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Extracellular Proteins of Tubercle Bacilli
I Zone-electrophoretic Purification of the Proteins of a Virulent Strain (H37Rv) of Mycobacterium tuberculosis*

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SUMMARY

Unheated culture filtrates of a virulent strain (H37Rv) of human tubercle bacilli were fractionated with ammonium sulfate and the protein constituents in some of the fractions were further purified by zone-electrophoresis using starch as the supporting medium. The degree of purity achieved by these procedures were measured in terms of the chemical, physical and immunochemical properties.

INTRODUCTION

Filtrates of unheated cultures of *M. tuberculosis* are known to contain a series of different protein antigens (Boyden and Sorkin, 1956). However, the true role of the extracellular protein antigens in tuberculous infection and acquired immunity is still obscure (Weiss, 1960). In the study of this problem, it seems important to isolate and purify the protein components by methods involving the least possible chemical alteration of the natural antigens, so that their true immunological and biological activities can be studied. Few investigators, including Seibert (1949), have attempted to fractionate the protein constituents of unheated culture filtrates of tubercle bacilli. Seibert succeeded in isolating several proteins by a combination of acid- and alcohol precipitation at low temperature. However, in view of the recent advances in protein chemistry, their method does not seem to be the best and indeed her products such as the A, B and C proteins, which were supposed to be the most purified protein preparations in terms of the physicochemical properties, were shown by the agar-gel diffusion technique to contain a variety of antigens (Seibert *et al.*, 1957). Since ammonium sulfate fractionation and electrophoretic separation are considered to be among the mildest methods for separating natural proteins, one may approach the problem of isolation and purification of the extracellular proteins of tubercle bacilli by using a combination of these methods.

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* An outline of this work was reported at the 6th Symposium on Baterial toxins (June 20, 1959), and at the 33rd Meeting of the Japanese Bacteriological Association (July 18, 1960).

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The studies reported here are concerned, in part, with a procedure which leads to products of considerable apparent purity. Information is also given of some of their biochemical properties.

MATERIALS AND METHODS

1. *Organism and culture medium*

Mycobacterium tuberculosis strain H37Rv was from the stock collection of the Department of Tuberculous Research of this Institute.

The organism was cultivated on a modified Souton's synthetic medium of the following composition per liter of solution: K_2HPO_4, 0.5 g, $MgSO_4$, 0.5 g, sodium citrate, 2.5 g, asparagine, 4.0 g, glycerol, 60 ml, KH_2PO_4, 2.5 g and 2.5 ml of 50 per cent $CaCl_2 \cdot 2H_2O$ solution. The asparagine, glycerol, sodium citrate, K_2HPO_4, $MgSO_4$ and KH_2PO_4 were dissolved in distilled water and made up to 1 liter. To remove the iron, 2.5 ml of $CaCl_2$ solution was added and the pH was adjusted to 7.3 with 5 N NaOH solution. The medium was then boiled for two or three minutes and filtered while hot. The filterate was cooled and tested for iron with *o*-phenanthrolene. It should give no detectable colour with this reagent and the final pH, if necessary, should be readjusted to 7.3. 1.5 ml of 0.1 per cent ferric ammonium citrate solution was then added and the medium dispensed in flasks and autoclaved at 120°C for 20 minutes.

2. *Preparation of unheated culture filtrates*

The stock culture of the organism on a glycerol-potato medium was usually transferred to a small flask of the above medium and incubated for two weeks at 37°C. This culture was used as a source of the inoculum. One loopful pericle was inoculated onto 80 ml aliquots of medium in 300 ml Erlenmeyer flasks and incubated at 37°C for three weeks. After incubation, the bacilli were filtered off aseptically through Tōyō No. 50 filter paper and then a Seitz filter in the cold without previous heating of the culture. The yield of the aseptic culture filterate at this stage was usually about 67 per cent of the original culture medium employed. Loss was mainly due to evaporation during incubation. The final filtrates were mixed with appropriate amounts of solid ammonium sulfate to give 80 per cent saturations of the salt, and kept in the cold before centrifugation.

3. *Zone-electrophoresis*

Zone-electrophoresis using starch as the supporting medium as described by Kunkel and Slater (1952) was employed for separation and purification of the fractions prepared with ammonium sulfate. The potato starch used for the experiments was thoroughly washed at least three times with cold water, three times with warm water (56°C) and twice with borate phosphate buffer at pH 8.3, ionic strength 0.1. Zone-electrophoretic separation was carried out at 2-5° C in borate phosphate buffer at pH 8.3 ionic strength 0.1 under constant conditions with a potential gradient of 2 mA/cm^2, usually for 16 hours.

After the electrophoresis, the starch block was cut into 1 centimeter sections and each section was eluted with two 10 ml aliquots of saline. The eluates were then each filtered through ordinary filter paper preparatory to chemical assay.

