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Extracellular Proteins of Tubercle Bacilli III. Column Chromatographical Purification of the Proteins of a Virulent Strain (H37Rv) of *Mycobacterium tuberculosis*[*]

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SUMMARY

Three proteins, mtp-1, mtp-3 and mtp-4, isolated by ammonium sulfate fractionation and zone-electrophoresis from unheated culture filtrates of a virulent strain (H37Rv) of *M. tuberculosis*, were purified further by column chromatography. Chemical analysis showed that these proteins were free from phosphorus, nucleic acids and sugars after chromatography. Mtp-1 and mtp-3 which gave a single homogeneous peak in free-electrophoresis and ultracentrifugation were both separated into three components on a DEAE cellulose column. The main component of each protein fraction gave a sharp homogeneous peak on recolumn chromatography by a gradient elution method. Antisera giving a single precipitation line in agar gel against crude concentrated culture filtrates of H37Rv strain were obtained by immunizing rabbits with this fraction.

INTRODUCTION

By using a combination of ammonium sulfate fractionation and zone-electrophoresis on a starch supporting medium, three serologically active protein fractions (mtp-1, mtp-3 and mtp-4) were isolated from unheated culture filtrates of a virulent strain (H37Rv) of *M. tuberculosis* (Yoneda and Fukui, 1961a). These protein fractions were purified by repeating the zone-electrophoresis and the final products seemed to be heat labile protein antigens of considerable apparent purity (Yoneda and Fukui b). However, they still contained appreciable amounts of heat stable antigens, which were presumably attributed to a small amount of the serologically active tuberculous polysaccharides unseparable from the protein portion by this procedure. Although they were suitable for a few serological studies (Fukui, Yoneda and Hori, 1961), further extensive purification of these antigens, particularly by removing the polysaccharides, was necessary to obtain precise information about their immunochemical properties and to prepare pure antisera for serological analysis of *Mycobacterial* extracellular protein antigens. Column chromatography using various ion-exchanger resins or adsorbents has recently been widely employed for the

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separation and purification of biologically active proteins (Keller and Block, 1960). Thus, this method was used in an attempt to purify these antigens to such degree that no minor components other than protein could be detected.

The present paper describes the purification of three protein antigens, mtp-1, mtp-3 and mtp-4 by column chromatography using DEAE cellulose and hydroxylapatite. The degree of purity achieved by this method is described in terms of the chemical, physical and immunological properties of the proteins.

MATERIALS AND METHODS

1. *Protein preparations*

The preparation of the three extracellular proteins (mtp-1, mtp-3 and mtp-4) of *M. tuberculosis* strain H37Rv used in this study is described in our previous paper (1961a). Concentrated solutions of these proteins in borate-phosphate buffer (pH 8.3, ionic strength 0.1) were stored at −20°C.

2. *DEAE cellulose and hydroxylapatite*

Hydroxylapatite, calcium phosphate, was prepared according to the method of Tiselius *et al.* (1956) with the slight modification of Halbert (1961). The DEAE cellulose employed was purchased from the Brown Company (Berline, New Hampshire) and ionized by Peterson's method (1956). All procedures of column chromatography were carried out at 4-6°C.

3. *Double diffusion precipitation in agar gel*

The double diffusion precipitation techniques of Oakley (1953) and of Ouchterlony (1948) were employed with the slight modification described in our previous paper (1961a, 1961b).

4. *Assays*

1. *Chemical analyses:* The protein content was determined by Lowry's method (1953), phosphorus by Fiske-Subbarow's method (1925), nucleic acids by Warburg and Christian's method (1941) and the sugars by Scott's method (1953). The nitrogen content was measured by a modified micro-Kjeldahl-Nessler method (1955).

2. *Dry weight measurement:* The dry weight of the specimens was estimated by drying measured volumes to constant weight at 120°C.

5. *Ultracentrifugation*

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

6. *Preparation of antisera*

The preparation of rabbit antisera of 0-30, 30-50% ammonium sulfate saturation fractions of H37Rv culture filtrates has been described previously (1961a). Rabbit antisera of mtp-1, mtp-3 and mtp-4 purified by column chromatography were also prepared. The method of immunization with these materials was described in our previous paper (1961a). In the present work, the first injection dose was 100μg of protein per rabbit and the booster dose was 50μg protein per rabbit.

