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Ribonucleic Acid, Deoxyribonucleic Acid, and Protein Content of Cells of Different Ages of *Mycobacterium tuberculosis* and the Relationship to Immunogenicity

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The amount of ribonucleic acid (RNA), protein, and deoxyribonucleic acid (DNA) was determined in pellicle cultures of different ages of the H37Ra strain of *Mycobacterium tuberculosis*, grown on a synthetic medium. We found that the highest content of RNA and protein was present in 2-week-old cultures, indicating that these cells were in the logarithmic phase of growth. DNA content was highest at 1 and 2 weeks. The amount of all three compounds then decreased about 50% during the following 6 weeks. Two-week-old cells should therefore be used for preparation of the immunogenic ribosomal fraction. The optimal concentration of zinc chloride increased RNA and protein synthesis, and also improved the appearance of the pellicle growth. Two-week-old cells, which contained the largest amount of RNA and protein, immunized mice significantly better than older cells. Since protein and DNA are not involved in the production of immunity, a correlation could be made between amount of RNA and the capacity of viable H37Ra cells to immunize mice. The immunizing capacity of these cells was not affected by ribonuclease, probably because the ribonuclease did not penetrate into the whole cells.

Viable cells of the attenuated strain H37Ra of *Mycobacterium tuberculosis* have been ruptured in this laboratory by use of French pressure cells, and a highly immunogenic particulate fraction has been obtained by differential centrifugation of the intracellular fluid (30). A ribosomal fraction was prepared from this particulate fraction by using sodium dodecyl sulfate (SDS) to remove the membranous components of the particulate fraction, and by recentrifuging at 144,000 × *g* for 3 hr to sediment the ribosomes. The ribosomal fraction, if incorporated into Freund's incomplete adjuvant, was equally, or more, immunogenic against tuberculous infection than the particulate fraction from which it was prepared (31). A crude preparation of ribonucleic acid (RNA) was prepared from the ribosomal fraction by use of a modification of the method described by Crestfield et al. (8), and this RNA, in Freund's incomplete adjuvant, proved to be as immunogenic as the ribosomal fraction (32). (Based on dry weight, this fraction was approximately as active as the viable whole cells from which it was prepared.) Crystalline trypsin had no effect on immunogenicity, but crystalline pancreatic ribonuclease reduced the immunogenic activity by at least 50% (32). These results suggested that RNA may be the labile immunogenic moiety present in viable tubercle bacilli, or it may be a part of a complex which produced the immune response.

It has been shown that the content of RNA in viable cells of other bacterial species varies with the age of the culture and is the highest during the logarithmic growth phase, as is the amount of protein. Deoxyribonucleic acid (DNA), on the other hand, appears to change very little during the different phases of growth.

From studies previously done in this laboratory, it was found that submerged H37Ra cells, grown in a modified synthetic Proskauer and Beck medium, have a generation time of approximately 24 hr (13). The ribosomal fractions were prepared from pellicle cultures of H37Ra grown on the same medium but, owing to a lack of precise measurements, neither the generation time nor the length of the growth cycle of these cells has been determined. The experimental evidence obtained over a 2-year period suggested that RNA may be responsible for the immunogenic activity of viable cells. It thus became important to determine whether the H37Ra cells, which were being used to prepare the ribosomal fractions, were in

the logarithmic growth phase. In addition, a correlation was made between the age of H37Ra cells and the capacity of these cells to immunize mice against tuberculosis.

The effect of ribonuclease on the immunogenicity of whole cells was also studied, and the influence of various concentrations of zinc on the amount of RNA, DNA, and protein formed was determined. Wegener and Romano (28) showed that small amounts of zinc added to cultures of *Rhizopus nigricans* increased growth, and that there was an immediate increase in RNA, followed by an increase in protein. DNA was less affected. Williston et al. (29) showed that trace amounts of zinc improve the pellicle growth of the BCG strain of *Mycobacterium bovis*.

