the number born alive is reported in Table I. Parturition was uncomplicated in all cases and 90% of the young were born alive. A diet which contains 5% hydrogenated fat has been shown to be too deficient in essential fatty acids to allow normal lactation(11). However, in the present study the diet was supplemented with enough corn oil to successfully overcome any essential fatty acid deficiency.

A recent report by Alfin-Slater *et al.*(13) that *trans* fatty acids are harmless was based on the results of feeding *trans* fatty acids to rats for 46 generations. The present results indicate that only traces of *trans* acids would be passed on to the fetus and each generation of rats would therefore start to accumulate them only after birth.

Johnston *et al.*(14) found human tissue to contain 2.0-14% of *trans* fatty acids. It would be of interest to know whether these *trans* fatty acids originated exclusively from dietary fat or were transferred through the placental wall, from a mother to her baby. Further studies in this regard are in progress.

Summary. Less than 0.5% of *trans* fatty acids were found in fat extracted from young born to mother rats which contained between 23.5 and 26.8% *trans* fatty acids in their carcass fats. The amount of *trans* fatty acids in the carcass fats of the young was markedly increased when they were allowed to suckle the maternal milk for 9 days.

1. Bertram, S. H., *Biochem. Z.* 1928, v197, 433.
2. Swern, D., Knight, H. B., and Eddy, R. C., *J. Am. Oil Chemists' Soc.*, 1952, v29, 44.
3. Hartman, L., Shorland, F. B., and McDonald, I. R. C., *Nature*, 1954, v174, 185.
4. Reiser, R., and Reddy, H. G. R., *J. Am. Oil Chemists' Soc.*, 1956, v33, 155.
5. Sinclair, R. G., *J. Biol. Chem.*, 1936, v115, 211.
6. Barbour, A. D., *ibid.*, 1933, v101, 63.
7. Kohl, M. F., *ibid.*, 1938, v126, 709.
8. Wesson, L. G., *Science*, 1932, v75, 339.
9. Johnston, Patricia V., Johnson, O. C., and Kummerow, F. A., 1957, to be published.
10. Jackson, F. L., and Callen, J. E., *J. Am. Oil Chem. Soc.*, 1951, v28, 61.
11. Kummerow, F. A., Pan, H. P., and Hickman, H., *J. Nutrition*, 1952, v46, 489.
12. McConnell, K., and Sinclair, R. G., *J. Biol. Chem.*, 1937, v118, 123.
13. Alfin-Slater, R. B., Wells, A. F., Aftergood, L., and Deuel, H. J., Jr., *J. Nutrition*, 1957, v63, 241.
14. Johnston, Patricia V., Johnson, O. C., and Kummerow, F. A., *Science*, 1957, v126, 698.

Received August 6, 1957. P.S.E.B.M., 1957, v96.

Immunogenicity of Particles Isolated from *Mycobacterium tuberculosis*. (23602)

ANNE S. YOUMANS, GUY P. YOUMANS AND IRVING MILLMAN
Department of Microbiology, Northwestern University Medical School, Chicago, Ill.

Youmans, Millman, and Youmans(1) have reported that mice immunized with very small enzymatically active particles (20-200 mμ) isolated by ultracentrifugation from extracts of *Mycobacterium tuberculosis* var. *hominis*, strain H37Ra, were as resistant to tuberculous infection as were mice immunized with whole living cells of the same strain. Since this first report, work has continued on the chemical(2,3), physical, and biological characteristics of these particles, and attempts have been made to isolate similar immunogenic particles from the BCG-4 strain of *Mycobacterium tuberculosis* var. *bovis*.

It has been found, using an extraction procedure similar to the one previously outlined (1), that consistently immunogenically active preparations can be obtained from the H37Ra strain. Greater difficulty has been encountered, however, in obtaining consistently immunogenically active preparations from the BCG strain.

The present paper will be concerned with

* This investigation was supported by grants from Illinois Tuberculosis Assn. and the Tuberculosis Inst. of Chicago.

some of the factors involved in the preparation of immunogenically active particles from both the H37Ra and BCG strains of *Mycobacterium tuberculosis,* with the chemical nature of the particles and with the effect of various treatments on the immunogenicity of the particles.