RESULTS

1. *DEAE cellulose chromatography of mtp-1*

Prior to chromatography, concentrated mtp-1 was dialyzed against the initial

concentration of phosphate buffer (0.001 M) at pH 7.0. DEAE cellulose was ionized by Peterson's method (1956) and first washed several times with the initial concentration of buffer on a filter. Non-sedimenting matter was removed by decantation and the cellulose was packed under slight positive pressure into a glass chromatographic column, 1.5 × 30 cm, fitted at the bottom with packed glass wool, to a height of 25 cm. The column was then mounted on an automatic fraction collector (Toyo Roshi, Model DFS-1) in a cold room and washed several time with 50 ml of the initial concentration of buffer. About 7 grams of the adsorbent were employed to pack the column. 10 ml of mtp-1 containing 119.3 mg of protein was applied to the column and then eluted by gradient elution with from 0.001 M to 0.2 M phosphate buffer at pH 7.0. 10 ml aliquots of the eluate were collected in the collector at a flow rate of 10 ml per hour per cm^2. Establishment of a linear gradient in this elution was checked by measuring the refractory index of aliquots from a similar control column. The protein content of each tube was determined spectrophotometrically, at 280 mμ. The elution pattern obtained is shown in Figure 1.

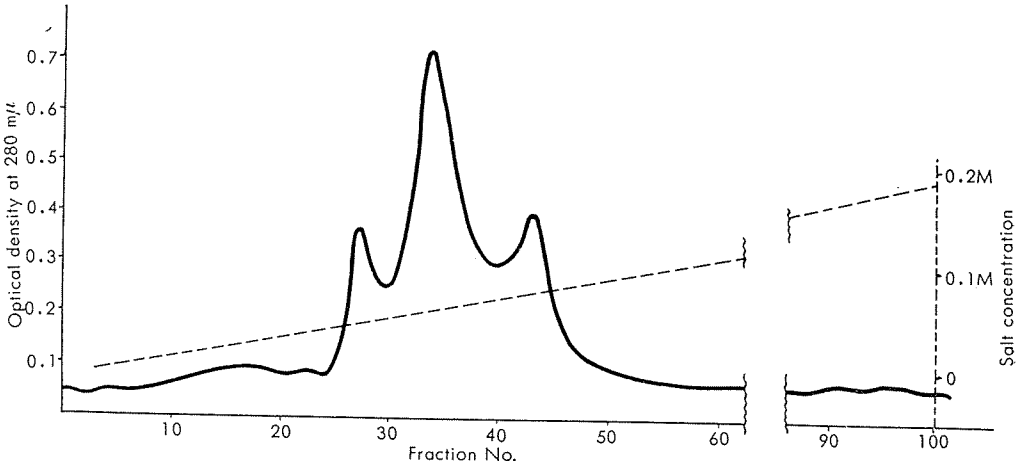

Fig. 1. DEAE Cellulose Column-Chromatography Pattern of mtp-1
Dotted line indicates the salt concentration of buffer determined from the refractive index

There were three peaks on the chromatogram, which did not separated completely. The total protein content of the pooled eluates from these three peaks was 60 mg, so that the recovery was about 50 per cent. Agar gel diffusion analysis showed that the first peak formed two precipitation bands against anti 0-30% Fraction serum, which fused with a single precipitation band of the other two peaks, in an Ouchterlony plate.

2. *DEAE cellulose chromatography of mtp-3*

Mtp-3 was chromatographed in the same way as mtp-1, but in this case 108.2 mg of protein was applied. The result is shown in Figure 2.

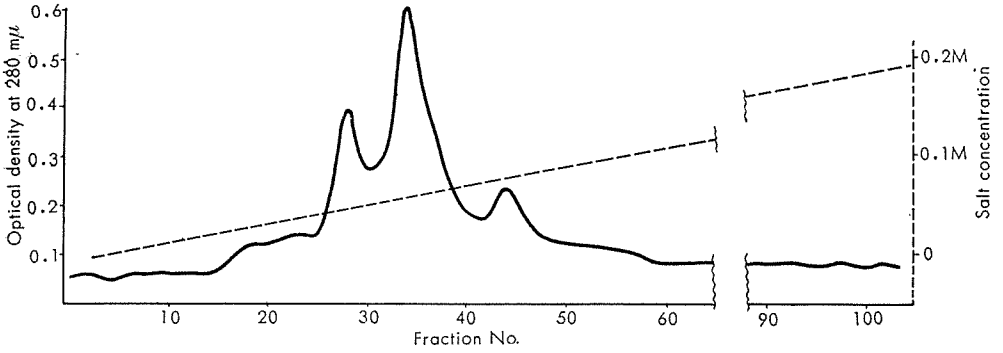

Fig. 2. DEAE Cellulose Column-Chromatography Pattern of mtp-3

Dotted line indicates the salt concentration of buffer determined from the refractive index