4. *Assay*

1. *The protein content* was determined by Lowry's method (1953) using crystalline bovine serum albumin as standard. The nitrogen content was determined by a modified micro-Kjeldahl-Nessler method (Yokoi and Akashi, 1955) on specimens dried at 120°C. The dry weight of the specimens were estimated by drying measured volumes to constant weight at 120°C.

2. *The nucleic acid content* was calculated from the 280/260 ratio (Warburg and Christian, 1941) measured in a Hitachi Beckmann type spectrophotometer.

3. *The carbohydrate content* was measured by the Anthrone method (Scott *et al.*, 1953) using glucose as a standard. Molish's method was sometimes employed for qualitative determination.

4. *Phosphorus* was determined in dried specimens by the Fiske-Subbarow's method (1925).

5. *The catalase activity* of the crude extract and fractions was measured by Euler-Josephson's method (1923) and expressed as the Katalase f value (Kat·f).

6. *The absorption spectra* of purified fractions were determined in a Hitachi Beckmann type spectrophotometer in the UV region using approximately 0.02 per cent solutions of the protein preparations.

5. *Analysis of the free solution by electrophoresis.*

Free electrophoresis was conducted in a Spinco, Model H at 4°C with purified protein fractions in veronal buffer, pH 8.6, ionic strength 0.1. The potential gradient was 3 mA/cm^2.

6. *Analysis by ultracentrifugation*

This was carried out in a Spinco analytical centrifuge Model E, at 20°C with purified protein fractions in M/20 phosphate buffer, pH 7.2. Samples were analysed with an analytical A rotor at a centrifugal force of 59,750 rpm. They were observed at intervals of 240 seconds.

7. *Preparation of antisera*

The fractions obtained from unheated culture filtrates by precipitation with ammonium sulfate at final saturations of 0-80 per cent, 0-30 per cent, 30-50 per cent and 50-80 per cent were used as sources of antigen. For preparing antigen specimens for immunizing rabbits, Freund's incomplete adjuvant was used. Thus, an appropriate mixture of Bayol F and Arlacel A was emulsified by adding an equal volume of antigen solution (Cohn, 1952). The entire schedule of immunization was as follows: Each rabbit was given by a single intramuscular injection of the antigen emulsion containing 30 mg protein before the test bleeding. Four weeks after the beginning of immunization, a test bleeding was made of each rabbit and then, a week later, the rabbits were boostered by giving another intramuscular injection of the same antigen emulsion containing 5 mg of protein. One week after the booster injection, the rabbits were sacrificed for total bleeding. The sera thus obtained were inactivated at 56°C for 30 minutes, mixed with merthiolate at a final concentration 0.01 per cent and then kept in the deep freeze. Because of the unexpected lethal action of the 50-80 per cent fraction, this antiserum was obtained by giving rabbits an intracutaneous injection of 100 μg of antigen solution, followed by intravenous injections of the same dose three times a week for 4 weeks, and then finally by a booster injection of the antigen emulsion containing 5 mg protein as described above.

8. *Double diffusion precipitation in agar gel*

The double diffusion precipitation technique of Oakley (1953) was employed with a slight modification. The technique was run in a specially made small plastic container (3.0 × 0.5 × 0.5 cm), covered with a thin plastic plate as illustrated in Figure 1.

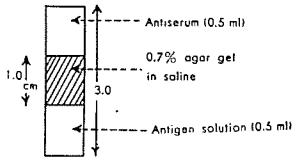


Fig. 1. Form and arrangement of reservoir

RESULTS

1. *Concentration and fractionation of unheated culture filtrates with ammonium sulfate and the chemical properties of the products*

Procedure

100 liters of chilled unheated culture filtrate containing 160 micrograms of protein per ml were added by 56.1 kg of solid ammonium sulfate to give a 80 per cent saturation of the salt. While dissolving the salt, ammonia was added periodically to maintain the pH at 6.8 - 7.2. The filtrate was then stored at 5° C in a cold room overnight. The supernatant was discarded in the following morning and the precipitate which had formed was collected by centrifugation, suspended in a small volume of borate-phosphate buffer (pH 8.3, ionic strength 0.1) and then dialyzed overnight against 40 liters of the same buffer. The dialyzed material was turbid and dark brown in color. This material was mixed with borate-phosphate buffer and made up to a final volume to one liter. The protein content of the supernatant was approximately 13.26 mg per ml and the yield was 83 per cent. A 100 ml aliquot was taken from this for *the 0-80 per cent fraction.* 900 ml of the material was mixed with 385 ml of saturated ammonium sulfate solution to give 30 per cent saturation. The resulting precipitate was *the 0-30 per cent fraction.* The supernatant was then mixed with 515 ml of saturated ammonium sulfate solution to give 50 per cent saturation. The precipitate formed was *the 30-50 per cent fraction.* The supernatant was further mixed with 3.6 liters of saturated ammonium sulfate solution to give a final saturation of 80 per cent. The precipitate formed was *the*