Methods. Particles to be used for immunization were extracted from 2 strains of mycobacteria: the attenuated human type strain, H37Ra, and the attenuated BCG strain, number 4.† The H37Ra cells were grown as surface pellicles on 300 ml of modified Proskauer and Beck medium(4) contained in one liter flasks, and were harvested at the time of mature growth (21-35 days) by pouring the contents of the culture flasks into coarse sintered glass filters, and washing the collected cells with 0.01 M phosphate buffer (pH 7.2). Growths from approximately 60 culture flasks were needed to give sufficient cells for the 6 grinding bottles usually employed for each preparation. The BCG-4 cells were grown on Sauton's medium in a manner similar to that used in the preparation of BCG vaccine(5). The age of these cells was approximately 14 days. Both freshly harvested and frozen BCG cells were employed to determine whether active particles could be obtained also from frozen cells which then would permit "stockpiling" of cells.

It was found as the studies progressed that more active immunogenic particles were obtained when smaller relative amounts of powdered glass were used for the grinding of the cells. Therefore, into each 250 ml heavy pyrex glass centrifuge bottle was placed 25 g of washed powdered glass, 200 g of stainless steel balls of different sizes, 40 g of the washed harvested cells, and 50 ml of sucrose buffer (0.25 M dissolved in 0.01 M phosphate buffer, pH 7.0). The rubber stoppered bottles were rotated for approximately 18 hours at 4°C on a ball mill. The extracts so prepared were fractionated by procedure described previously in detail(1). The high speed (40.000 rpm) sediment which appeared was composed of particles which were used to immunize the mice. This sediment after being carefully scraped from the edge of the tubes with a glass rod and homogenized gently by hand with a Teflon homogenizer was diluted with 0.25 M sucrose buffer to a volume of 5 ml for each grinding flask used, since this concentration protected approximately 50% of the mice. Throughout the preparations, it was necessary to keep all materials cold (4°C), and to use chemically cleaned glassware since trace amounts of detergent appeared to inactivate the particles. The sediment, when prepared from H37Ra cells, appeared as a red pellet of about 1 cm in diameter in each centrifuge tube and consisted of 2 layers: a loose top layer and a darker gelatinous bottom layer which adhered tightly to the tube. Because of this red color it has been termed the "red fraction." In contrast, however, the sediment prepared from BCG cells, while also consisting of the 2 layers, appeared grey in color. The immunogenicity of the individual layers was measured, and all except the bottom gelatinous layer prepared from BCG cells were found to be active. Groups of 20 mice, "Strong A" strain, ranging in weight between 18 and 22 g, were vaccinated by injecting intraperitoneally 0.2 ml of each of the homogenized suspensions of particles within 2 to 3 hours after their preparation. Four weeks later the mice were challenged intravenously with 1 mg, wet weight, of a standardized ground suspension of the highly virulent human strain, H37Rv(6). The time of death, in days, of each mouse was recorded until the 30th day, and an autopsy done to confirm that death was due to pulmonary tuberculosis. At 30 days all living mice were sacrificed. Two methods were used to evaluate the immunogenic activity of the fractions, and the choice of method was determined by the rapidity with which the mice died. When the mice died before 30 days a normal distribution curve of the deaths is obtained and Litchfield's method of determining the median survival time (St_{50}) can be used(7). Ninety-five % confidence limits then can be calculated and the results compared statistically. However when the immunized mice tended to

† BCG-4 cells were kindly supplied by Dr. Sol R. Rosenthal, Tice Clinic, University of Illinois, Chicago.

TABLE I. Immunogenic Effect of Whole Cells and of "Red Fraction" from Extracts of H37Ra Cells against Tuberculous Infection.

	Red fraction				Whole cells	
Age of cells in days	mg N/ml†	No. organisms per 0.2 ml (× 1000)	No. of mice	% No. of mice S-30*	No. of mice	% No. of mice S-30*
35	1.84	4.4	20	45.0	20	50.0
27	1.31	3.2	18	88.0	20	75.0
25	1.32	3.0	20	55.0	19	68.4
20	2.82	2.0	18	33.3	20	75.0
30	1.56	4.4	20	50.0	20	70.0
32	2.23	2.0	19	26.3	18	38.8
32	1.43	.4	18	44.4		
32	2.66	1.4	20	55.0	18	11.7
22	2.11	2.6	9	55.5	19	73.6
28	1.58	1.4	19	57.8	20	85.0
27	1.31	4.2	18	50.0	19	63.1
23	1.89	6.0	19	52.6	17	82.3
26	1.74	6.8	17	47.0		
			16	56.2		
26	2.01	4.8	18	61.1	16	43.7
		Total	269		226	
		Mean		51.8		61.4

* No. of mice which survived 30 days.