Although the pattern was always slightly different from that of mtp-1, there were also three peaks on the chromatogram. The recovery from these particular peaks was about 47 per cent of the protein applied. Protein from the first peak formed two procipitation bands against anti 30-50% Fraction serum and, as in the case of mtp-1, these bands fused with a single precipitation band formed between the serum and the protein from the other two peaks.

3. *DEAE cellulose chromatography of mtp-4*

Mtp-4 was chromatographed on a DEAE cellulose column by the same procedure as for mtp-1 and mtp-3. In this case, 106.9 mg of protein was applied to the column. As can be seen in Figure 3, a single broad peak with considerable tailing

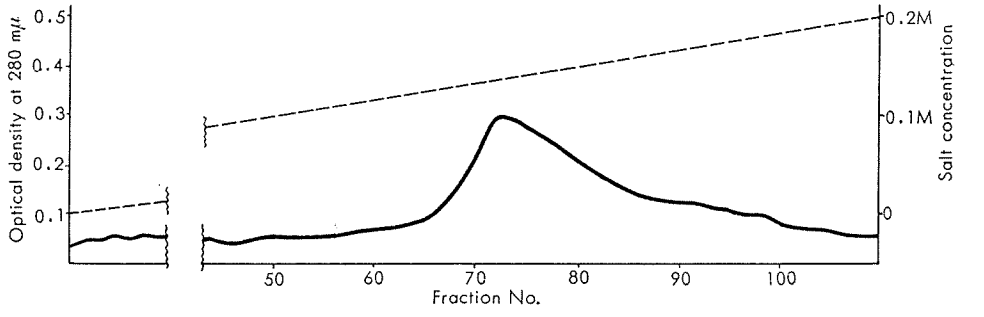

Fig. 3. DEAE Cellulose Column-Chromatography Pattern of mtp-4

Dotted line indicates the salt concentration of buffer determined from the refractive index

appeared on the chromatogram. The recovery of protein from the peak was 35 per cent of the total protein applied. The eluate from this peak was collected, precipitated with half saturated ammonium sulfate, dialyzed against the initial

concentration of buffer an then rechromatographed on DEAE cellulose. However, no peak could be detected. The peak seen on the first chromatogram formed a single precipitation line against anti 30-50% Fraction serum in an Ouchterlony plate.

4. *Rechromatography of mtp-1 and mtp-3.*

Eluates from the main peaks of mtp-1 and of mtp-3 on the chromatogram described in the previous sections were collected, precipitated by half saturated ammonium sulfate and then dialyzed against the initial phosphate buffer (0.001 M, pH 7.0). Ten ml of each solution containing 35.6 mg protein for mtp-1 and 34.2 mg for mtp-3 were applied to the DEAE cellulose column described previously. The results are illustrated in Figure 4. As can be seen from the figure, a single sharp protein peak was obtained in both cases, which appears to be homogeneous and they were in the same place on the rechromatograms as had been on the first chromatograms. There was 87 per cent recovery for mtp-1 and 90 per cent for mtp-3. In Oakley's tubes, they formed single sharp precipitation lines against anti 0-30 or 30-50% Fraction serum (Photograph 1).

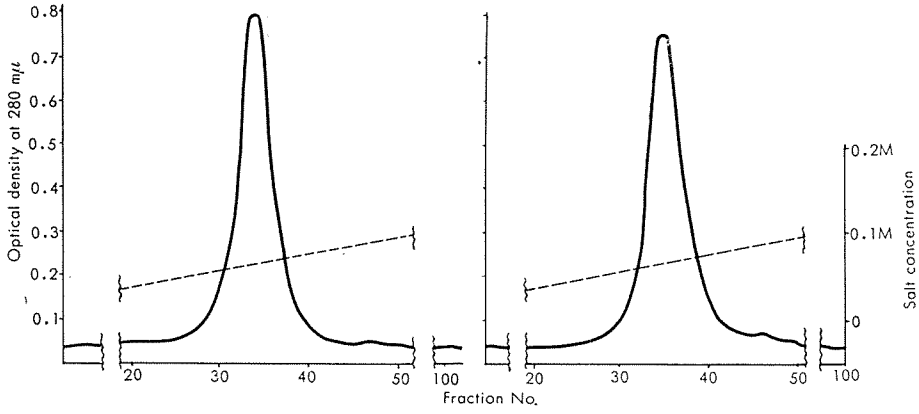

Fig. 4. DEAE Cellulose Rechromatography Patterns of mtp-1 and mtp-3
Dotted line indicates the salt concentration of buffer determined from the refractive index