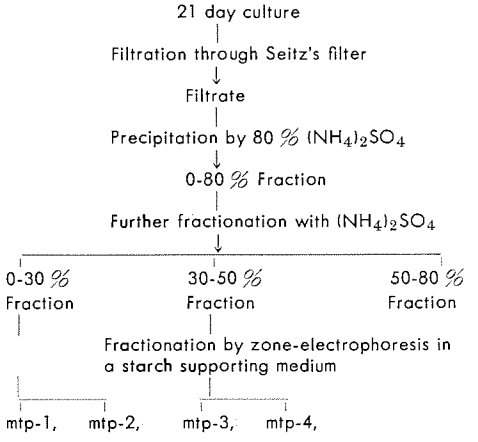


Fig. 2. Flow chart for preparation of protein fractions (unheated culture filtrate of *M. tuberculosis*)

Fractionation was carried out in the cold.
All soluble protein fractions were stored in a deep freezed state (-20°).

50-80 per cent fraction. These three precipitates were each dissolved in minimal amounts of borate phosphate buffer and then dialyzed in cellulose tubing against

buffer until free from ammonium sulfate. The dialyzed *0-30 per cent fraction* was turbid and dark brown colored while the other fractions were transparent. Estimation of the protein contents of the clear supernatants obtained by centrifugation at 12,000 rpm for 30 minutes showed that the yields were 37.7, 29.2 and 9.6 per cent respectively. *The 0-80 per cent fraction* was dialyzed in a similar way before experiments. The fractionation scheme is given in Figure 2.

Some chemical properties of fractions 0-80, 0-30, 30-50 and 50-80 per cent

Nitrogen, phosphorus, nucleic acids and sugar contents were measured in the four fractions obtained by ammonium sulfate precipitation by the methods described in a preceding section. The results are shown in Table 1.

Table 1. Chemical characterization of the four fractions obtained by $(NH_4)_2SO_4$ fractionation

Fraction	Nitrogen* content (%)	Phosphorus* content (%)	P/N ratio	Nucleic* acids content (%)	Sugar* content (%) (as glucose)
0-80 %	9.35	0.60	0.064	3.5	13.3
0-30 %	12.12	0.41	0.034	3.1	7.3
30-50 %	12.70	0.40	0.031	0.8	8.4
50-80 %	7.10	0.73	0.103	2.9	35.2

The weight of samples was estimated by dring measured volumes to constant weight at 120° C.

* The nitrogen, phosphorus, nucleic acid and sugar content of samples were measured by the modified Nessler's, Fiske-Subbarow's, Warburg-Christian's and Scott's methods, respectively.

From the data of yields, one may conclude that proteins in unheated tuberculous culture filtrates are mostly (87.3 per cent) precipitable by 50 per cent ammonium sulfate, and, from the nitrogen contents shown in Table 1, fraction by half saturation of ammonium sulfate resulted in some degree of purification. Thus, considerable amounts of sugars most of which may be from polysaccharides appear to be removed from the product by half saturation with ammonium sulfate. Comparison of the two fractions, 0-30 and 30-50 per cent, which contain about the same amount of nitrogen, phosphorus and sugar, shows that the nucleic acids content of the 30-50 per cent fraction is much smaller than that of the 0-30 per cent fraction. It is of considerable interest that catalase activity was present exclusively in this 30-50 per cent fraction. This will be described in the following section.

2. *Separation and purification of proteins from the products obtained by ammonium sulfate fractionation by zone-electrophoresis*

The data in Table 1 show that fractionation by ammonium sulfate resulted in partial purification of extracellular tuberculous proteins. However, the grade of purity of the products appeared to be very low in terms of their nitrogen content

even in fractions 0-30 and 30-50 per cent. Therefore, these three fractions were further fractionated by zone-electrophoresis.

Zone-electrophoresis using starch as a supporting medium was carried out in a horizontal-type apparatus as described in the section on *MATERIALS AND METHODS*. About 7.5 ml of 1-2 per cent protein solution after dialysis against borate-phosphate buffer, was used for each experiment. There was no appreciable loss of protein after zone-electrophoresis, and more than 90 per cent recovery of the separated material indicating that no singificant adsorption of the proteins on starch took place. The electrophoretic patterns of the fractions (0-30, 30-50 and 50-80 per cent) in packed starch plates are presented in Figures 3, 4 and 5. Because of the rather large quantity of material to be fractionated, this procedure were repeated many times and always gave similar patterns. It is evident from Figures 3 and 4 that the two fractions (0-30 and 30-50 per cent) contain at least two major protein fractions respectively, while the pattern of the 50-80 per cent fraction appears to be rather complicated. It should be noted here that there was always a fraction at the side of cathode near the origin which was detectable by its absorption at 260 mμ. The eluate from this fraction was opalescent and strongly fluorescent. The chemical nature of this fraction is now being studied.

