased CO_2 tension for growth at 37 C even on media containing large amounts of biotin. This strain, however, was able to grow at atmospheric CO_2 tension, and without added biotin, when incubated at 34 C. A similar observation has been reported by Borek and Waelsch (1951) for a strain of *L. arabinosus*. This strain required increased CO_2 tension for growth at 37 C on a medium lacking phenylalanine and tyrosine, and for growth at 39 C on a medium lacking aspartic acid. The fact that the requirement for CO_2 at the higher temperatures could be replaced by specific amino acids was interpreted as evidence that the synthesis of these amino acids from CO_2 was inhibited at the higher temperatures. In the case of the tubercle bacillus no such replacement for the CO_2 requirement was found. The cause of this CO_2 requirement at higher temperature is unknown.

Soltys *et al.* (1952) reported that Stanley Griffith isolated a strain of tubercle bacilli which could grow in primary culture only in the presence of added CO_2. Soltys has also reported that growth of tubercle bacilli may be enhanced by CO_2. Our own observations stress the beneficial effect of increased CO_2 tension and of oleic acid medium of acid reaction for the primary isolation of tubercle bacilli from their natural sources. No evidence was obtained from our studies that the requirement of tubercle bacilli for biotin or increased CO_2 tension is related to drug resistance to streptomycin or isoniazid.

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The authors wish to express their appreciation to Mr. William Jones for his technical assistance in these studies.

SUMMARY

Strains of *Mycobacterium tuberculosis* are described which required biotin for their growth on an oleic acid-albumin agar medium of acid reaction (pH 6.3–6.8). Incubation under increased carbon dioxide tension (1–5 per cent) abolished the requirement of these strains for biotin.

Mutants which did not require biotin or increased CO_2 tension could be isolated from such strains.

The inhibitory effect of moderate concentrations of the biotin analogue, 4-(imidazolidone-2) caproic acid, on the growth of biotin-independent strains was reversed not only by biotin but also by increased CO_2 tension. However, in the presence of high concentrations of the inhibitor only biotin reversed the inhibition, suggesting that small amounts of biotin are essential for CO_2 "fixation".

The presence of oleic acid in albumin agar medium of acid reaction enhanced the requirement for biotin or carbon dioxide. The majority of strains obtained on primary culture from pathological materials manifested fastest growth on incubation under 1 to 5 per cent carbon dioxide on an oleic acid-albumin agar medium of pH 6.6.

One strain required increased CO_2 tension for growth at 37 C but was able to grow at atmospheric CO_2 tension, and without biotin, when

incubated at 34 C. A CO_2-independent mutant was readily isolated from this strain.

The significance of these findings for the role of increased CO_2 tension and biotin in the growth of *M. tuberculosis* is discussed, and the advantage of incubation under increased CO_2 tension and of oleic acid-albumin medium of pH 6.6 for the primary isolation of tubercle bacilli is pointed out.

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