MOLECULAR WEIGHT, ELECTROCHEMICAL AND BIOLOGICAL PROPERTIES OF TUBERCULIN PROTEIN AND POLYSACCHARIDE MOLECULES*

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PLATE 13

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It is of fundamental importance to obtain as nearly complete an understanding as possible of the size and other physical properties of the molecule or molecules in tuberculin which are able to elicit the tuberculin reaction. Not only is such knowledge of great value in the preparation of a pure product and in further attempts to explain the cause of the tuberculin reaction itself and its relationship to the disease, but also it will be helpful in interpreting a whole series of similar immunological reactions of which the tuberculin reaction is a prototype. The differences observed between the tuberculin reaction (the delayed type of reaction) and typical protein sensitization reactions (the immediate type) may well depend upon some physical property of the molecule, for both are obviously elicited by constituents in the tubercle bacillus culture filtrates.

Throughout this study, therefore, the relationship in various preparations between tuberculin potency and antigenicity, on the one hand, and molecular weight or shape and electrochemical properties, on the other, was noted, and the aim was to prepare homogeneous fractions having certain of these different properties. The separations were rendered difficult because of the large amount of polysaccharide

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and in some cases of nucleic acid present with the protein. Neither of these diluting components, however, seemed to be responsible for any of the physiological reactions noted and obtained when the fractions were injected. The polysaccharide has been studied in considerable detail in order to understand its relationship to the protein and methods for its elimination.

Methods

Studies of the sedimentation velocities in the Svedberg ultracentrifuge (1), rotating at a velocity of 60,000 to 70,000 r.p.m. (centrifugal force of 285,000 to 360,000 times gravity) were made on the various tuberculin preparations. The diffusion constants of those fractions which appeared to be homogeneous in the centrifuge were then determined by the method developed by Lamm (2), using the cell designed by him. The refractive index scale method was used in both centrifuge and diffusion procedures. Specific volume determinations were also made on representative fractions. All sedimentation constants reported in this paper have been expressed in units of $10^{-12}$, and diffusion constants in units of $10^{-7}$.

The sedimentation constant was calculated from the formula given by Svedberg (3)

$$ s_m = \frac{dx}{dt} \cdot \frac{1}{\omega^2 \cdot \eta/\eta_0} \cdot \frac{1 - V\rho_0}{1 - V\rho} $$

where \( dx/dt \) = the sedimentation velocity observed, \( \omega \) = the angular velocity, \( \eta/\eta_0 \) = the ratio of the viscosity of the solution to that of water at 20°C., \( V \) = the partial specific volume of solute, and \( \rho \) and \( \rho_0 \) the densities of solution and of water at 20°C. A brief description of the technique involved has been given recently (4).

The diffusion constant was calculated as an average of the constants determined for seven or eight points on each curve of four to six exposures taken during the diffusion, using the formula (equation 4) in the paper of Lamm and Polson (5).

From the diffusion constant thus obtained and the sedimentation constant found on the same solution the molecular weight may be calculated according to the formula given by Svedberg (6),

$$ M = \frac{RT_s}{(1 - V\rho)D} $$

where \( R \) is the gas constant, \( T \) the absolute temperature, and \( \rho \) the density of the solution.

The molar frictional constant, \( f \), of the molecule may also be calculated from these data, thus:

$$ f = \frac{RT}{D} = \frac{M(1 - V\rho)}{s} $$
If, however, the molecule is compactly spherical and unhydrated the following formula,

\[ f_0 = 6\pi \eta N \left( \frac{3MV}{4\pi N} \right)^{\frac{1}{2}}, \]

where \( N \) is the Avogadro number, will be valid. For such a molecule, therefore, the frictional ratio \( f/f_0 \) should be unity. A larger value of \( f/f_0 \) indicates that the molecule either deviates from the compact spherical shape or that it is hydrated.

The degree of molecular weight homogeneity of a fraction could be judged to some extent by observing the symmetry of the sedimentation curves made from exposures taken at different intervals during a run, and also by noting the agreement between the \( D_{20} \) constants determined for a number of points on the diffusion curves made from various exposures. In several cases a further criterion of the homogeneity of a fraction was gained by comparing the \( D_{20} \) obtained from a real diffusion experiment with the \( D_{20} \) calculated from the sedimentation curves. A small degree of heterogeneity would reveal itself as a marked deviation between the two and in such case the diffusion constant computed from the sedimentation diagram would be only an apparent one.

The concentration of the sedimenting molecules may be calculated from the sedimentation diagrams, certain constants for the apparatus, and the refractive index increment for the solution (4, 7). The refractive index increment of a fraction which was practically homogeneous in the centrifuge (TPA-21b, see later), was measured in the Pulfrich refractometer and found to be 0.001948 at wave length \( \lambda = 579 \) m\( \mu \).

Electrochemical homogeneity was determined by means of electrophoresis in the Tiselius apparatus (9). There is, however, a noteworthy difference to be considered between electrochemical homogeneity and molecular weight homogeneity as determined by sedimentation and diffusion. Earlier investigations (10) have given examples of cases of homogeneous sedimentation and diffusion but inhomogeneous electrophoresis (e.g. the serum globulins), on the one hand, and of inhomogeneous sedimentation and diffusion but homogeneous electrophoresis (e.g. dissociation of proteins into particles of nearly unchanged chemical nature) on the other. In this investigation the criterion for electrophoretic homogeneity was limited to the number of individual boundaries migrating, as representative of separate components, and not to the purity of the individual boundaries.