MATERIALS AND METHODS

Microorganisms and cultural conditions. The attenuated strain H37Ra of *M. tuberculosis* was used both for chemical determinations and for immunization of mice. This strain was subcultured once a week, on the same day, by placing a piece of pellicle (10 to 15 mm in diameter) from a 2-week pellicle culture onto the surface of 300 ml of modified Proskauer and Beck (P & B) medium (33) in 1-liter Erlenmeyer flasks. The cells were incubated at 37 C on a medium consisting of magnesium citrate, 1.5 g; potassium monobasic phosphate, 5.0 g; asparagine, 5.0 g; potassium sulfate, 0.5 g; zinc chloride, 10 mg (1.5×10^{-4} M); glycerol, USP (Star Brand, Proctor and Gamble); and glass-distilled water to a volume of 1 liter. The *p*H was adjusted to 7.0 with 40% NaOH, and the medium was measured into flasks and sterilized by autoclaving (at 15 psi for 20 min).

The pellicles remained on the surface of the medium until approximately the sixth week; by the eighth week, growth was submerged, a characteristic of older mycobacterial cells. Since conditions for growth should change under these circumstances, the chemical determinations were made on cells only through the sixth week, and only on cells from pellicles which were still on the surface of the medium. A portion of each pellicle was collected with a large loop, placed in a test tube, washed with 0.01 M phosphate buffer (*p*H 7.0), and then ground in a mortar to make a fine suspension of cells (a saline-sodium citrate buffer, containing 0.15 M NaCl plus 0.015 Na citrate, was used to inhibit endogenous deoxyribonuclease). The suspension was standardized by measuring 1.0 ml into a calibrated Hopkin's tube and centrifuging this sample for 20 min at 2,500 rev/min. The amount of cells (mg/ml) was measured from the calibrated tip of the Hopkin's tube, each division of which equals 10 mm^3 or 10 mg of cells (moist weight). The suspension then was diluted to the desired concentration by use of the phosphate buffer. All chemical determinations on the cultures of different ages were done in duplicate on the same day.

In the experiments in which the effect of zinc on growth was studied and the amounts of nucleic acids and proteins were determined, measured amounts of zinc chloride was added to zinc-free P & B medium (the medium was made with bottled white glycerol USP [Fisher Scientific Co., Pittsburgh, Pa.] instead of tinned glycerol). Two-week cultures were used for the chemical determinations and, again for each experiment, all determinations were done on the same day.

The virulent H37Rv strain of *M. tuberculosis*, also maintained as pellicle growths on the synthetic P & B medium, was used to challenge the mice.

Chemical methods. RNA was measured by use of the orcinol reaction, as modified by Dische et al. (1). Orcinol (Eastman Kodak Co., Rochester, N.Y.) was purified by the method described by Schneider (22) and was recrystallized twice. Crystalline yeast RNA (Sigma Chemical Co., St. Louis, Mo.) was used as the standard in the initial experiments, but later mycobacterial RNA prepared in this laboratory was substituted. The reason for the change will be given in the Results.

DNA was measured by Dische's method (10), using calf thymus DNA (Mann Research Laboratories, New York, N.Y.) as the standard. Protein was measured by the method of Lowry et al. (14), and crystalline bovine albumin (Armour and Co., Chicago, Ill.) was the standard. The protein determinations were done after the cells were hydrolyzed by the following procedure: to 1 ml of a 10 mg/ml suspension of washed cells, an equal volume of 10% trichloroacetic acid was added and this mixture was heated at 90 C for 15 min. The tubes were centrifuged at 4,000 rev/min for 10 min to sediment the precipitated protein. The supernatant fraction was removed with a capillary pipette, and 10 ml of 0.01 M phosphate buffer (*p*H 7.0) was added to give a 1 mg/ml concentration (*p*H 7.0). The contents of the tubes were poured into separate small tubes and the precipitate was homogenized with a matching Teflon pestle. A portion of this solution was tested for the amount of protein present. All chemical determinations were done in duplicate.

Dry weights were done on three equal samples of cell suspensions and ribosomal fraction by drying at 100 C, until a constant weight was achieved.

Evaluation of the immune response. CF-1 male mice were vaccinated intraperitoneally with 10-fold dilutions of viable H37Ra cells and were challenged intravenously 4 weeks later with 1.0 mg of the virulent H37Rv strain of *M. tuberculosis*. The immune response was measured by recording the percentage of mice which survived for 30 days, as all or most of the controls were dead by that time. We have reported this method in detail elsewhere (34).