† Nitrogen determinations made on unwashed particles so traces of supernatant may be present.

die over a prolonged period of time and a normal distribution of deaths was not obtained, neither the median survival time nor the mean survival time could be used as a measure of the response to infection. For this reason, the number of mice which survived 30 days was used as a measure of immunity to tuberculous infection since 95% or more of the control infected mice are dead by this day. By employing the chi square test, mice vaccinated with various fractions can be compared statistically with the controls, or with each other. The methods used for the vaccination of mice and for the evaluation of the results have been described in detail in separate publications(6,8). Nitrogen content was determined on each sample of the standard dilutions of the "red fraction" by the micro-Kjeldahl technic(9) and the mg of nitrogen per ml of each "red fraction" was calculated. Bacterial counts were made also on each "red fraction" by spreading 0.05 ml of the standard dilution over an area of 5 x 1 cm on a microscope slide. The number of acid fast bacteria in 1,000 fields was counted and from this figure the number of whole cells remaining in the preparation could be calculated.

Results. Table I lists the results of 14 consecutive experiments in which "Strong A" mice were immunized with the "red fractions" obtained from freshly harvested H37Ra cells, and the living H37Ra cells from which the particles were isolated. It is apparent that both the "red fraction" and the whole cells were highly immunogenic in that they protected mice against a large challenging dose of virulent tubercle bacilli, and, moreover, the results were remarkably consistent, especially in mice vaccinated with the "red fraction."

Also included in the Table is the age of the cells employed in each experiment, the nitrogen values of each of the "red fractions," and the number of whole cells in the 0.2 ml of the "red fraction" injected into each mouse. There was no direct correlation between the immunogenic activity of the particles with either the nitrogen value, or the age of the cells from which they were prepared. The number of microorganisms present in each sample was far too small to immunize alone, as it had been found previously(1) that between 1,610,000 and 17,381,000 organisms of the H37Ra strain were needed to produce a detectable immune response in this strain of mice.

The effect of dilution, heat, lyophilization,

TABLE II. Effect of Physical Factors on the Immunogenic Activity of the "Red Fractions" Obtained from Extracts of H37Ra Cells.

Treatment	No. of mice	No. of replicate exp.	D-30*	S-30‡	% S-30	Median survival times (St_{50}) (95% confidence limits)	
Red fraction	269	14	130	139	51.8		
1- 5 dilution	18	1	18	0	.0	19.5 (18.4-20.6)	S†
1- 10 "	146	8	124	22	15.0	16.0 (15.5-16.5)	S
1- 20 "	20	1	18	2	10.0	16.0 (15.2-16.9)	S
1-100 "	53	3	50	3	5.6	15.0 (14.3-15.8)	
Red fraction—heat (126°C for 15 min.)	70	4	66	4	5.7	16.5 (16.0-17.0)	S
1- 5 dilution	20	1	19	1	5.0	18.5 (17.5-19.6)	S
1- 10 "	73	4	71	2	2.7	16.0 (15.6-16.5)	S
1- 20 "	20	1	20	0	.0	16.0 (15.4-16.7)	S
1-100 "	37	2	37	0	.0	13.5 (12.9-14.2)	
Red fraction—lyophilized							
0.25 M sucrose buffer	66	4	43	23	34.8		
1- 5 dilution	19	1	16	3	15.8	17.0 (14.9-19.4)	S
1-10 "	57	3	54	3	5.2	16.5 (16.0-17.0)	S
1-20 "	19	1	18	1	5.3	16.5 (15.9-17.2)	S
0.88 M sucrose buffer	59	3	21	38	64.4		
Red fraction—sonic vibration, 2 hr, 9000 cycles	37	2	23	14	39.1		
Red fraction—Berkefeld filtered	40	2	37	3	9.2	15.5 (14.6-16.5)	
Control, non-immunized mice	251	14	243	8	3.1	14.5 (14.1-14.9)	