Chemical analyses were made upon most of the fractions as follows: Total concentration of a solution was determined by means of the dipping refractometer. Nitrogen determinations were made by means of a modification of the micro Kjeldahl method of Pregl. Carbohydrate was determined at first by the orcin-sulfuric acid method of Tillmans and Philippi (11), but later better results were obtained using the carbazol reaction of Dische (12). The tuberculin polysaccharide prepared by one of the authors and described later, was used as a standard, and by means of the shade of color obtained in this carbazol reaction it was possible to distinguish immediately between the presence of real tuberculin polysaccharide
which gave a slightly brownish pink color because of the mannose present (13),
and that of nucleic acid carbohydrate which gave a reddish pink color. Standards
containing 0.1, 0.2, and 0.3 mg. polysaccharide and blank determinations were
made with each set of analyses.

Nucleic acid was determined by means of the diphenylamine reaction of Dische
(12), using a very highly purified preparation of thymus nucleic acid1 as standard,
since the nucleic acid of the tubercle bacillus is known to contain thymine (14).
A blank and standards containing 0.1, 0.2, and 0.3 mg. nucleic acid were run
with each set of analyses. The reaction was highly specific for the tubercle bacil-
lus nucleic acid when the proportion of reagents recommended by Dische were
used (1 gm. diphenylamine in 100 cc. glacial acid plus 2.74 cc. concentrated sulfuric acid. Two parts of this reagent and one part of the unknown solution were
heated in a boiling water bath for 10 minutes). The tuberculin polysaccharide
gave no reaction.

The biological potency of the fractions was tested by means of intracutaneous
reactions with 0.1 cc. amounts on tuberculous guinea pigs. The reactions were
measured in three dimensions 24 and 48 hours after the injection, and these read-
ings were then averaged in all of the animals tested. Toxicity tests for tuberculous
guinea pigs were made by intraperitoneal injections. In those cases where the
product was lethal the animal was dead within 24 hours and at autopsy showed
the pathological changes of typical tuberculin death, namely, inflation of the
lungs and fluid, fibrin and congestion in the splanchnic area. Precipitin tests
for the protein fractions were made with rabbit antiserum2 to the whole mixed
protein fraction from tuberculin, containing a very low content of polysaccharide,
and for polysaccharide with the anti-tubercle bacillus horse serum.3

Two groups of materials were used in these experiments. The first consisted
of the unchanged culture medium filtrates, where the molecules would be found
in their most natural state. The second group included OT (old tuberculin)
which had been heated during its preparation and which would, therefore, be
expected to contain molecules markedly changed from their natural state. This
substance, as well as fractions isolated from it, such as the purified protein deriva-
tive of tuberculin (hereafter designated as PPD) (15), are of especial interest
because of their practical value in medical practice.

EXPERIMENTAL

Original Tuberculin Culture Medium Filtrates.—The first studies
were made on the unchanged culture medium filtrates produced by

1 This preparation of nucleic acid was kindly given to us by Professor E. Ham-
marsten, Stockholm.

2 We are indebted to Dr. P. Kallós for facilities and assistance in obtaining this
serum.

3 Prepared by the Mulford Laboratories of Sharp and Dohme, and sent to us
by Dr. Michael Heidelberger.
growing two human strains (PN and H 37) and one bovine (523) strain of tubercle bacilli and the Moeller strain of timothy grass bacillus on the Long synthetic medium for 8 weeks, filtering off the bacilli through the Seitz filter, concentrating this filtrate on the ultrafilter, and washing it until it was salt-free. These fractions were called TPU, prefixed to the name of the strain, meaning tubercul protein ultrafiltered.

Table I gives the sedimentation constants obtained. In all cases the sedimentation diagrams showed one main component and the presence of some smaller and larger molecules, as evidenced by the asymmetry of the curves which developed as sedimentation progressed. The TPU-bovine curves, however, seemed to be the most symmetrical throughout the entire run. The $s_{20}$ determinations on the TPU-human (PN) fractions were made at pH 6.8; all the others were made at pH 8.0. In all cases phosphate buffer plus 0.2 M NaCl, giving a total molarity of 0.235–0.25, was used.

The three determinations upon TPU-PN, namely, unpreserved, preserved with 0.5 per cent phenol, or merthiolate$^4$ (1:10,000), indicated that very little change was caused by the preservatives used, except that slightly more of the quickly sedimenting components (probably denatured protein) were present. Carbohydrate analyses showed the presence of considerable polysaccharide in all cases.

These fractions were studied in the Tiselius electrophoresis apparatus at 0°C. in the presence of phosphate buffer at pH 8.0 and of

\begin{table}
\centering
\caption{Sedimentation of Tuberculin Filtrates}
\begin{tabular}{l|c|c|c}
\hline
Substance & $s_{20}$ & Per cent of the total as carbohydrate & Per cent of the total as nucleic acid \\
\hline
TPU-human (PN)-unpreserved & 1.7 & & \\
+ 0.5 per cent phenol & 1.7 & & \\
+ merthiolate (1:10,000) & 1.8 & 35.7 & 0.54 \\
TPU-human (H 37) & 1.8 & 72.5 & 1.10 \\
TPU-bovine (523) & 1.7 & 32.1 & 0.26 \\
TPU-timothy (Moeller) & 1.4 & 26.8 & 0.49 \\
& 1.6 & & \\
\hline
\end{tabular}
\end{table}

$^4$ The merthiolate was obtained from Eli Lilly and Company.
ionic concentration 0.1 or 0.2, with the corresponding appropriate voltages of 300 or 200. The TPU-human (PN) was found to have three boundaries migrating toward the positive pole. The fastest and slowest of these boundaries were thin, while the intermediate one was very heavy. In the cases of TPU-bovine and TPU-timothy only two mobile boundaries appeared, but both were equally heavy. In all preparations an extra very heavy immobile band was present, which proved to be caused by polysaccharide. Since the mobilities of the protein and polysaccharide components were so different electrophoresis seemed a promising method for separating them.