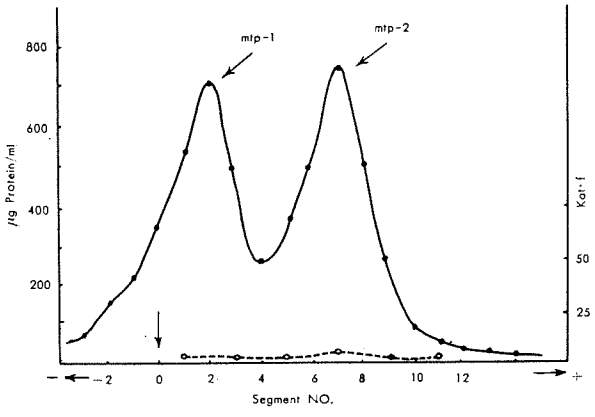


Fig. 3. Electrophoretic pattern of the 0-30 % $(NH_4)_2SO_4$ fraction.

—•——•— Protein concentration of separate eluates

–o– – – –o– Distribution of catalase activity expressed as Kat·f.

Conditions: Borate-phosphate buffer ph 8.3, ionic strength 0.1

Protein concentration	1.5% (7ml)
Electric current	2 mA/cm^2
Time	16 hrs
Temperature	4° C
Cell (plastic)	40×10×1.5cm

0 – – Origin

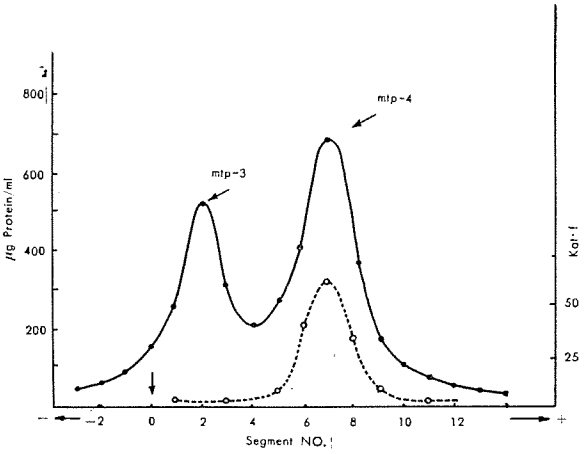


Fig. 4. Electrophoretic pattern of the 30-50 % $(NH_4)_2SO_4$ fraction

—•——•— Protein concentration of separate eluates

—○— — — —○— Distribution of catalase activity expressed as Kat·f.

Conditions : Borate-phosphate buffer pH 8.3, ionic strength 0.1

Protein concentration	1.5% (7 ml)
Electric current	2 mA/cm²
Time	16 hrs
Temperature	4° C
Cell (plastic)	40×10×1.5 cm

0 – – Origin

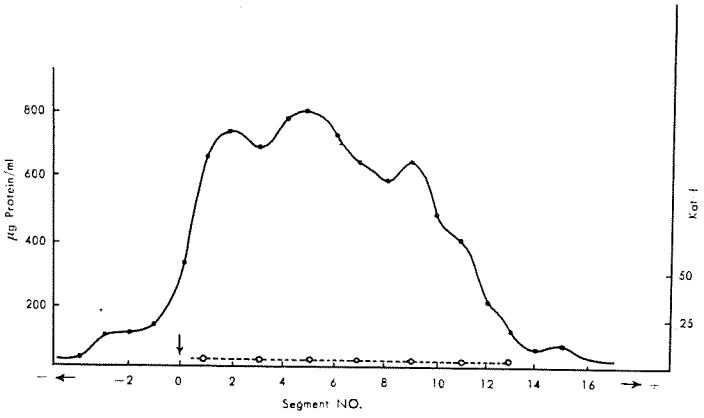


Fig. 4. Electrophoretic pattern of the 50-80 % $(NH_4)_2SO_4$ fraction on starch supporting medium

—•——•— Protien concentration of separate eluates

—○— — — —○— Distribution of catalase activity expressed as Kat·f.

Conditions : Borate-phosphate buffer pH 8.3, ionic strength 0:1

Protein concentration	1.5 % (7m)
Electric current	2 mA/cm²
Time	16 hrs
Temperature	4° C
Cell (plastic)	40×10×1.5 cm

0 – – Origin

Since the two fractions (0-30 and 30-50 per cent) each appeared to consist of two major protein fractions, these latter are tentatively named as follows; mtp-1, mtp-2, mtp-3 and mtp-4 as shown in Figures 3 and 4.

