

# STUDIES ON AUTOLYSIS OF MYCOBACTERIA

## II. AUTOLYSIS OF VARIOUS PATHOGENIC AND NONPATHOGENIC STRAINS GROWN IN A MEDIUM DEFICIENT IN NITROGEN

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A rapid decline in optical density, representing autolysis, has been observed to follow growth of *Mycobacterium tuberculosis* (H37Rv strain) in a semisynthetic liquid medium containing an excess of glucose and a limiting amount of nitrogen in the form of ammonium chloride. Schaefer *et al.* (1949) suggested that autolysis was due to autodigestion of cellular elements followed by disruption of the cellular membrane by the "tween 80" used in the medium to produce dispersed growth of the bacilli. As a result of studies on autolysis of the H37Rv strain, Redmond (1955) and Redmond and Bowman (1955) have suggested that the lytic effect observed may be the result of a lysogenic state of the bacilli. During preliminary tests on various strains a saprophytic strain, *Mycobacterium lacticola*, was observed not to lyse. These results suggested the possibility that virulence might be correlated with the ability to undergo autolysis. The present paper records the results obtained from studies on various virulent, avirulent, attenuated, "atypical," and saprophytic cultures.

### MATERIALS AND METHODS

The culture methods and the procedures employed previously (Redmond, 1955; Redmond and Bowman, 1955) were used. The organisms were grown in a basal medium consisting of a mixture of buffer salts supplemented with 10 mg per ml glucose and 15  $\mu$ g per ml ammonium chloride as the only available carbon and nitrogen sources, respectively. Polyoxyethylene sorbitan monooleate (tween 80) and bovine albumin, Fraction V (Armour) were added in order to obtain diffuse growth. The development of the cultures was followed turbidimetrically. Duplicate tests were run on all strains. The various species and strains on which observations were made are characterized in table 1. The 5

"atypical" strains are representative of a large number of strains isolated from patients with pulmonary disease and described by Timpe and Runyon (1954). Twelve other "atypical" strains were tested during these studies with results similar to those of the 5 recorded. Before being tested for lysis on the nitrogen deficient medium, the bacilli of each strain were grown for one or more subcultures on the same medium containing 1000  $\mu$ g per ml  $\text{NH}_4\text{Cl}$ . The organisms were washed in the basal medium (without  $\text{NH}_4\text{Cl}$ ) before being used as inocula.

### EXPERIMENTAL RESULTS

The results assembled in table 2 indicate the optical densities obtained by each strain at the time of maximal growth and also the densities after 28 days. The difference ( $\Delta$ , Column 3) represents the relative extent of autolysis of each strain.

The mycobacteria listed fall roughly into three groups. The first group is comprised of strains that showed a decrease in optical density of more than 0.20 and with a mean of 0.28. The behavior of strains of this group is illustrated in figure 1. The failure of such strains to lyse when provided with 1,000  $\mu$ g per ml  $\text{NH}_4\text{Cl}$  is illustrated by means of H37Rv (curve 1), whereas the lysis of this and other strains on the nitrogen deficient medium is shown in curves 2 to 5.

Twelve pathogenic strains recently isolated from patients with active tuberculosis and a streptomycin resistant variant of H37Rv were found to lyse to the same extent as did the H37Rv strain.

The strains listed in group 2 (table 2) lysed much less than did the strains of group 1. The results of tests on 4 of these are represented graphically in figure 2. Only 2 strains, *Mycobacterium smegmatis* (curve 4) and 607 (curve 1),