5. *Chromatography and rechromatography of mtp-4 on hydroxylapatite columns*

Since mtp-4 could not be rechromatographed on DEAE cellulose, probably due to denaturation of the protein on the column, attempts were made to chromatograph it on hydroxylapatite. This was poured into a glass chromatographic column, 3 × 10 cm, fitted at the bottom with packed glass wool and was allowed to settle by gravity to a column height of 7 cm. Ten ml of mtp-4 solution, containing 73.3 mg of protein, was applied to the column and eluted by gradient elution

with 0.001 M to 0.2 M phosphate buffer, pH 6.8. The elution method was the same as described in the preceding sections.

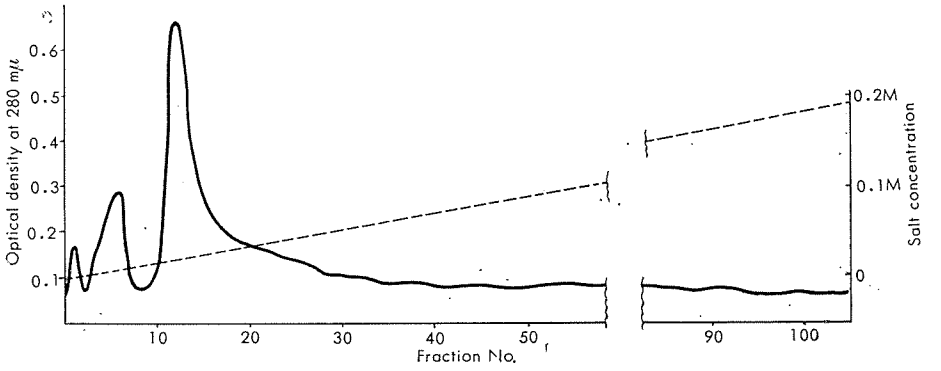

Fig. 5. Chromatography Pattern of mtp-4 on a Hydroxylapatite Column
Dotted line indicates the salt concentration of buffer determined from the refractive index

As is seen in Figure 5, three separate peaks appeared which were all eluted at a rather low concentration of the buffer. Agar gel diffusion analysis showed that only the main peak. which was eluted at the slowest rate, could react with the anti 30-50 % Fraction serum and formed a single sharp precipitation line in an Ouchterlony plate. The recovery from this main peak represented about 48 per cent of the protein applied.

The protein of the main peak was concentrated from the eluate by salting out with ammonium sulfate. It was dialyzed against the initial phosphate buffer (0.001 M, pH 6.8) and then applied to a fresh hydroxylapatite column. 53.2 mg of protein was applied.

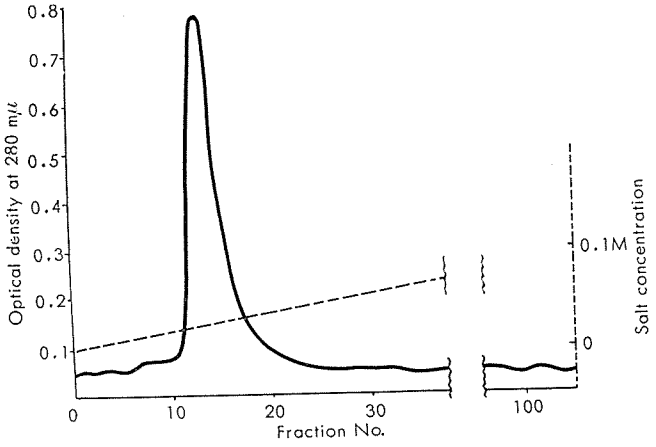

Fig. 6. Hydroxylapatite Rechromatography Pattern of mtp-4
Dotted iine indicates the salt concentration of buffer determined from the refractive index

Figure 6 illustrates the rechromatography of mtp-4 on the column. From the figure, it will be seen that only a single sharp peak appeared on the chromatogram at the salt concentration expected from the first chromatogram. However, the pattern was considerably skewed. The recovery was about 92 per cent of the total protein applied. By using Oakley's technique it was found that the final protein preparation still gave two precipitation lines against anti 30-50% Fraction serum, although one of these lines was very faint.