Fractions Separated by Electrophoresis.—The experiments were so arranged that the current was allowed to flow until the top positive compartment (see Text-fig. 1) was filled with the solution exhibiting the two mobile components. By compensation in the opposite direction it was possible to move the carbohydrate boundary down into the U tube and thus to fill the two compartments on the positive side with the protein solution. This solution, from these two compartments on analysis was found to contain a much lower percentage of carbohydrate than the original solution and correspondingly higher content of protein. It was separated from the remainder of the
solution and run again in the apparatus in the same way, using fresh buffer, except in the case of TPU-timothy. In this manner, it was possible to obtain fractions with very low carbohydrate content (see Table II).

In the final TPU-human and timothy fractions two components were, therefore, still present, as shown by the presence of two electrophoretic boundaries. When run in the centrifuge the TPU-human (PN) showed the presence of two clearly defined components with $s_{20} = 1.9$ and 4.9 in two different experiments 1 and 2, as shown in Table II.

Table II. The larger molecule does not seem to appear in the original medium and may, therefore, have been formed during the separation of protein and polysaccharide. The TPU-timothy, however, gave on sedimentation only one main gradient, but it was markedly heterogeneous.

In the case of the TPU-bovine, during the final separation, it was possible, by running for a considerable length of time, to separate the two boundaries a half cell distance and, therefore by pipetting out the upper half, to obtain about 2 cc. of a solution with only one electrophoretic component. This was sufficient for centrifuge and diffusion.
runs and analyses. It proved to be a single homogeneous substance in the centrifuge and also in diffusion, with constants of $s_{20} = 1.6$ and $D_{20} = 12.0$ respectively. Assuming a specific volume of 0.700 (the same as found for the human strain protein), a molecular weight of about 10,000 was calculated. See Table X.

Biological tests were made upon some of the fractions separated from the TPU-human (PN). The summary of the skin reactions in tuberculous guinea pigs and of the precipitin tests is given in Table III. The protein fraction was that fraction migrating into the top positive compartment and described in Table II. The carbohydrate-protein fraction was that which remained behind on the negative side of the U tube with the polysaccharide and, therefore, was probably the fraction with slowest migration and most closely associated with the polysaccharide. The carbohydrate fraction was obtained by electrophoresis as described in the section under polysaccharide.

Table III shows that the tuberculin protein separated from the carbohydrate by electrophoresis had about the same potency as the standard PPD which has been used in extensive tuberculin testing (15). Furthermore, the protein which was most closely associated with the carbohydrate was probably somewhat more potent per unit of protein than the free protein itself and slightly less antigenic, as demonstrated by the precipitin tests. Toxicity tests on tuberculous animals also suggested (Table IV) that the free protein may be less

**Table III**

**Biological Potency of Fractions Separated by Electrophoresis**

| Substance          | Per cent carbohydrate | Average dimensions of skin reactions (Five tuberculous animals each) | Precipitin with least amount protein |
|--------------------|------------------------|---------------------------------------------------------------------|-------------------------------------|
|                    |                        | First set 0.005 mg. protein | Second set 0.003 mg. protein | Third set 0.005 mg. protein |                                     |
|                    |                        | mm.                     | mm.                        | mm.                      | mg.                                  |
| Protein            | 3.6                    | 13 x 14 x 1.7           | 12 x 12 x 1.3              | 10 x 10 x 1.3            | 0.0008                               |
| Carbohydrate-protein | 40.0                  | 13 x 14 x 1.7           | 15 x 17 x 1.9              | 2 x 2 x 0.25            | 0.0012                               |
| Carbohydrate       | 85.6                   | 4 x 4 x 0.4             | 2 x 2 x 0.25              | 9 x 11 x 1.3            | 0.0011                               |
| Standard PPD       | 24.0                   | 13 x 14 x 1.6           | 17 x 13 x 1.4              | 15 x 18 x 2.4           |                                      |
| TPU-human (PN)     | 35.0                   |                         |                          |                        |                                      |
toxic than that protein associated with carbohydrate. In Table IV the numbers in circles indicate the animals which died from the injections. That the increased toxicity was not due to a toxic carbohydrate is indicated by the fact that even as much as 10 mg. of the polysaccharide, isolated in this way in its most nearly native form, was not toxic. Previous studies on the polysaccharide isolated in quantity by chemical means showed that even as much as 50 mg. given intraperitoneally would not kill tuberculous guinea pigs. Probably, therefore, the protein fraction most closely associated with the carbohydrate may be somewhat more potent than the separated protein, but the differences observed are only suggestive and should be confirmed before they can be considered really significant.