These four protein fractions were purified by repeated zone-electrophoresis in the following way: According to the diagrams obtained, the eluates of high protein content from three segments of one fraction were combined, precipitated with ammonium sulfate of the same saturation as that at which the original fraction was precipitated and the precipitates were dissolved in borate-phosphate buffer. The dialyzed material was then subjected to zone-electrophoresis as described above. This procedure was repeated twice on each protein fraction.

The yield of protein at each step during the procedure is shown in Table 2

Table 2. Yield of protein at each fractionation step

Fraction	Total protein mg	Yield %
Culture filtrate (100 l)	16000	100
0-80 % Fraction	13260	83
0-30 % Fraction	5431.5	37.7
30-50 % Fraction	4207.5	29.2
50-80 % Fraction	1375.2	9.55
mtp-1 (Zone -1)	1368.0	9.5
mtp-2 (Zone -1)	1521.0	10.6
mtp-3 (Zone -1)	1107.0	7.7
mtp-4 (Zone -1)	1161.0	8.1
mtp-1 (Zone -2)	587.7	4.1
mtp-2 (Zone -2)	613.8	4.3
mtp-3 (Zone -2)	484.2	3.4
mtp-4 (Zone -2)	475.2	3.3
mtp-1 (Zone -3)	382.5	2.7
mtp-2 (Zone -3)	307.8	2.1
mtp-3 (Zone -3)	306.0	2.1
mtp-4 (Zone -3)	291.6	2.0

and the electrophoretic patterns of the final fractions on the strach supporting medium are shown in Figures 6-a, -b, -c and -d. It may be seen from the diagrams that they each show a single peak in their electrophoretic patterns and that all the patterns appear to be fairly symmetrical. Careful inspection, however, shows that there is some skewness in all the patterns, particularly in those of mtp-2 and mtp-4. These final purified protein preparations are dissolved very easily in water or neutral buffer solutions, and 1-2 per cent solutions of mtp-1 and mtp-3 are almost colorless,

while solutions of mtp-4 and particularly of mtp-2 are brown colored.

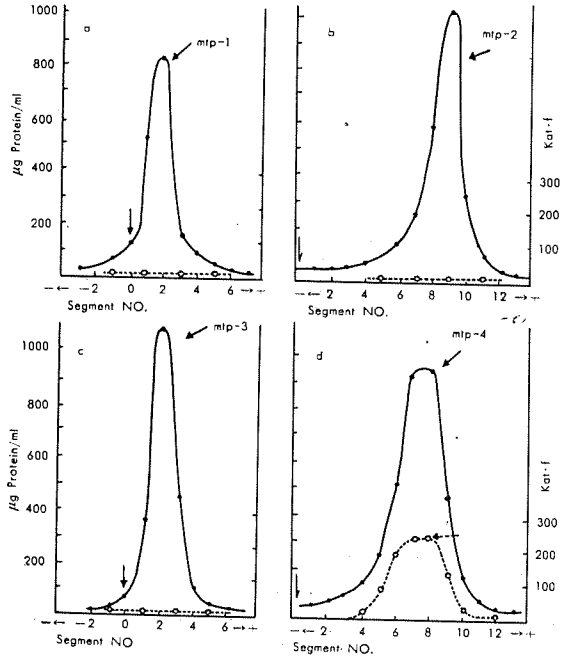


Fig. 6. Electrophoretic pattern of fractions mtp-1, mtp-2, mtp-3, and mtp-4 on starch supporting medium

—•——•— Protein concentration of separate eluates

–o– – – –o– Distribution of catalase activity expressed as Kat•f.

Conditions : Borate-phosphate buffer pH 8.3, ionic strength 0.1

Protein concentrations	mtp-1, 0.8 %, mtp-2, 0.9 %
	mtp-3, 0.9 %, mtp-4, 1.0 %
Electric current	2 mA/cm²
Time	16 hrs
Temperature	4° C
Cell (plastic)	40×10×1.5 cm

0 – – Origin

3. *Properties of purified fractions*

Since the electrophoretic patterns on a starch supporting medium of the four purified fractions were reproducible and clear cut, these fractions were then subjected to chemical, physical and immunochemical analyses.

UV absorption spectrum

Figure 7 shows the absorption spectra for approximately 0.02 per cent solutions of the purified protein fractions. All fractions showed a similar pattern and their maximal absorption was at 278 mμ, the typical absorption maxima of proteins.