6. *Chemical analyses*

A summary of certain chemical analyses of mtp-1, mtp-3 and mtp-4 purified

Table 1. Chemical Characterization of mtp-1, mtp-3 and mtp-4 Obtained after Purification

Materials	Nitrogen content (%)	Phosphorus content (%)	Nucleic acids content (%)	Sugar content (%)
mtp-1	15.9	<0.1	<0.1	<0.1
mtp-3	16.6	<0.1	<0.1	<0.1
mtp-4	16.2	<0.1	<0.1	<0.1

The weight of sample was estimated by drying measured volumes to constant weight at 120° C. Samples for phosphorus determination were 1 mg dry weight and 10 mg dry weight for sugars. 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.

by chromatography is presented in Table 1. It is evident from these data that further purification by column chromatography does indeed increase the purify of these materials in terms of their nitrogen contents. Moreover, minor components such as phosphorus, nucleic acids and particularly sugars, which still remained after zone-electrophoresis, were scarcely detectable after chromatographical purification.

7. *Ultracentrifugal analyses*

Ultracentrifugal examination of these chromatographically purified materials showed that both mtp-1 and mtp-3 were single homogeneous components, while mtp-4 was still comprised of two components. Photograph 3 illustrates the patterns of these proteins in the ultracentrifuge.

8. *Immunological properties*

Analyses by agar gel diffusion as well as chemical and physical studies clearly indicated that the final samples of both mtp-1 and mtp-3 were highly purified proteins which could be considered homogeneous. Therefore attempts were made to obtain pure antisera of these proteins by injecting the samples into rabbits. The method used is described in the section on *Materials and Methods*.

To test the purity of the antisera thus obtained, analyses by agar gel diffu-

sion by Okaley's method with a slight modification (Yoneda and Fukui, 1961a) were employed. The arrangement and form of the reservoirs were the same as described in our previous papers. Thus, in the specially made small plastic containers (3.0 × 0.5 × 0.5 cm) undiluted antisera of mtp-1 and mtp-3 were set against concentrated culture filtrates of strain H37Rv and incubated at 37°C for three days. Then the precipitation patterns were photographed. As controls, anti 0-30 or 30-50% Fraction serum was employed in place of the test antisera. They are shown in Photograph 3. It may be seen from the photograph that anti mtp-1 and mtp-3 serum gave only single precipitation lines against the crude culture filtrate while the control antisera formed many precipitation lines. Antisera were also prepared for mtp-4 by the method used for mtp-1 and mtp-3, but these sera gave several faint lines besides a main strong precipitation line against the concentrated culture filtrate.

DISCUSSION

As was described in our previous paper (1961a), purification by repeated zone-electrophoresis failed to remove appreciable amounts of phosphorus, nucleic acids and particularly sugars from three protein antigens (mtp-1, mtp-3 and mtp-4). The results presented in this paper clearly show that chromatography on DEAE cellulose and hydroxylapatite columns removes these components from the protein antigens. The values of 15.9～16.6 for the nitrogen content and of 1.70～1.75 for the 280/260 mμ ratio of UV absorption indicate that these final products are highly purified forms of these proteins. The absence of detectable amounts of sugars, phosphorus and nucleic acids further suggests that they are probably of a simple protein nature. In our previous paper, it was noted that considerable amounts of heat stable antigens were still present in the original mtp-1, mtp-3 and mtp-4 preparations. However, by the ring test, they were scarcely detectable in these final products after chromatography. The absence of these heat stable antigens may probably be accounted for by the removal of serologically active tuberculous polysaccharide from the original preparations.

The finding that both mtp-1 and mtp-3 are comprised of three components separable from each other by DEAE cellulose chromatography is of considerable interest. It is still uncertain if these compontents are merely artifacts produced by DEAE cellulose chromatography. However, since when their main components were rechromatographed, they were eluted in the buffer solution of the expected salt concentration and were recovered in high yield (87～90 per cent), these components may not be mere artifacts but true constituents of both mtp-1 and mtp-3, unseparable by zone-electrophoresis. Analysis by agar gel diffusion suggests that one of the three components, the first component elutable at the lowest salt concentration, may be comprised of both cross reacting and identical antigens with the other two component. Whether this is true must be tested by further im-

munochemical studies of these components.