**TABLE IV**

*Lethal Potencies*

| Electrophoretic fractions                      | Number of animals injected and number killed by the following doses in terms of mg. protein injected |
|-----------------------------------------------|---------------------------------------------------------------------------------------------------|
| TPU-human (PN)                                | 10 mg. 3 mg. 2 mg. 1.5 mg. 1 mg. 0.7 mg. 0.5 mg.                                                 |
| Protein                                       | 1 1 1 1                                                                                           |
| Carbohydrate-protein                          | 1 1 1 1                                                                                           |
| Carbohydrate                                  | 1 1 1 1                                                                                           |

*This animal must have died of complications since five others receiving more did not die.*

*Tuberculin Polysaccharide.*—The polysaccharide was isolated in electrophoresis by pushing the immobile component into the top compartment on the negative side by the compensation process, and allowing the protein to migrate away from it, and then re-running in the same way the solution from the two compartments on the negative side. The final solution in the top negative compartment had a total concentration of 0.64 per cent, all of which proved to be polysaccharide on analysis. The next lower compartment contained a solution with total concentration 1.07 per cent, of which 90 per cent was polysaccharide and about 7.5 per cent protein. This fraction was analyzed in the centrifuge in two different experiments and in both was found to have one main gradient, with $s_{20} = 1.76$ and 1.81, and a very small amount of a more quickly sedimenting substance, probably protein.
This polysaccharide was then compared with one isolated in quantity in 1931 by the method described by Renfrew (13) and modified by Masucci, McAlpine, and Glenn (16). The latter contained only 0.09 per cent nitrogen and was completely soluble in water, giving a colorless solution. It caused no skin reaction in tuberculous guinea pigs and also had no toxicity, as mentioned above. When studied in the centrifuge in concentrations between 0.2 and 2.0 per cent in phosphate buffer at pH 8 containing 0.2 molar sodium chloride and a total molarity of 0.235, it showed a single homogeneous component. The values for the sedimentation constant showed no drift with change in the concentration but fluctuated between 1.6 and 1.9, around the mean $s_{20} = 1.75$. It was, furthermore, practically homogeneous when tested in diffusion, with a constant of $D_{20} = 11.0$. The specific volume at 20°C. was found to be 0.619, and from these data a molecular weight of 9000 was calculated (see Table X). Apparently the tuberculin polysaccharide differs remarkably from polysaccharides hitherto studied in exhibiting molecular homogeneity.

This latter preparation, when compared with the one isolated by electrophoresis, was found to have the same precipitating power for anti-tubercle bacilli serum, both giving a definite precipitate in a final antigen dilution of 1:6,000,000. It is significant that the sedimentation constant of the polysaccharide is so nearly like that of the main protein component in the tuberculin from the human type tubercle bacillus. Therefore, in the sedimentation graph of the original culture the curves for the two would be practically superimposed upon one another, presenting one curve that may give the false impression of a single homogeneous substance.

Fractions Separated by Chemical Procedures from the Culture Filtrates of Human Tubercle and Timothy Strains of Bacilli.—Fractions isolated from the human type tubercle bacillus (H 37) culture filtrate by trichloroacetic acid precipitation (17), by repeated half or complete saturation with ammonium sulfate (18), by electrodialysis of the half-saturated fraction, by fractional ultrafiltration (18), and by chromatographic adsorption (19) to aluminum hydroxide and elution at pH 8,

*Recent investigations (Svedberg and Gräfen on plant juices show that the polysaccharides of native liquids are as a rule homogeneous (private communication from Professor Svedberg on unpublished results).*
all gave asymmetrical curves when studied in the ultracentrifuge, indicating more or less molecular heterogeneity.

However, during these studies it was found that a considerable amount of an extremely polydisperse denatured fraction precipitated when the original culture medium or the half-saturated ammonium sulfate fractions were brought to pH 4.6–4.8 with acetate buffer. Therefore, this fraction was removed, and then it was possible to obtain a small amount of a fraction, which was practically homogeneous in the ultracentrifuge and also in diffusion, by one-tenth to one-fourth saturation with ammonium sulfate at pH 4.8. It was necessary to repeat the precipitation at least four times. Four preparations were made in this way (see Table V). The TPA-30b gave the most ideal curves in the centrifuge, and since the buffer concentration (0.235 molar) was sufficient to eliminate all possible Donnan effect, the molecular weight calculated from these constants, 32,000, is probably the most accurate figure.

The TPA-30K, while not quite so homogeneous in the centrifuge, was studied further, since it was the only fraction obtained in sufficient quantity; about 28 cc. of a 1.25 per cent solution was obtained. It was found to have 16.3 per cent nitrogen, based on the dry weight,
and 2 per cent polysaccharide. After being either frozen at -8° to -10°C. or dried in vacuo with its buffer at pH 8, there was no evidence of alteration in its sedimentation characteristics.
When studied in electrophoresis it showed a single boundary. By rewashing and reconcentrating the solution on the ultrafilter between each determination with different buffers of \( \mu = 0.03 \), it was possible to obtain the migration velocities at different pH's, as indicated in the curves (Text-fig. 2, heavy line), and thus to determine the isoelectric point, which proved to be at pH 4.3.

Furthermore, a portion of the solution was washed free of buffer on the ultrafilter and then electrodialyzed. A small amount of a precipitate formed. This was filtered off and then the solution was titrated\(^6\) electrochemically, using the glass electrode. A blank titration in water was first made by adding 0.25 cc. one-tenth normal hydrochloric acid in a total volume of 2.25 cc. and then titrating back with small additions of one-tenth normal sodium hydroxide. Thus the correction to be added to the hydrogen ion concentration at every volume increment was determined. Similar titration was then made with the protein solution, and, using the blank corrections determined, the equivalents of base bound at each pH were calculated as described in a previous publication (20). In this way the isoionic point was found to be at pH 4.7. (See Text-fig. 2, dotted line.) When the pH 4.3 was reached during the titration, a sharp and heavy isoelectric precipitate occurred, which again disappeared as this point was passed. This difference noted between the isoelectric and isoionic points is in accord with the finding of Tiselius (9) on egg albumin and, furthermore, Adair and Adair (21) reported a difference of 0.4 pH in their studies on hemoglobin.