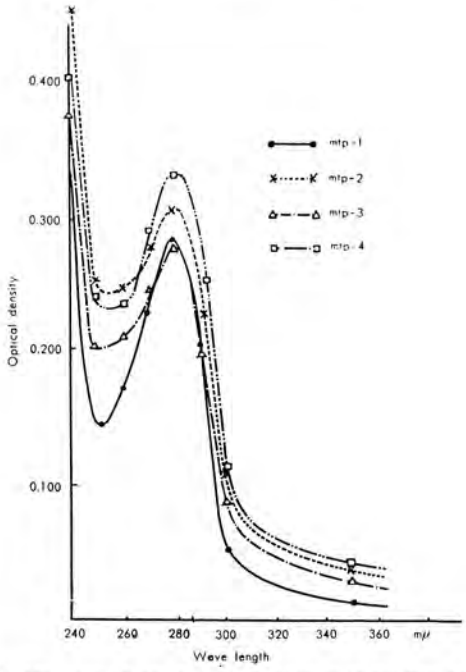
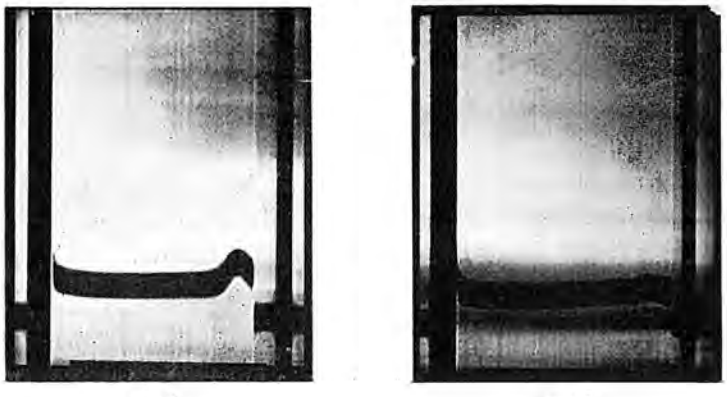


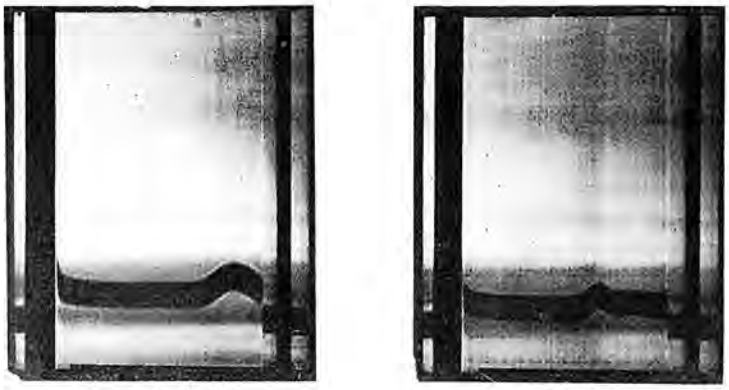
Fig. 7. Ultraviolet light absorption spectrum of the four protein fractions separated by Zone-electrophoresis
Protein concentration in M/20 phosphate buffer pH 7.2
mtp-1, 200μg/ml, mtp-2, 210 μg/ml,
mtp-3, 200μg/ml, mtp-4, 230 μg/ml,

Sedimentation and free-electrophoresis

Ultracentrifugal examination of the purified fractions showed that both mtp-1 and mtp-3 consisted of a single, homogeneous component respectively, while mtp-4 still contained a slower moving minor component as an impurity. Figure 8 illustrates



mtp-1 mtp-2



mtp-3 mtp-4

Fig. 8. Sedimentation patterns of the four purified protein fractions

The solutions contain of fractions mtp-1, mtp-2, mtp-3, mtp-4, contained 9, 12, 10 and 8 mg/ml protein in 0.05 M phosphate buffer respectively. Rotor speed: 59,780 (250,000 × g) Temperature : 20° c

the patterns of these fractions in the ultracentrifuge after a 75 minutes run at 59,780 rpm at 20° C. Of particular interest is the ultracentrifugal behavior of mtp-2. Thus, a typical polydispersed pattern was observed during centrifugation. Sedimentation constants calculated from serial photographs are given in Table 3. The free-electrophoretic analyses of the four fractions showed that each of the protein preparations was a single, slightly asymmetrical electrophoretic component. The electrophoretic mobilities of these fractions are also given in Table 3.