Formation of a single precipitation line in agar gel even against concentrated crude culture filtrates clearly indicates that the anti mtp-1 or mtp-3 serum contains no detectable amounts of precipitating antibodies which react with the antigens in the filtrate other than mtp-1 or mtp-3. It may also provide further strong evidence of the high degree of purity of these antigens purified on DEAE cellulose. Although further separation of the components of mtp-4 is needed, successful preparation of two pure protein antigens and their antisera has permitted us to carry out precise immunochemical analyses of some *Mycobacterial* extracellular proteins. Thus, using mtp-1 or mtp-3 and their antisera, investigations are now in progress on the cross reacting materials produced by strains of "unclassified *Mycobacteria*" (Fukui, Yoneda and Hori, 1960).

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purification of the proteins of a virulent strain (H37Rv) of *M. tuberculosis*. *Biken's J.* **4,** 25-40.
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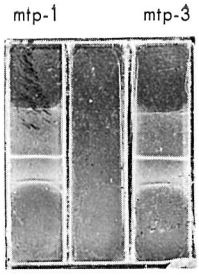
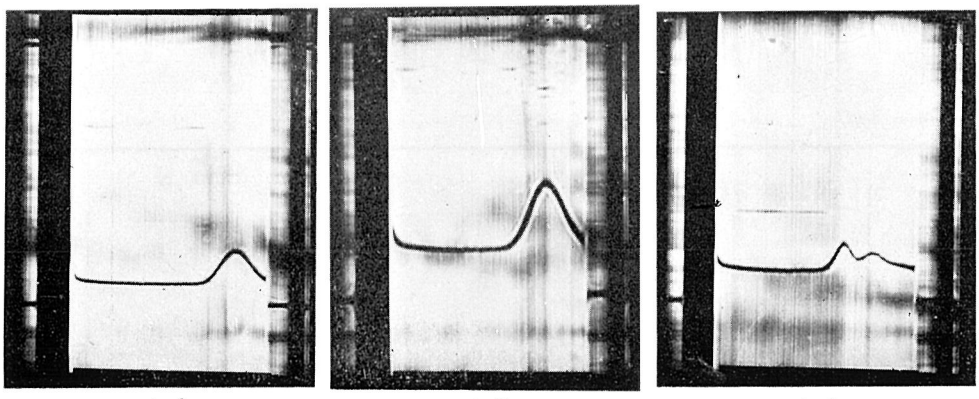


Anti 0～50 fraction rabbit serum

Photograph 1. Oakley's Double Agar Diffusion Precipitation Patterns of mtp-1 and mtp-3 Purified Chromatographically

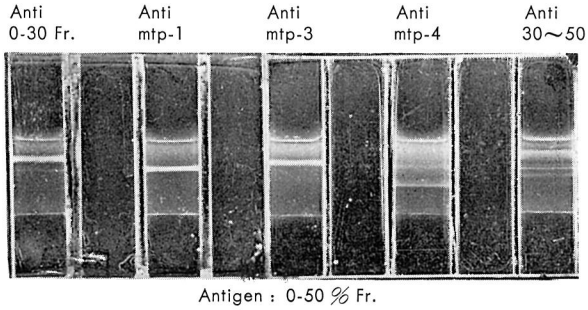
Antisera : Undiluted anti 0-50 % fraction rabbit serum (0.3 ml)
Antigens: 0.3 ml of 500 μg protein/ml



mtp-1 mtp-3 mtp-4

Photograph 2. Sedimentation Patterns of mtp-1, mtp-3 and mtp-4

The solutions of mtp-1, mtp-3 and mtp-4 contained 10, 15 and 10 mg protein/ml respectively in 0.05 M phosphate buffer, pH 7.2.
Rotor speed : 59,780 (250,000×g), Temperature : 20°C.
Angle : mtp-1, 60° mtp-3, 70° mtp-4, 60°



Antigen : 0-50 % Fr.

Photograph 3. Oakley's Double Agar Diffusion Precipitation Patterns of Various Antisera

Antisera : 0.3 ml of undiluted sera.
Antigen : 0.3 ml of 5 mg/ml protein of 0-50 % fraction.