Only 0.0009 mg. of this TPA-30K fraction was required to give a definite precipitate with 0.1 cc. rabbit antiserum to the whole mixed protein fraction.

When tested intracutaneously in tuberculous guinea pigs a surprising demonstration of toxicity and of the local anaphylactic type of reaction was obtained. One normal and nine tuberculous guinea pigs were given simultaneous tests with 0.05 mg. doses of several fractions, among which was the TPA-30K, representing the large molecule, and PPD-b3, which will be described later and which represented the small molecule (see Table VI). The total dosage of

\(^6\) We are indebted to Dr. Torsten Teorell for kindly making this titration.
all the fractions injected was only 0.25 mg. of which 0.15 mg. was comparatively inactive material. Nevertheless four of the nine guinea pigs were dead at 24 hours and one more died at 48 hours. Therefore, this substance was much more effective than any product previously studied in producing death in the tuberculous guinea pig, since the usual lethal dose of tuberculin protein is about 1 mg. All of those guinea pigs surviving for 24 hours showed very large edematous, diffuse, and deep reddish or purple local reactions at the site of the TPA-30K injections, in contrast to the comparatively small

| Guinea pig No. | TPA-30 K (0.05 mg.) | PPD-b 3 (0.05 mg.) |
|----------------|---------------------|---------------------|
|                | 24 hrs. | 48 hrs. | 24 hrs. | 48 hrs. |
| Normal         | mm.     | mm.     | mm.     | mm.     |
| TB 1           | 30 x 35 x 4 (purple) | 15 x 25 x 3 | 15 x 20 x 2 | 28 x 20 x 5 |
| TB 2           | 30 x 22 x 3 (red) | 13 x 15 x 2 | 7 x 7 x 1 | 21 x 21 x 3 |
| TB 3           | 30 x 22 x 3 (purple) | 12 x 20 x 3 | 20 x 11 x 2 | 25 x 18 x 3 |
| TB 4           | 12 x 13 x 2 (purple) | 12 x 12 x 2 | ? | 25 x 30 x 4 (center) |
| TB 5           | 20 x 30 x 3 (purple) | Dead | ? | ? |
| TB 6           | Dead | | | |
| TB 7           | " | | | |
| TB 8           | " | | | |
| TB 9           | " | | | |

and more localized or typical tuberculin reactions, at the site of the PPD-b3 injections. At 48 hours the relative sizes of these two reactions in each guinea pig were reversed and the typical delayed reaction with PPD-b3 was the larger of the two. Moreover, the edematous and reddish or purple character of the TPA-30K reactions had disappeared.

*Fractionation of the Protein from the Timothy Bacillus.*—Since one-tenth to one-fourth saturation with ammonium sulfate yielded an homogeneous fraction from the human type tubercle bacillus
tuberculin an attempt was made to secure a similar fraction from the timothy type bacillus filtrate. The result was complete failure, however, for when tested in the centrifuge the fraction was far from homogeneous. It was, therefore, redissolved in citrate buffer at pH 3 and reprecipitated three times by 0.25 saturation with ammonium sulfate and then washed on the ultrafilter with acetate buffer at pH 4.8. This fraction appeared practically homogeneous in the centrifuge and diffusion and, with the determination of the specific volume, as recorded in Table V, it was possible to calculate a molecular weight of 17,000. (See also Table X.) It is probable, therefore, that the original molecules in the culture filtrate are even smaller, since the sedimentation constants (see Tables I and II) when determined on the filtrates were lower.

Studies on Old Tuberculin and Purified Protein Derivative Fractions Isolated from It.—Attempts were then made to study the smallest potent molecule. The PPD was chosen (15), since it exhibited those properties thought to be evidence of small molecular size. For example, it had been found to pass through membranes of smaller pore size, it was not coagulable by heat, it had a much higher osmotic pressure than the ammonium sulfate fractions, it was non-antigenic with respect to producing the Arthus reaction and gave a very low titer in the precipitin test, while at the same time it was highly potent and a very satisfactory standard diagnostic tuberculin in the skin test (15).

The PPD is a trichloroacetic acid precipitate of old tuberculin (OT) which is prepared by heating in the Arnold sterilizer the entire tubercle bacillus culture, filtering off the dead bacilli through the Seitz filter, adding glycerine and then concentrating the filtrate on the steam bath to one-fifth of the original volume. It seemed advisable, therefore, to study first the original OT, the liquid from which the PPD is isolated. Since large quantities of salts and glycerine were present the solution was thoroughly washed on the ultrafilter before testing in the ultracentrifuge. Much polydispersity was evident, but one main component was found with a mean $s_{20} = 1.37$ (see Table VII). It was apparent that large quantities of very small molecules were also present.