RESULTS

RNA determinations. In the initial experiments, crystalline yeast RNA was used as the standard in the orcinol reaction to determine the amount of RNA present in the ribosomal fraction and whole cells. With the orcinol method, the values of RNA in the ribosomal fractions were approximately 100% higher than the values found by measuring

the amount of ultraviolet (UV) absorption at 260 mμ by use of a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Moreover, the orcinol values plus the protein content exceeded the dry weight of the ribosomal fraction; when the values obtained at 260 mμ were used, the RNA plus protein approximately equaled the dry weight. It was apparent, therefore, that yeast RNA was not a suitable standard for measuring the amount of RNA in mycobacteria. A standard curve was obtained by use of mycobacterial RNA which was prepared in this laboratory, and this curve differed from the yeast RNA curve. When mycobacterial RNA was used as a standard, similar values for the amount of RNA present in the ribosomal fraction were obtained with both the orcinol method and by UV absorption. We therefore felt that the orcinol method would give fairly accurate values of the total amount of RNA present in whole mycobacterial cells.

The results of the RNA determinations on cells of different ages are given in Table 1 and Fig. 1. The standard deviation and standard error between determinations on different cultures of the same age and the percentage of the dry weight also are given. The amount of RNA varied according to the age of the cultures. Two-week-old cultures were used for subculturing, and the amount of RNA in the inoculum was high and remained at about the same level through the first 2 weeks. The standard deviation was very low for the 2-week-old cells, indicating consistent amounts of RNA in different cultures of this age. Since 2-week-old cells have been used routinely for vaccination during the past 3.5 years a great number of RNA determinations were made and the standard error was very low.

At 3 weeks, the amount of RNA decreased by approximately 20%. The large standard deviation indicated that different cultures of the same age varied considerably in their content of RNA and stage of growth. The stationary phase of growth appeared to start sometime during the third week.

By the fourth week, the amount of RNA had fallen to 30.8 μg/mg of cells, and the decrease from 2 to 4 weeks appeared to be linear. By the sixth week, the RNA had decreased to approximately one-half of the amount measured at the 2-week period. At this time, the pellicles were wet on the surface and portions began to sink into the medium, and it was assumed that the cells were in the declining or death phase of growth.

Protein determinations. Table 1 gives the amounts of protein present in cells of different ages. The protein dropped approximately 30% by the end of the first week, but increased by 60% at the second week and again dropped at the third week by approximately 30%. The protein then gradually decreased through the sixth week and was 50% less at 6 weeks than at the 2-week period. The protein curve from 3 to 6 weeks paralleled the RNA curve (Fig. 1). The standard deviation between determinations was greater than was ob-

TABLE 1. *Amount of nucleic acids and protein in cells of different ages of the H37Ra strain of Mycobacterium tuberculosis*[a]

	Inoculum (0)	Age of cultures (weeks)				
		1	2	3	4	6
RNA						
Mean of 7 determinations	53.3	52.0	54.0	43.7	30.8	26.7
Standard deviation		±4.7	±2.6	±8.47	±5.1	±6.8
Standard error		1.8	1.0	3.2	2.1	2.8
Percentage of dry weight		25.9	27.0	19.7	15.4	15.3
Protein						
Mean of 5 determinations	81.2	55.0	81.2	55.6	43.0	40.7
Standard deviation		±8.8	±13.4	±9.5	±10.4	±8.2
Standard error		3.9	6.0	4.2	5.2	4.7
Percentage of dry weight		27.4	40.6	25.0	21.5	23.4
DNA						
Mean of 5 determinations	1.31	1.34	1.31	1.30	0.95	0.66
Standard deviation		±0.38	±0.60	±0.26	±0.30	±0.15
Standard error		0.17	0.27	0.13	0.15	0.09
Percentage of dry weight		0.66	0.66	0.58	0.47	0.37
Dry weight		201.0	200.0	222.0	200.0	174.0
Total RNA, protein, DNA		108.3	136.5	100.6	74.8	68.1
Percentage of dry weight		53.7	68.3	45.3	37.4	39.1

[a] Amounts are expressed as micrograms per milligram (moist weight) of H37Ra cells.