Table 3. Sedimentation constants and electrophoretic mobilities of fractions mtp-1, mtp-2, mtp-3 and mtp-4

Fraction	Sedimentation constant $S_{20} \times 10^{-13}$ cm,sec^{-1}	Electrophoretic mobility cm^2, volt^{-1}, sec^{-1} $\times 10^{-5}$
mtp-1	2.83	−6.12
mtp-2	—*	−9.17
mtp-3	2.88	−5.87
mtp-4	8.50 (5.62)**	−16.45

* This could not be measured because of its polydispersed pattern

** Sedimentation constant of a minor component.

Chemical analyses

A summary of certain chemical analyses of these fractions is presented in Table 4. It is evident from this data that separation and purification by zone-electrophoresis does indeed raise the grade of purity of the protein fractions in terms of their nitrogen contents. As can be seen in the table, the nitrogen content

Table 4. Chemical characterization of the four purified protein fractions

Fraction	Nitrogen* content (%)	Phosphorus* content (%)	P/N ratio	Nucleic* acids content (%)	Sugar* content (%) (as glucose)
mtp-1	14.40	0.40	0.028	0.28	4.69
mtp-2	14.87	0.21	0.014	1.25	1.79
mtp-3	15.20	0.37	0.024	1.00	4.68
mtp-4	15.87	0.17	0.011	0.68	1.08

The weight of samples was estimated by drying measured volumes to constant weight at 120° C.
* The nitrogen, phosphorus, nucleic acids and sugar content of samples were measured by the modified Nessler's, Fiske-Subbarow's, Warburg-Christian's and Scott's method, respectively.

varies in the fractions from 14.4 to 15.9 per cent. However, assuming a value 16.0 for the percentage of nitrogen in tuberculin protein (Seibert *et al.*, 1938), the final fractions contain at least 90 per cent protein and, in fractions mtp-3 and mtp-4, 95 and 99 per cent respectively. The preparations were all almost free from phosphorus and contained very little nucleic acid. Since the latter were calculated from the 280/260 ratio, it may not represent the true nucleic acids content, which might indeed be much smaller. On the other hand, considerable sugar is present in all preparations particularly in fractions mtp-1 and mtp-3. Difficulty was always encountered in removing traces of starch from the eluates and, for this reason, saline eluates of washed starch treated in the same way as the fraction-eluates were used as controls during determination of the sugar content. Tuberculous polysaccharides remained near the origin during electrophoresis and caused for the difficulty. Eluates from the slower moving protein portions were contaminated with these polysaccharides. The rather higher sugar content of fractions mtp-1 and mtp-3 could mostly be accounted for by contamination with such tuberculous polysaccharides.

Catalase activity

Since qualitatively, unheated culture filtrates of this organism showed catalase activity, this activity was measured in each fractionation step by the method as described in a preceding section. The results are summarized in Figure 9. The catalase activity is expressed as the Kat·f according to the following equation:

$$k = \frac{1}{t} \log_{10} \frac{a}{a\text{-}x} \quad (\text{min}^{-1})$$

$$\text{Kat} \cdot f = \frac{2k_{3.0} - k_{1.5}}{\text{dry weight in 50 ml}}$$

a ---- initial concentration of peroxide

x ----the amount of peroxide which has reacted

It can be seen from the figure that the catalase activity is exclusively in fraction mtp-4 and the intermediate fractions from which mtp-4 is derived. The activity was quite heat labile and these fractions lost their activity by heating at 60° C for 10 minutes. Assuming no alteration or denaturation of the enzyme took place during the fractionation procedure, this enzyme was purified about 16 times at the final

step, in terms of its specific activity. Although the activity in fraction mtp-4 was very low, all attempts to dissociate the activity from the particular protein fraction failed.

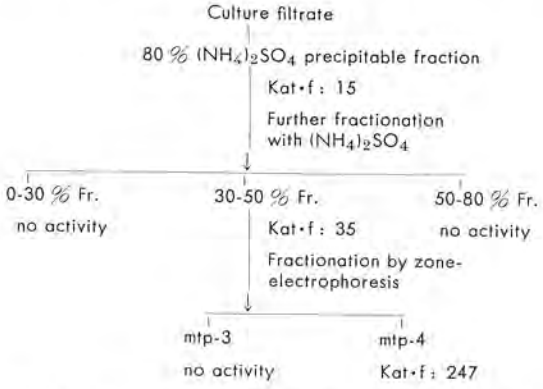


Fig. 9. Distribution of catalase activity in the various protein fractions of the culture filtrate of H37Rv

Catalase activity was measured by the method of von Euler-Josephson (1923) and was expressed as Kat•f.

Immunochemical analyses

Since all evidence so far obtained indicates considerable purity of the final protein fractions in terms of their chemical and physical properties, their grade of purity was tested immunochemically by the agar gel diffusion technique. Figure 10

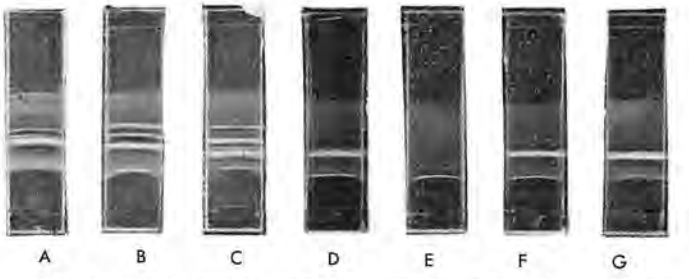


Fig. 10. Oakley's double agar diffusion patterns produced by crude and fractionated pretein fractions of unheated culture filtrate of H37Rv