On analysis this washed OT was found to contain 72 per cent
| Substance | Per cent concentration used | Centrifuge and diffusion data | Molecular weights | Per cent nucleic acid | Per cent concentration of solution | Ionic strength of solution | Potential gradient | Electrophoretic data |
|-----------|-----------------------------|------------------------------|-------------------|----------------------|-----------------------------------|---------------------------|-------------------|---------------------|
|           | NaCl | NaHPO₄ | Na₂HPO₄ | ε₉₀ | Dₑ₀ |                           |                           |                     |                     |                     |                     |
| OT        | 1.0  | 0.2   | 0.03   | 0.02 | 1.4  |                           |                           | 72.1               | 10.0                | 3.8                 | 8                   | 0.2                | 5.6                | 15.9 |
| PPD       | 1.0  | 0.2   | 0.03   | 0.02 | 1.1  |                           |                           | 24.0               | 21.8                | 1.0                 | 8                   | 0.02               | 7.8                | (49.2) Two boundaries |
| PPD-a     |      |       |        |      |      |                           |                           | 12.8               | 13.0                | 2.35                | 8                   | 0.02               | 9.1                | (29.3) Two boundaries |
| PPD-30d   | 1.0  | 0.033 | 0.002  | 0.002| 1.5* | 10.2* | 0.739 | 19,000              | 4.9                 | 3.0                 |                     |                     |                     |                     |
|           | 1.0  | 0.033 | 0.002  | 0.002| 1.8  | 9.0               |                           |                     |                     |                     |                     |                     |                     |
| PPD-b2    | 1.0  | 0.2   | 0.033  | 0.002| 1.1  |                           |                           |                     |                     |                     |                     |                     |                     |
|           | 0.95 | 0.2   | 0.033  | 0.002| 1.5* | 2.1†             | 5.7               | 0.65                | 8                   | 0.1                 | 8.2                | 10.1 |
|           | 0.48 | 0.2   | 0.033  | 0.002| 2.8  |                           |                           |                     |                     |                     |                     |                     |                     |
| PPD-b3    | 0.96 | 0.0066| 0.0004 | 0.004| 0.9* | 9.3*              | 4.4               | 3.0                 | 1.0                 | 8                   | 0.1                | 13.8               | 5.6 |
|           | 0.69 | 0.033 | 0.002  | 0.002| 1.1* | 9.4*              | 0.5               | 8                   | 0.03                | 8.3                | 10.7 |
|           | 1.00 | 0.2   | 0.03   | 0.02 | 1.3  |                           |                           |                     |                     |                     |                     |                     |                     |
|           | 1.00 | 0.2   | 0.033  | 0.002| 1.2  | 6.7               | 16,000              |                     |                     |                     |                     |                     |                     |
|           | 0.20 | 0.2   | 0.033  | 0.002| 6.2  |                           |                           |                     |                     |                     |                     |                     |                     |
|                | 1.0 | 0.2 | 0.033 | 0.002 | 1.0 | 10.0 | 9000 | 1.2 | 1.6 | 2.0 | 0 | 1.23 | 8 | 0.05 | 10.6 | 5.8 | 100.0 | 100.0 | 1.00 | 8 | 0.1 | 16.8 | 14.0 |
|----------------|-----|-----|-------|-------|-----|-----|------|-----|-----|-----|---|------|---|------|------|-----|-------|-------|-----|-----|------|-----|-----|
| PPD-b3b        |     |     |       |       |     |     |      |     |     |     |   |      |   |      |      |     |       |       |     |     |      |     |     |
| TPA-30K        |     |     |       |       |     |     |      |     |     |     |   |      |   |      |      |     |       |       |     |     |      |     |     |
| Tubercle bacillus nucleic acid |     |     |       |       |     |     |      |     |     |     |   |      |   |      |      |     |       |       |     |     |      |     |     |

* Molecular weights were not calculated from these values because of a possible charge effect.
† Molecular weight was not calculated from these values since its significance would be doubtful on account of inhomogeneity and sedimentation and diffusion anomalies.
polysaccharide and 10 per cent nucleic acid. An attempt was made to separate it into its components by electrophoresis. As can be seen in Table VII, even in the presence of as high a concentration of buffer as $\mu = 0.2$, a boundary migrated to the positive pole with the great velocity of $15.9 \times 10^{-5}$ cm$^2$ sec.$^{-1}$ volt$^{-1}$. Because of the concentration and therefore almost black color of the solution it was impossible to determine whether there was more than one additional very heavy and comparatively immobile band.

The analyses of the solution in the various compartments at the end of electrophoresis are given in Table VIII. From these data it is evident that the fast moving component in the top positive compartment was practically all nucleic acid.

| Substance                          | Per cent total concentration | Per cent of total as nucleic acid | Per cent of total as polysaccharide minus nucleic acid |
|-----------------------------------|-----------------------------|----------------------------------|------------------------------------------------------|
| Original OT solution ................| 3.8                         | 10.0                             | 72.1                                                 |
| Top positive compartment ..........  | 0.07                        | 100.0                            | 0.0                                                  |
| Lower positive compartment ........ | 0.25                        | 76.5                             | 23.5                                                 |
| Bottom U compartment ................| 2.67                        | 8.3                              | 68.6                                                 |
| Lower negative compartment ........| 2.17                        | 11.5                             | 67.7                                                 |
| Top negative compartment .......... | 1.92                        | 10.2                             | 89.8                                                 |

PPD was then studied and the sedimentation graph showed it to consist of one main component with $s_{20} = 1.07$, but the curve was asymmetrical. In electrophoresis two components moving with the extremely high velocity of 49.2 and 36.2 cm$^2$ sec.$^{-1}$ volt$^{-1}$ (see Table VII), and one immobile boundary were seen. Considering the foregoing experiment and the apparent concentration of nucleic acid in the PPD, this high mobility may be due to its presence.