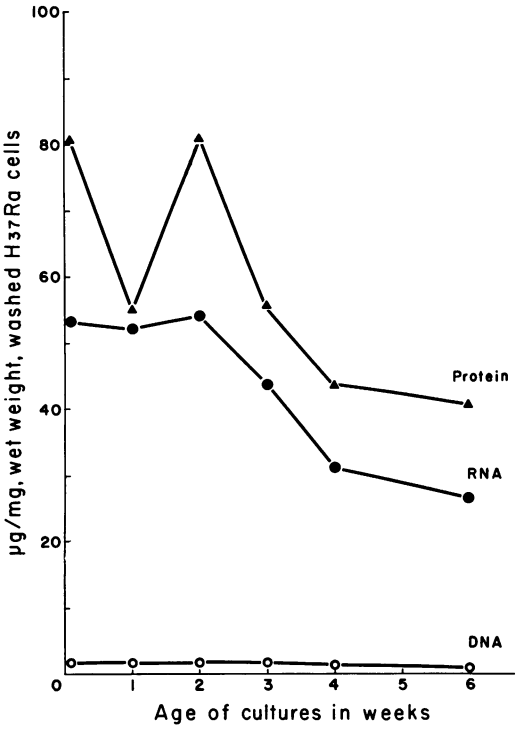

FIG. 1. *RNA, protein, and DNA in cells of different ages from pellicle growths of the H37Ra strain of Mycobacterium tuberculosis.*

tained with RNA, and, in marked contrast to RNA, it was the greatest at 2 weeks. In spite of this variation between experiments, the same type of protein curve was obtained in each experiment.

DNA determinations. Table 1 also illustrates the amounts of DNA present in these cultures. Although the amounts were approximately the same through the third week, the values started decreasing after the first week. By the fourth week, the amount of DNA decreased by approximately 27% and by the sixth week it was approximately 50% less than the DNA content at the first week. The variation was greatest with the 2-week-old cells, as was noted with protein, since the DNA values varied between 0.62 and 2.1 μg/mg of cells (wet weight).

Relationship of amount of RNA, protein, and DNA to the dry weights of the cells of different ages. As is shown in Table 1, the ratio of wet weight to dry weight was different with cultures of different ages. Thus, it was valuable to consider the relationship of RNA, DNA, and protein to dry weight of cells. The dry weights were similar for cells from cultures at 1, 2, and 4 weeks but were 10% higher with 3-week-old cells, a significant difference. By the sixth week, the dry weight decreased to 174 μg/mg of cells. Total nucleic acids plus protein equaled 54% of the dry weight of 1-week-old cells, and this amount increased to 68% in 2-week-old cells. However, by the third week, nucleic acids plus protein accounted for only 45% of the dry weight, and this percentage decreased still further, to approximately 40% of the dry weight in cells, in the fourth and sixth week cultures.

RNA amounted to 26% of the dry weight at the first week and 27% at the second week. By the third week, the percentage of RNA had decreased to 19.7% of the dry weight (a decrease of 26%), and, by the fourth and sixth weeks, only 15% of the dry weight was RNA, approximately 50% of the RNA present at the second week. DNA followed a similar pattern, being highest at the first and second weeks and then falling approximately 50% by the sixth week. Protein, with the exception of the first week, followed a similar pattern.

As would be expected from these results, the yield of the particulate fraction, obtained from mechanically ruptured cells, was the greatest with 2-week-old cells (60.4 mg/g of cells). Three-week-old cells produced an average of 41.4 mg/g of cells, whereas 4-week-old cells produced an average of 38.5 mg/g of cells. After the particulate fraction was treated with SDS and the ribosomes were collected, the amount of ribosomes generally was about 25% of the particulate fraction. Data were available only on ribosomes from 2-week-old cells, and the average amount from the results of many experiments was 18.6 mg/g of cells (moist weight).

Effect of different concentrations of zinc ions on the amount of RNA, protein, and DNA. The results from the chemical determinations done on 2-week-old cells which had been grown on P & B synthetic media, containing various concentrations of zinc ions, are given in Table 2. The data are the average of determinations from three separate experiments. The table gives both the molar concentration of the zinc ions present and the amount of zinc chloride added. Dry weights, total RNA, protein, and DNA also are given as the percentage of the dry weight.