TABLE 1  
Cultures of acid-fast bacilli studied

Culture or Species	Pathogenicity	Source	History or Characterization
H37Rv.....	Human	Trudeau Foundation	Isolated in 1905
H37RvR.....	Human	Trudeau Foundation	Resistant to SM
H37Ra.....	Avirulent	Trudeau Foundation	Variant of H37Rv
Spears.....	Human	Lawson VA Hospital	Enhanced by SM
Bovine (Ravenel).....	Rabbit	Trudeau Foundation	
BCG (Birkhaug).....	Attenuated	H. Hillbae	Vaccine # B-22
BCG (Holm).....	Attenuated	J. Holm	Vaccine # 768
BCG (Rosenthal).....	Attenuated	C. Palmer	Vaccine
Avian (Kirschberg).....	Fowl	Trudeau Foundation	Optimum temp 40 C
Vole.....	Vole	England	
Atypical 42.....	Mouse pos.	Kansas City	Buff-yellow
Atypical 79.....	Mouse pos.	Kansas City	Buff-yellow
Atypical 118.....	Mouse pos.	Lawson VA Hospital	Buff-yellow, rough
Atypical 163.....	Mouse neg.	Atlanta VA Hospital	Yellow-orange
Atypical 181.....	Mouse neg.	Batthey Hospital	Buff, smooth
ATCC #607.....	Avirulent	Trudeau Foundation	Doubtful TB
R1Ra.....	Avirulent	Trudeau Foundation	Variant of R1Rv
<i>Mycobacterium phlei</i> .....	Saprophyte	Trudeau Foundation	Timothy bacillus
<i>Mycobacterium butyricum</i> .....	Saprophyte	C. D. C.*	
<i>Mycobacterium smegmatis</i> .....	Saprophyte	C. D. C.	
<i>Mycobacterium lacticola</i> .....	Saprophyte	R. Gordon	ATCC #8150
<i>Mycobacterium ranae</i> .....	Amphibia	D. Yegian	
Red saprophyte.....	Saprophyte	Copenhagen	Red, smooth
White saprophyte.....	Saprophyte	Copenhagen	White, smooth
Radish.....	Saprophyte	Columbus, Ga.	From radish

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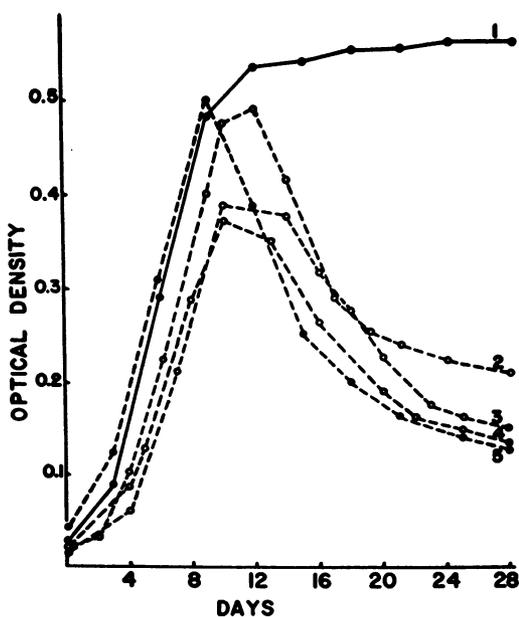


Figure 1. Nitrogen depletion and autolysis of mycobacteria (Group 1). 1. CONTROL. H37Rv in 1000 µg per ml NH<sub>4</sub>Cl. 2-5. In 15 µg per ml NH<sub>4</sub>Cl. 2. Bovine; 3. BCG(H); 4. BCG(B); 5. H37Rv.

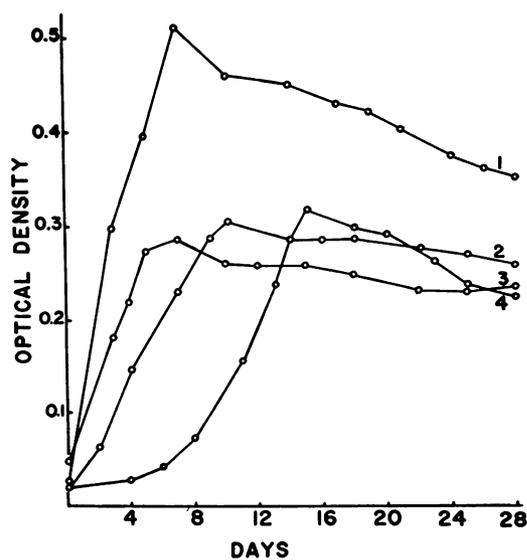


Figure 2. Growth and autolysis of mycobacteria (Group 2). In 15 µg per ml NH<sub>4</sub>Cl. 1. ATCC #607; 2. Vole; 3. BCG(R); 4. *Mycobacterium smegmatis*.

exhibit any marked degree of lysis. With the exception of 607 the peak growth of the organisms in this group is below the peak growth of the H37Rv strain in the nitrogen deficient medium.