Since in previous ultraviolet absorption studies (22) of tuberculin, it had been found that treatment of the PPD with ammonium sulfate removed the substance having the characteristic thymine absorption band, a preparation treated in this way, PPDa, was studied in electrophoresis. The content of nucleic acid was reduced to nearly half
and the mobilities of the boundaries in electrophoresis were correspondingly reduced. These facts, together with the data showing that in general (Table VII) when the content of nucleic acid was low, the mobility was also low, would seem to be good evidence that nucleic acid was responsible for the high mobilities observed. A sample of human type tubercle bacillus nucleic acid was found to have a mobility of $14 \times 10^{-6}$ cm$^2$ sec$^{-1}$ volt$^{-1}$ in buffer of one-tenth ionic strength.

Attempts were then made to purify the PPD further by means of ammonium sulfate fractionation. A fraction taken off at one-tenth saturation at pH 4.8, instead of being homogeneous in the centrifuge as was the case with the large molecule, TPA-30b, was found to have a high degree of polydispersity. Finally a fraction, PPD-50d, obtained between 0.2 and 0.5 saturation, proved to be no more homogeneous in the centrifuge than the original PPD, but since the analyses showed that most of the nucleic acid had been removed, it was studied further (see Table VII). It appeared to be nearly homogeneous in diffusion and, therefore, had a probable average molecular weight of about 19,000. Fractionation of the PPD by electrophoresis has not so far proved to be successful in yielding a homogeneous fraction, probably because of the presence of the nucleic acid.

Finally the most promising separation or purification of PPD was made by fractionally precipitating with hydrochloric acid, by use of the hydrogen electrode, as follows. That fraction, precipitating at pH 4.0 and designated as PPD-b2 in Table VII, was removed, precipitated by half-saturation with ammonium sulfate, and then precipitated during electrodialysis. The filtrate from PPD-b2 gave no further precipitate with hydrochloric acid even at pH 3.0, and was therefore precipitated with trichloroacetic acid. It was called PPD-b3 and precipitated when electrodialyzed. During this electrodialysis a fraction, PPD-b3b, passed through the parchment membrane to the cathode. It was recovered by precipitation with trichloroacetic acid.

These three fractions were then studied extensively, as shown in Table VII, since the analyses indicated that a reasonably good purifi-

7 Prepared by and given to us by Dr. Robert Coghill.
cation had been accomplished. The sedimentation constants of all of them were very low even in the presence of a sufficient concentration of buffer to suppress all Donnan effect. The sedimentation graphs and results calculated from the diffusion curves indicated considerable homogeneity, in the case of PPD-b3 and PPD-b3b, but not for PPD-b2.

The very low diffusion constant of $D_{20} = 2.1$ for PPD-b2, accompanying such a low sedimentation constant, is noteworthy and strong indication that the molecule behaves abnormally, like thread-like or hydrated molecules. This fraction was difficult to put into solution in buffer at pH 8.0, swelling at first and then showing indication of gelling. However, it showed but one boundary in electrophoresis.

Change in concentration of PPD-b3 altered its diffusion constant very little, whereas change in buffer concentration caused a great difference. At the very low molar concentration of 0.007, a marked charge effect was evident from the fact that the curves tilted to one side (see Lamm, 2). Undoubtedly, of the data obtained, the calculated weight of 16,000 is probably the most nearly correct. This molecule also showed only one component in electrophoresis and an attempt was made to determine its isoelectric point. (See Text-fig. 2, dashed line.) It was not possible to reach the isoelectric point, if there was one, because a precipitate occurred when the solution became more acid than pH 4.4. However, the character of the curve indicates that this molecule bound considerably more base than did the larger molecule, TPA-30K, and that the difference between the two became more rapid around a pH of about 5. The significance of this will be discussed in a future publication, but at present the result may be taken as evidence of the presence of more polar groups in this small molecule than in the larger molecule and fits well with the observation repeatedly made that the conductivity of a concentrated solution of PPD-b3 was greater than that of the buffer in which it was contained.

The PPD-b3b was apparently an homogeneous molecule with a molecular weight of 9000 and the smallest one so far isolated from tuberculin. It is not only an interesting molecule because of its...
homogeneity and small size but also because it has considerably less potency than the other molecules separated from PPD and therefore represents the first stage of breakdown in the molecule associated with loss in potency. This effect is shown in Table VI, referred to earlier. It is also seen in Table IX in another series of experiments, where comparative tests with all the PPD molecules are reported as average reactions observed simultaneously on the same six tuberculous guinea pigs.

PPD-b3 was found to be a poor antigen in the precipitin reaction since 0.009 mg. was required to give a definite minimal precipitate with the same antiserum for which one-tenth this amount was required of the large molecule, TPA-21b.

**TABLE IX**

Potency of Purified PPD Fractions

| Fraction | Average size of skin reactions on six tuberculous guinea pigs |
|----------|-------------------------------------------------------------|
|          | 24 hrs. | 48 hrs. |
| PPD      | 16 x 16 x 3.1 | 15 x 15 x 3.1 |
| PPD-b2   | 15 x 14 x 3.2 | 13 x 14 x 3.0 |
| PPD-b3   | 18 x 17 x 3.0 | 17 x 16 x 3.5 |
| PPD-b3b  | 12 x 11 x 2.3 | 8 x 9 x 1.8 |

**DISCUSSION**

It can be seen from these results that there exist in the original human tubercle bacillus culture filtrate molecules with two different sedimentation constants, including a large amount with a constant of 1.9 and a small amount with a constant of 4.9. These may represent molecules respectively of about 15,000 to 18,000 and 70,000 molecular weight. Since an homogeneous substance was isolated with a weight of 32,000, by ammonium sulfate precipitation, it is probable that this molecule is an aggregate of two original ones formed during the isolation. It is significant that this larger molecule exhibits such high antigenic properties, as demonstrated by the striking local anaphylactic type of reaction in tuberculous guinea pigs.