We found that, as the amount of zinc chloride in the medium increased to 10 mg/1,000 ml, the amount of RNA and protein increased. There was also a slight, but probably insignificant, increase in DNA. At higher concentrations of zinc, the amounts of RNA, DNA, and protein decreased to values similar to those obtained with none or very few zinc ions. These values were reflected in the total amounts of RNA, protein, and DNA as a percentage of the dry weights. The

TABLE 2. *Effect of different concentrations of zinc ions on the RNA, protein, and DNA in 2-week-old cells of Mycobacterium tuberculosis*

Concn of zinc (M)	$ZnCl_2$ (mg/1,000 ml)	RNA (μg/mg of cells)[a]	Protein (μg/mg of cells)[a]	DNA (μg/mg of cells)[a]	Dry wt of cells (μg/ml)	Total RNA, protein, DNA content (μg/mg)	Percentage of dry wt
0	0	46	56	0.95	192	103	54
1.5×10^{-5}	1	46	56	0.81	200	103	52
7.7×10^{-5}	5	51	63	0.82	194	115	59
1.5×10^{-4}	10	53	69	1.0	184	123	67
3.1×10^{-4}	20	49	61	0.9	182	111	61
6.2×10^{-4}	40	49	54	0.9	198	104	53

[a] Moist weight.

greatest percentage (67%) was present in cells grown on medium containing 10 mg/1,000 ml of zinc chloride. Therefore, as was shown by Wegener and Romano (28), zinc ions are important in the synthesis of RNA and protein in bacteria.

The chemical findings were correlated with the appearance of pellicles grown on these various media. At 2 weeks, all the pellicles were dry and floating on the surface of the media. However, the media which contained the two highest concentrations of zinc had pellicles which consisted not only of horizontal growth on the surface of the medium but also of vertical growth from the pellicle into the medium. At 3 weeks, a more marked change in the pellicles occurred. The pellicles were wet and were submerged in the media containing zinc chloride in concentrations of 0, 1, 20, and 40 mg/1,000 ml. However, the pellicles were dry and floating on the surface of the media which contained 5 and 10 mg of zinc chloride. As Williston et al. (29) pointed out, zinc ions markedly influence the type of growth obtained with mycobacteria, in that small amounts improve pellicle cultures. Whether this is a reflection of the content of RNA and protein is not known, but the best pellicles occurred with the highest amount of RNA and protein.

Relationship between age of cultures and the immunogenic response produced in mice. The pooled data from many experiments are given in Table 3 (the immunogenic response was measured in mice vaccinated with viable H37Ra cells of 3 different ages). The large numbers of mice vaccinated with 1.0 mg of H37Ra cells, as well as the large numbers of nonvaccinated mice, resulted from the use of these mice as controls in each of the experiments in which ribosomal fractions obtained from the H37Ra cells were tested for immunogenicity.

Cells from the 2-week cultures immunized mice significantly better than cells from either the 3-week or 4-week cultures, using 1.0, 0.1, and 0.01 mg vaccinating doses. Owing to the large number of mice used, statistically the 2-week 0.001-mg vaccinating dose was also significantly better than the 3-week 0.001-mg dose, but it was not better than the 0.001-mg dose from 4-week cells.

The mycobacterial cells from the 3- and 4-week cultures produced an immune response which was similar in mice vaccinated with the 1.0- and 0.1-mg doses. However, the 0.01-mg dose of the 3-week-old cells produced a significantly better immune response than was obtained with the 4-week-old cells. The small immunogenic response obtained with both the 0.01- and 0.001-mg doses of 4-week cells was real and probably was revealed by the large numbers of mice used.

Therefore, cells of all ages were slightly immunogenic in mice vaccinated with the 0.001-mg dose. This low degree of immunogenic activity may be a reflection of the time of challenge. Although these cells have been shown not to multiply in CF-1 mice (23), the 0.001-mg dose of 3-week-old cells will immunize 50 to 60% of the mice, if the mice are challenged at 10 or 12 weeks after vaccination (Youmans and Youmans, *unpublished data*). It is possible that the 0.01-mg dose of the 4-week-old cells, which contained less RNA, might produce a higher immune response in the animals if a later challenge period was used.