* No. of mice which died before 30 days. † S = A statistically significant difference between this median survival and that of the controls. ‡ No. of mice which survived 30 days.

sonic vibration and Berkefeld filtration on the immunogenic activity of the particles prepared from H37Ra cells is given in Table II. Dilution of the "red fraction" with 0.25 M sucrose buffer markedly reduced its immunogenic activity, although a 1-20 dilution produced in the mice a median survival time which was significantly longer than was found with the controls. The effect of other diluents is being investigated.

Since autoclaving H37Ra whole cells at 126°C for 15 minutes reduced their immunogenic activity about 50% (8,10) the particles were autoclaved in a similar manner. The immunogenic activity of the particles also was reduced markedly. The dilutions through 1-20 gave a slightly longer survival time in the mice than was found with the control mice, although the number of mice which survived 30 days was similar to the number of control mice which survived for the same period.

In each of 4 experiments when the particles were lyophilized in 0.25 M sucrose buffer significant immunogenic activity remained. However, lyophilization caused a decrease in activity from 51.8 to 34.8%, and this difference was found to be statistically significant at the 95% level when analyzed by the chi square test. Dilutions of the lyophilized material through 1-20 produced a slightly longer median survival time than was found with the controls. When the particles were lyophilized in 0.88 M sucrose buffer there was no decrease in activity.

Sonic vibration at 9,000 cycles for 2 hours decreased the immunogenic activity (S-30) of the particles from 51.8 to 39.1%. This was found also to be statistically insignificant. Filtration of the suspension of particles through a (German) Berkefeld filter, normal porosity, eliminated the immunogenic activity, possibly by retention of the particles. In addition, a number of experiments were conducted in which the "red fraction" was kept at 4°C for 24 and 48 hours, and one week before being used to vaccinate the mice. There was no loss of immunogenic activity following this treatment, nor following washing of

TABLE III. The Immunogenic Activity of Whole BCG Cells, Both Frozen and Freshly Harvested, and Particles Prepared from These Cells.

Vaccine	No. of mice	No. of exp.	D-30*	S-30†	% No. of mice, S-30
Particles prepared from fresh BCG cells	183	10	150	33	18.0
Original whole BCG cells	180	10	59	121	67.2
Particles prepared from frozen BCG cells	296	16	215	81	27.3
Original whole frozen BCG cells	280	15	96	184	65.7
None (controls)	380	20	363	17	4.4

* No. of mice which died before 30 days. † No. of mice which survived 30 days.

the particles 3 times by centrifugation with 0.25 M sucrose buffer at 40,000 rpm for 3 hours.

In contrast to the rather consistent results obtained with the "red fraction" prepared from H37Ra cells, particles prepared from freshly harvested BCG cells and frozen BCG cells varied widely in immunogenic activity. A summary of the results is given in Table III. These combined data indicate a very low degree of activity for the particles isolated from BCG cells although actually in individual experiments the percent of the mice which survived 30 days ranged from 0.0 to 42.1% when the particles were prepared from freshly harvested cells, and from 0.0 to 57.8% when the particles were prepared from the frozen cells.

In 2 experiments, particles prepared from frozen BCG cells and which permitted 49.5% of the mice to survive 30 days, were sonically vibrated at 9,000 cycles for 2 hours. These treated particles permitted only 20.0% of the mice to survive 30 days. This change was found to be statistically significant at the 99% level when analyzed by the chi square test. In addition, the immunogenic activity of the particles washed three times with 0.25 M sucrose buffer was reduced significantly. Thus, the immunogenic activity of the particles prepared from BCG cells appears to be affected more easily by these procedures than the activity of these particles prepared from H37Ra cells. It was noted also that the immunogenic activity of the particles prepared from BCG cells was reduced significantly when the particles were suspended in distilled water instead of sucrose buffer.

Nitrogen determinations and bacterial counts were made on each preparation and were similar to the values given for the "red fraction" prepared from H37Ra cells. With fractions from both H37Ra and BCG there was no direct relationship between nitrogen value and activity.