The third group of organisms (table 2) did not

TABLE 2

Lysis in medium containing 15 µg/ml NH<sub>4</sub>Cl. The extent of growth and autolysis of various strains of mycobacteria as shown by optical density readings at the peak of growth (Maximal), and after 28 days (Terminal). Δ indicates the extent of lysis (Maximal—Terminal)

Culture or Species	Maximal	Terminal	Δ
<i>Group 1</i>			
H37Rv*	0.51	0.13	0.38
Spears.	0.36	0.11	0.25
Bovine*	0.48	0.19	0.29
BCG(B)*	0.39	0.13	0.26
BCG(H)*	0.40	0.15	0.25
			Mean 0.28
<i>Group 2</i>			
BCG(R)*	0.29	0.21	0.08
H37Ra.	0.16	0.11	0.05
<i>Mycobacterium phlei</i> .	0.16	0.13	0.03
<i>Mycobacterium ranae</i> .	0.33	0.26	0.07
<i>Mycobacterium smegmatis</i> *.	0.25	0.15	0.10
RIRa.	0.26	0.18	0.08
Red saprophyte.	0.14	0.11	0.03
White saprophyte.	0.20	0.16	0.04
Vole*	0.32	0.27	0.05
607*	0.51	0.35	0.16
			Mean 0.07
<i>Group 3</i>			
Avian*	0.24	0.22	0.02
Atypical 42.	0.20	0.20	0.00
Atypical 79*	0.32	0.32	0.00
Atypical 118.	0.18	0.17	0.01
Atypical 163.	0.22	0.21	0.01
Atypical 181.	0.23	0.23	0.00
<i>Mycobacterium butyricum</i> *.	0.52	0.52	0.00
<i>Mycobacterium lacticola</i> *.	0.53	0.53	0.00
Radish.	0.11	0.10	0.01
			Mean 0.005

\* Data for these strains plotted on graphs (Figures 1, 2, 3).

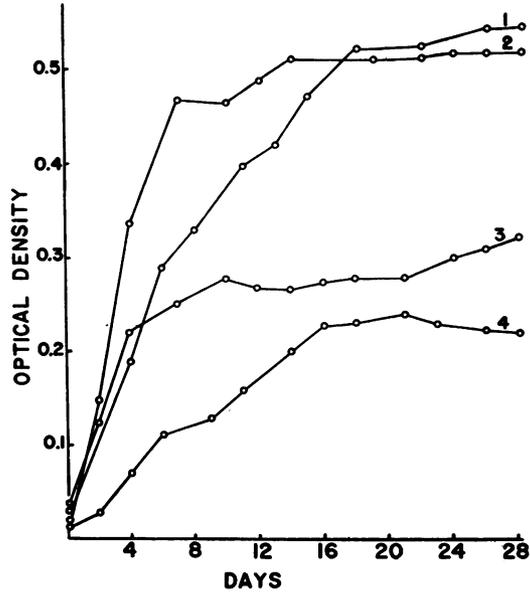


Figure 3. Growth and no autolysis of mycobacteria (Group 3). In 15 µg per ml NH<sub>4</sub>Cl. 1. *Mycobacterium butyricum*; 2. *Mycobacterium lacticola*; 3. Atypical 79; 4. Avian.

lyse. Optical density curves of 4 strains of this group are shown in figure 3. Except for two strains, *Mycobacterium butyricum* and *M. lacticola*, all bacilli of this group failed to show growth comparable to H37Rv on the nitrogen deficient medium.

The observation that most of the nonlysing strains did not grow to the same extent as the H37Rv strain suggested that a fraction of the NH<sub>4</sub>Cl may not have been utilized and could then act to prevent autolysis. To check this hypothesis two sets of tubes were set up, each containing 10 ml of the "lysis inducing medium." One set was inoculated with the Vole strain, the other with *M. lacticola*. Just prior to peak growth, as determined by the optical densities, the medium was removed following centrifugation and the organisms washed and resuspended in fresh medium containing no NH<sub>4</sub>Cl. Observations for four weeks showed that the absence of NH<sub>4</sub>Cl from the medium did not lead to autolysis of either strain.