Another interesting comparison was made by use of the 0.01-mg vaccination dose, since the larger doses immunized to a similar degree, by plotting the percentage of mice which survived for 30 days against the amount of RNA present in those cells. A straight line was obtained. A straight line was not obtained if the percentage of mice surviving was plotted against either protein or DNA.

We also found, when evaluating the data from a great number of experiments, that the mice vaccinated with 1.0 mg of the 2-week-old H37Ra cells responded immunogenically in a much more uniform manner between experiments (range of variation, 50 to 100% survivors) than mice vacci-

TABLE 3. *Immunogenicity of H37Ra cells of different ages*

Size of vaccinating dose (mg)[a]	Age of H37Ra cultures								
	54 μg of RNA/mg,[a] 2 weeks			43.7 μg of RNA/mg, 3 weeks			30.8 μg of RNA/mg, 4 weeks		
	No. of mice	No. of S-30 mice[b]	Percentage of S-30 mice	No. of mice	No. of S-30 mice	Percentage of S-30 mice	No. of mice	No. of S-30 mice	Percentage of S-30 mice
1.0	1,973	1,699	86	1,761	1,139	65	546	331	61
0.1	256	222	87	170	116	68	115	87	76
0.01	265	210	79	175	111	63	157	43	27
0.001	178	51	28	276	52	19	116	24	21
Controls	2,016	241	12	1,824	109	6	601	32	5

[a] Moist weight.
[b] S-30 = number of mice which survived >30 days.

nated with the same dose, using either the 3- or 4-week old cells (range of variation, 11 to 95% survivors). The variation in the immune response found with the older cells was marked, and the reason for this variation has not been known. The variation could not be related to the appearance of the cells at the time of harvest. Now it appears that the variation may be related to the RNA content of the cells. As was noted in Table 1, the variation in RNA content between different 2-week-old cultures was very small, and this consistent amount of RNA may be responsible for the much more uniform immune response obtained. In cells, however, from 3- and 4-week cultures, the variation in RNA content was much greater, and this fluctuation may be responsible for the variable immune response obtained with these cells.

Determination of the effect of crystalline pancreatic ribonuclease on the immunogenic activity of 2-week-old viable H37Ra cells. The cells were suspended in distilled water, and 2 mg of ribonuclease per mg of H37Ra cells was added to one portion of this solution. Another portion was untreated and both were incubated at 37 C for 18 hr. The ribonuclease was not removed from the suspension before the cells were injected into mice, but parallel tests showed that this amount of ribonuclease alone did not produce an immune response. The pooled results of two to three experiments are given in Table 4. Ribonuclease, under these conditions, appeared to have no effect on the immunogenic response obtained in the mice vaccinated with doses of the H37Ra treated with ribonuclease compared with the mice vaccinated with the same doses of the control H37Ra cells. Only at the lowest dose, 0.01 mg, was there a suggestion that ribonuclease may have affected the immunogenicity of whole cells, and this difference was not significant ($P = 0.1$). The amount of RNA was not decreased in the cells treated with ribonuclease.

TABLE 4. *Effect of pancreatic ribonuclease on the immunogenic activity of 2-week-old viable H37Ra cells*

Immunizing prepn	Amt injected (mg)[a]	No. of mice	No. of S-30 mice[b]	Percentage of S-30 mice
H37Ra cells	1.0	55	45	82
	0.1	88	72	82
	0.01	58	50	86
H37Ra cells treated with ribonuclease	1.0	58	51	88
	0.1	88	66	75
	0.01	59	44	75
Controls		90	5	6

[a] Moist weight.
[b] S-30 = number of mice which survived >30 days.

DISCUSSION

One of the interesting findings of this study was that crystalline yeast RNA cannot always be used as a standard when the orcinal method is used to determine the amount of RNA in other species of microorganisms. This test revealed that mycobacterial RNA is different from yeast RNA.