To determine the chemical composition of the immunizing "red fraction," the entire yield of particles from 14.0 g (dry weight) of *M. tuberculosis* var. *hominis*, strain H37Ra, was washed 5 times by centrifugation at 40,000 rpm for 3 hours in 0.25 M sucrose. The fraction was washed and resuspended in 0.25 M sucrose because it had been found that washing with distilled water liberated nitrogenous material and therefore reduced the yield. The washed fraction was lyophilized and weighed. The total yield, corrected for estimated sucrose, was 223.0 mg, dry weight. The fraction then was analysed according to the procedure of Schneider(11), and Table IV shows the results of this analysis. More than 70% of the fraction was composed of lipid. No desoxyribonucleic acid (DNA) was detected. Actually, over 90% of the DNA known to be present in whole mycobacterial cells could be found in the supernatant frac-

TABLE IV. Chemical Analysis of Immunogenic "Red Fraction" from *M. tuberculosis* var. *hominis* Strain H37Ra.

Chemical fractions	Wt (mg)	% of total
Total acid soluble P	.22	.1
" " " N	1.08	.48
Phospholipid A (alcohol soluble)	133.8	60.0
Phospholipid B (alcohol-ether 3-1 soluble)	28.0	12.6
Desoxyribonucleic acid	.0	.0
Pentosenucleic "	11.6	5.2
Protein	29.5	13.2
Total	203.98	91.58

tion, indicating almost complete solubilization of the nuclei. A quantitative determination of polysaccharides could not be made because of the presence of sucrose in the suspending medium. However, paper partition chromatography of the "red fraction" showed the presence of several carbohydrate components the nature of which is being studied at the present time.

Preliminary results of a similar chemical study of the immunizing fraction from BCG cells have produced essentially the same results.

Discussion. The frequent low degree of immunizing activity of the particles isolated from BCG cells when compared to those isolated from H37Ra strain is difficult to explain. Particularly since whole cells of BCG appear to be equal, if not better, immunizing agents than the H37Ra strain(8). The BCG fractions were prepared in the same manner as were fractions of H37Ra. However, there is a suggestion that the particles prepared from BCG cells may be more labile than the particles prepared from H37Ra cells.

The susceptibility of the immunogenic activity of the particles to various physical treatments was not unexpected in view of previous results which indicated that stabilizing agents were necessary to maintain maximum enzymatic activity of these same particles(2,3). This provides further evidence that these particles are "mitochondrial-like" since it has been shown that suspending fluids of high tonicity preserve the integrity of mammalian mitochondria. However, Marr and Cota-Robles(12) and Robrish and Marr(13) have shown that cell wall preparations from certain other microorganisms also might contain enzymes (hydrogenase and cytochrome). Unfortunately, the results of the chemical analysis of the isolated washed particles give little information concerning the relation of structure to immunogenic activity, but the relatively high lipid content of the immunogenic fraction might indicate that these particles may have consisted of cell wall fragments. However, Mudd *et al.*(14) have demonstrated by electron microscopy the presence in tubercle bacilli of intracellular "rosettes" which in size and susceptibility to electron bombardment resemble the immunogenic particles isolated in this laboratory. These findings appear to be evidence against the possibility that the particles found in the immunizing "red fractions" consist of cell wall fragments. Actually the enzymatic activity of the cell wall preparations mentioned above(12, 13) possibly may have been due to contamination by adsorption with components of disrupted intracellular particles. Since no DNA was found, it would appear that the immunizing fraction did not contain nuclei.

The marked difference in color noted between the immunogenic fractions obtained from the BCG strain and the H37Ra strain might be related to a difference in the amount of cytochrome. Since the chemical analyses performed showed no difference between the strains H37Ra and BCG, work is now in progress to determine whether immunogenic particles obtained from different strains of tubercle bacilli may differ in their content of cytochromes, and of purine and pyrimidine bases.