A : 0-80 % fraction (1 mg/ml)
B : 0-30 % fraction (1 mg/ml)
C : 30-50 % fraction (1 mg/ml)
D : mtp-1 (250 μg/ml)
E : mtp-2 (250 μg/ml(
F : mtp-3 (250 μg/ml)
G : mtp-4 (250 μg/ml)

shows precipitation reactions of various fractions in agar gel with anti 0-80 per cent

rabbit sera, using Oakley's technique. It will be seen that fractions mtp-1 and mtp-3 both give a single line and that fraction mtp-4 gives only two lines one of which is rather faint. A number of lines, however, are found in the 0-80 per cent, 0-30 per cent and 30-50 per cent fractions. Fraction mtp-2 is of particular interest. The photograph shows that this fraction gave no precipitation reaction with crude antisera suggesting that it might consist of denatured proteins. Its peculiar polydispersed pattern in ultracentrifugation was described above. It was found that the precipitating activity of fractions mtp-1, mtp-3 and mtp-4 was lost completely by heating these fractions at 70° C for 10 minutes in neutral buffer.

DISCUSSION

A combination of ammonium sulfate fractionation and zone-electrophoresis of unheated culture filtrates of *M. tuberculosis* strain H37Rv on a starch supporting medium, yielded four products which appear electrophoretically to be highly homogeneous. All evidence indicates that these products are highly purified protein fractions. These have been disignated as follows; mtp-1, mtp-2, mtp-3 and mtp-4. The grade of purity achieved by this procedure differed considerably in each fraction depending upon the criteria used. Thus on a chemical basis, particularly from the nitrogen contents, fraction mtp-4 is apparently the purest, followed by fractions mtp-3, mtp-2 and mtp-1. But this may not be true on physical and immunochemical criteria. Ultracentrifugal analyses showed that fraction mtp-4 was apparently slightly heterogeneous and this fraction was shown to be contaminated by an antigen which gave a faint but distinct precipitation line with crude antiserum in agar gel, while fractions mtp-3 and mtp-1 whose nitrogen contents were the lowest were fairly homogeneous on ultracentrifugal analyses and gave only a single precipitation line with crude antisera in agar gel. Although the electrophoretic mobilities and the sedimentation constants for both fractions mtp-1 and mtp-3 suggest that these proteins are essentially the same, they differ significantly in their nitrogen contents. Whether this is a true difference in the nitrogen contents of the proteins of the two fractions or whether it indicates the presence of a small amount of some non-protein components in fraction mtp-1 is not yet known and requires further chemical analyses. Fraction mtp-2 which was found to be fairly homogeneous electrophoretically showed a peculier polydispersed pattern on ultracentrifugation. The results indicate that this fraction must be comprised of numerous components with various sedimentation constants, but the same electric charge. Moreover, since it gave no precipitation with crude antisera, it seems that these components might be denatured proteins. Chemical analyses showed that all the final fractions and particularly fraction mtp-1 and mtp-3 contained considerable amounts of sugar. As was mentioned in a previous section, such sugars could mostly be tuberculous polysaccharides which still contaminated the fractions after electrophoresis. Thus, with material that contains such large amounts of polysaccharides as tuberculous

culture filtrates, it is apparent that great difficulty would be encountered in separating the protein portion of slow mobility on a starch supporting medium without getting contamination from the polysaccharides. This is one of the limits of the electrophoretic separation method. Removal of the polysaccharides, therefore, should be achieved by other methods such as a column chromatography using DEAE cellulose.

The heat lability of the precipitation reactions in agar gel of fractions mtp-1, mpt-3 and mtp-4 shows that these are probably due to protein antigens in the frations. However, the ring tests showed that they contained a small amount of heat stable antigen indicating that these fractions are still heterogeneous. However, available data suggest that these three purified fractions will be valuable for serological and biological investigations. It may be possible to use these purified protein antigens in the antigen analysis of *Mycobacteria*, as suggested by a preliminary study. Information on the serological relationship between these antigens and proteins A, B and C will be reported in the next paper.

From Figure 9, it will be seen that catalase activity is always associated with fraction mtp-4. However, this activity is very low and, assuming a value for Kat·f of 30,000 of cow liver catalase (Sumner *et al.*, 1937), the final activity of fraction mtp-4 corresponds to only one per cent of the latter activity. One may conclude from this that the activity of fraction mtp-4 is due to a very minor component which contaminates this protein fraction. However, it may be that fraction mtp-4 is comprised mostly of the apo-protein of mycobacterial catalase. This is only a speculation and further immunochemical studies using the purified catalase of this organism are needed to prove this.

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