When mycobacterial RNA was used as a standard, however, the RNA results obtained with the attenuated H37Ra strain of *M. tuberculosis* were similar to the results found with several other microorganisms (3, 4, 6, 7, 12, 15, 16, 18, 20, 22, 24, 26, 27). The RNA content was highest with cells from 2-week-old pellicle cultures and decreased about 50% through the next 4 weeks. Judging from the results of other investigators, who used microorganisms where growth was followed either turbidimetrically or by plate counts, and who made correlations with the content of RNA and protein, the greatest amount of RNA and protein was found in cells in the logarithmic phase of growth. Therefore, our results seemed to

indicate that the 2-week-old pellicle cultures of the H37Ra strain were in the logarithmic phase of growth, whereas the stationary phase began in the third week. Thus, if H37Ra cells are to be used for vaccination and for the preparation of the ribosomal fractions, then they should be no older than 2 weeks, when grown as pellicles on the synthetic P & B medium. Rosenthal (19) found that cells from pellicle growths of the BCG strain of *M. bovis*, which were harvested at 10 days, were more immunogenic than older cells.

Protein in the H37Ra strain followed the pattern of that found by Webb (27), when he meassured the protein content of cells of different ages of *Clostridium perfringens* (*welchii*). There was a decrease in protein in the initial phase of growth and then a marked increase of protein during the logarithmic phase. Using mycobacteria, the highest protein concentration was found during the 2-week period. Since protein was found to increase during the logarithmic phase of many other species of microorganisms (11, 16, 20), this supports the inference made from the RNA results that the cells were in the logarithmic phase at 2 weeks. Protein then gradually decreased about 50% through the fourth week, and this decrease paralleled the decrease of RNA during the same time period.

The amount of DNA in the H37Ra cells was found to be lower than the amount found in most microorganisms (5, 12) and in *M. bovis* (25). Stuy (24), however, found only 1% of the dry weight of *Bacillus cereus* to be DNA. In our experiments, the amount of DNA varied considerably between experiments, and in one case it was as high as 1% of the dry weight. The amount of DNA appeared to be highest just before or during the logarithmic phase of growth, again at 1 and 2 weeks, and then decreased by about 50% through the following 4 weeks. This has been shown to occur in other bacteria (2, 16, 18, 24, 27). However, some investigators have found DNA to remain fairly constant throughout the growth cycle (6, 7, 9, 12, 20).

These results show, therefore, that the changes in the amounts of RNA, protein, and DNA, in cells of the attenuated strain H37Ra of *M. tuberculosis*, follow a pattern similar to that observed in other bacteria. Even though this strain of mycobacterium has a very slow growth rate, the amount of RNA is approximately the same during the logarithmic phase (about 25% of the dry weight) as is found with bacteria having a faster rate of growth. The amount of protein appears about the same, but the amount of DNA appears to be lower than is found with most microorganisms. Whether this is related to the lower rate of growth is not known, but amber mutants of DNA may form, and thus decrease the amount of DNA and restrict the rate of growth.

The results presented in this paper confirm Wegener and Romano's (28) finding that the correct concentration of zinc ions increased the content of RNA and protein in bacteria and may affect the DNA content slightly. These results also confirm the finding of Williston et al. (29) that similar concentrations of zinc ions markedly improve the pellicle growth of the BCG strain of *M. bovis*. There appears to be a relationship between the two findings, since the same concentration of zinc ions that gave the highest RNA, DNA, and protein content also gave the best pellicle growth.

This study has shown a correlation between the amount of RNA in viable mycobacterial cells and the immunogenicity of these cells. In addition, the possibility that the quality of the RNA may be different in cells of different ages should be considered. Mitchell and Moyle (17), who followed the normal growth cycle of *Micrococcus pyogenes* var. *aureus*, found that the percentage of nucleic acid fragments varied inversely with the rate of synthesis of RNA. Therefore, nucleic acid fragments appeared when the stationary phase of growth began. A similar mechanism may operate in mycobacteria. RNA does break down and is used as an energy source by microorganisms during the stationary and resting phases of growth (9).

The finding that ribonuclease had no effect on the immunogenicity of viable whole H37Ra cells was not surprising since Schlenk and Dainko (21) found that ribonuclease could not penetrate the pores of the yeast cell walls but was effective on yeast spheroplasts.

The results presented in the paper suggest a relationship between RNA and immunogenicity of viable mycobacterial cells.

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