DISCUSSION

The number of strains of bacilli included in this study on the effect of available nitrogen on autolysis is too small to justify any general conclusions regarding separation of the acid fast

organisms on the basis of autolysis. Nevertheless, the H37Rv strain, the pathogenic Spears strain, two strains of BCG, one bovine strain and 12 strains of recently isolated tubercle bacilli are extremely susceptible to lysis. The failure of the "atypical" myobacteria from human lesions to undergo lysis in the nitrogen deficient medium is a feature which assists in setting them apart from the tubercle bacilli.

The lytic tendency in two strains of BCG and its absence in another strain indicates that the significance of the different autolytic patterns observed in various strains of mycobacteria cannot be understood until the mechanism causing disintegration of the bacilli is known.

In view of the results presented here it is apparent that an autolytic mechanism, whatever may be its nature, is active in many pathogenic tubercle bacilli following growth in a nitrogen deficient medium, and that under the same conditions it is inactive or only slightly active in most atypical and saprophytic strains.

The important problem, therefore, is not the correlation between autolysis and pathogenicity of the mycobacteria, but, the elucidation of the mechanism of autolysis and the effect of this mechanism on the total biology of the bacilli. Information regarding its association with other properties of the acid fast bacilli may lead to a better understanding of the phenomenon.

Since the initiation of our studies on autolysis experimental data have accumulated which indicate that the H37Rv strain may be lysogenic, (Redmond, 1955, and Bowman and Redmond, 1956). In support of this theory are unpublished results which show that a number of agents known to be inducing agents of lysogenic bacilli produce lysis of the H37Rv strain.

In discussing the cause of, and factors involved in, induction of phage in lysogenic bacilli, Jacob and Wollman (1953) have suggested that ". . . the unbalance of any metabolic process" may initiate phage development and bacterial lysis. It would seem that exhaustion of available nitrogen from the medium during the reproductive phase of the culture might be considered to result in such an unbalance.

Lysogenicity has been demonstrated by Freeman (1951) to be an important factor in production of toxin by diphtheria bacilli. Also, it has been indicated by Fisk (1942) that lysogenicity is closely correlated with pathogenicity of *Staphylococcus aureus*.

Lysis of tubercle bacilli has been observed by other workers who have offered various explanations. Spontaneous disintegration of tubercle bacilli grown on solid medium was observed by Steenken (1935, 1938) who suggested that the lytic agent was probably enzymic in nature. Marshak and Schaefer (1952) suggested that some of the enzymes of the bacilli were converted biologically to other enzymes capable of using the nitrogenous compounds of the protoplasm thus bringing about the disintegration of the bacilli. In this laboratory, attempts to demonstrate directly such enzymes in lysing cultures have not been successful. In a previous paper, Schaefer *et al.* (1949) suggested that the tween 80 was possibly the factor that brought about the final breakdown of the cell membrane which had been weakened by the autodigestive processes. Choremis *et al.*, (1955) demonstrated a lytic factor that caused disintegration of pathogenic tubercle bacilli. They referred to the factor as being "virus-like" but gave no further evidence of bacteriophage. Hnatko (1953) has reported the first bacteriophage isolated from a strain of mycobacteria. Strain 607 was used as the indicator strain. The same author reported finding three other lysogenic strains from which he was unable to isolate the phage. In these cases he suggested that the phage was adsorbed by the susceptible strain used for its demonstration and consequently was not capable of producing sufficient lysis to cause plaque formation.

#### SUMMARY

Fourteen pathogenic strains of human tubercle bacilli, one bovine strain and two strains of BCG, were observed to undergo marked autolysis following growth in a semisynthetic liquid medium containing 10 mg glucose and 15  $\mu$ g ammonium chloride per ml as the only available carbon and nitrogen sources, respectively. Peak growth is attained on about the tenth to twelfth day and is followed by rapid reduction in optical density.

No autolysis was detectable for a period of three months in medium containing 1000  $\mu$ g per ml  $\text{NH}_4\text{Cl}$ .

Two saprophytic strains, *Mycobacterium smegmatis* and strain 607, lysed approximately one third as much as the H37Rv strain. Other saprophytic strains and 17 "atypical" strains showed little or no tendency to lyse in the "lysis inducing medium."

The hypothesis is advanced that the autolysis observed in the mycobacteria is the result of a lysogenic state.

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