Summary. 1. By following a carefully standardized procedure highly immunogenic particles uniformly were obtained by ultracentrifugation from ground extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. These particles, which ranged in size from 20-200 mμ, were just as effective as the living whole cells of this strain in protecting mice against a severe tuberculous infection. 2. The sediment comprised of the particles prepared from H37Ra cells was red in color and therefore has been termed the "red fraction." The immunogenic activity of this fraction was almost eliminated when the particles were diluted 5 times or more with 0.25 M sucrose buffer, autoclaved at 126°C for 15 minutes, or filtered through a Berkefeld filter. However, lyophilization in 0.25 M sucrose buffer significantly reduced their immunogenic activity while lyophilization in 0.88 M sucrose buffer did not. Sonic oscillation appeared to have no significant effect on the activity of the particles. When the cells were washed 3 times with sucrose buffer the immunogenic activity was not affected. 3. In contrast to these findings, even though the same preparation procedures were followed,

the fraction containing the particles when prepared from BCG cells was not always immunogenically active. The sediment containing the particles appeared grey in color, and the immunogenic activity was reduced by sonic vibration and by washing with sucrose buffer. 4. A chemical analysis of the particles from both H37Ra and BCG showed that they contain large amounts of lipid and no DNA. The significance of these findings is discussed.

1. Youmans, G. P., Millman, I., and Youmans, A. S., *J. Bact.*, 1955, v70, 557.

2. Millman, I., and Darter, R., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 271.

3. Darter, R. W., and Millman, I., *ibid.*, 1957, v95, 440.

4. Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, v55, 629.

5. Rosenthal, S. R., *Acta Tuberc. Scandinav.*, 1952, v26, 106.

6. Youmans, G. P., and Youmans, A. S., *Am. Rev. Tuberc.*, 1951, v64, 541.

7. Litchfield, J. T., Jr., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 399.

8. Youmans, G. P., and Youmans, A. S., *J. Immunol.*, 1957, v78, 318.

9. Ma, T. S., and Zuazaga, G., *Ind. Eng. Chem.*, Anal. Ed., 1942, v14, 280.

10. Youmans, A. S., and Youmans, G. P., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 566.

11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

12. Marr, A. G., and Cota-Robles, E. H., *Bact. Proc.*, 1956, p113.

13. Robrish, S. A., and Marr, A. G., *ibid.*, 1957, p130.

14. Mudd, S., Takeya, K., and Anderson, H. J., *J. Bact.*, 1957, v72, 767.

Received August 8, 1957. P.S.E.B.M., 1957, v96.

Vit. D and Citrate Metabolism. Inhibition of Vit. D Effect by Cortisol.* (23603)

HELEN C. HARRISON, HAROLD E. HARRISON AND EDWARDS A. PARK
Department of Pediatrics, Johns Hopkins Univ. School of Medicine and Pediatric Division, Baltimore City Hospital

The search for metabolic effects of Vit. D which might throw light on its mechanism of action both in promoting bone salt deposition and in regulating serum calcium levels has led to studies of changes in concentrations of citrate in plasma, urine, and tissue associated with deficiency of Vit. D and its correction. Serum citrate levels are reduced in Vit. D deficient infants and are increased by Vit. D administration as is also urinary excretion of citrate(1). If large doses of Vit. D are given to rachitic children serum citrate levels are increased above normal range and hypercitricemia is seen as a manifestation of excessive Vit. D dosage both in patients(2) and experimental animals(3). Administration of Vit. D to Vit. D deficient rats increases the concentration of citrate in serum and in certain tissues such as kidney, bone and intestine although not liver(4). According to Carlsson and Hollunger(5) there is a slight initial decrease of citrate in serum of the Vit. D deficient rat during the first few hours post Vit. D and then a progressive increase which can be detected by 24 hours following Vit. D. The rise of serum citrate after Vit. D administration to some extent parallels the evidences of the antirachitic effect of Vit. D such as increase in serum calcium and phosphorus concentration and bone salt deposition. On the other hand feeding of calcium salts to Vit. D deficient rats given low calcium diets rapidly increases concentration of calcium but concentration of citrate in serum does not show a parallel rise(5). More recently DeLuca, Gran and Steenbock(6) have found that citrate is utilized more rapidly by rat kidney homogenates prepared from Vit. D deficient rats than from animals given Vit. D and they suggest that Vit. D retards conversion of citrate to α keto glutarate, thus leading to intracellular citrate accumulation. The asso-

* This work was supported by grant from the N.I.H., P.H.S.