It is possible that an analagous aggregation takes place with the
protein from the timothy bacillus, although in this case the aggregated molecule is only 17,000, and the molecules in the original solution have a still lower sedimentation constant ($s_{20} = 1.4$).

However, the denaturation process, which evidently occurs during this method of purification, as well as the gelling which sometimes appears during simple concentration on the ultrafilter, may be due to continued successive aggregations until particles of many sizes and even of huge dimensions may be formed (as high as $s_{20} = 46$ was observed.) A similar explanation may be made for the denaturation observed during the crystallization of the tuberculin protein. At each resolution of the crystals a considerable amount of insoluble material remained on the filter paper. Furthermore, a batch of crystals which had been recrystallized (23) ten times and which consisted of pure needles and burrs, on standing in the ammonium sulfate medium in the ice box, gradually transformed completely into clearly defined hexagonal plates as shown in the photographs. It is possible that the needles, because of the close proximity in the compact burrs, coalesced into hexagonal plates, as is suggested by Fig. 2 taken during the period of transformation. Many of these plates, while having the appearance of beautiful crystals, were insoluble in water and even in phosphate buffer at pH 8.0.

The small molecular size of about 10,000 for the original protein molecules of the bovine bacillus tuberculin may be of significance in connection with two immunological facts previously noted; namely, (a) that the bovine bacillus tuberculin protein is more potent in tuberculous guinea pigs inoculated with human tubercle bacilli than is the human bacillus tuberculin protein (24), and (b) that antiserum to the human bacillus tuberculin protein sometimes gives little or no precipitate with the bovine bacillus antigen, whereas the antiserum to bovine bacillus tuberculin protein usually reacts with the human bacillus antigen (25).

The properties of those molecules considered to be most nearly homogeneous were examined further (see Table X) with the hope of determining their shape and also their degree of homogeneity. Among other procedures the molar frictional ratios were calculated. The concentrations of the molecules in solution were also calculated from the sedimentation curves. Furthermore, the diffusion constants
were calculated from the sedimentation curves, using the time measured from when the centrifuge reached the speed of the experiment. Because of the slow sedimentation only one of the later exposures was used in this calculation. In view of these possible errors, the agreement between calculated and determined diffusion coefficients in all cases except those of the PPD-b2 and PPD-b3 was reasonably close and, therefore, indicated homogeneity. Note in Table X the discrepancy between the actual diffusion constant of PPD-b2 and the apparent one computed from the sedimentation diagram, which indicates heterogeneity.

**TABLE X**

*Homogeneous Molecules Isolated from Tuberculin*

| Substance          | Per cent concentration used | Per cent concentration (calculated from sedimentation diagrams) | $D_20$ (found in diffusion) | $D_20$ (apparent, calculated from sedimentation diagrams) | $s_m$ | $f/f_0$ | Molecular weight |
|--------------------|-----------------------------|---------------------------------------------------------------|-----------------------------|-----------------------------------------------------------|-------|----------|-----------------|
| Polysaccharide     | 1.0                         | 0.95                                                          | 11.0                        | 13.5                                                      | 1.6   | 1.5      | 9000            |
| TPU-Bovine (523)   | 0.5                         | 0.42                                                          | 12.0                        | 11.5                                                      | 1.6   | 1.3      | 10,000          |
| TPA-30b (human)    | 0.87                        | 8.2                                                           | 10.2                        | 13.6                                                      | 1.8   | 1.2      | 32,000          |
| TTPA-A (timothy)   | 1.0                         | 0.46                                                          | 10.0                        | 10.3                                                      | 1.0   | 1.6      | 17,000          |
| PPD-b3b            | 1.0                         | 0.81                                                          | 10.0                        | 10.3                                                      | 1.0   | 1.6      | 9000            |
| PPD-b3             | 1.0                         | 0.63                                                          | 6.7                         | 11.1                                                      | 1.2   | 1.9      | 16,000          |
| PPD-b2             | 0.95                        | 0.48                                                          | 2.1                         | 14.5                                                      | 1.5   | *        | *               |

* These values were not calculated since their significance would be doubtful on account of inhomogeneity and sedimentation and diffusion anomalies.

Moreover, the $f/f_0$ ratios were similar to those found for most proteins previously studied, except for the PPD molecules. In the case of the latter, the deviation in shape from the normal compact or unhydrated molecule was marked, especially in the case of PPD-b2. This fits well with the difficulties encountered during work with this fraction, namely, swelling, gelling, and loss in solubility. Probably a distortion of the molecule occurred during the heating in the presence of salts and glycerine and preparation of OT from which the PPD was isolated. Thus an elongation of the molecule was probably produced with exposure of extrapolar groups as is evident from the
base-binding properties of PPD-b3 (see Text-fig. 2, broken line) and a consequent aggregation of these elongated molecules, through the exposed groups to molecules like PPD-b2, as described by Astbury, Dickinson, and Bailey (26) for heated egg albumin. Dr. O. Snellman of the Physical Chemistry Institute at Upsala kindly made a determination for us on the PPD-b3 preparation by means of the method of double refraction in flow and found a marked effect, indicating very asymmetrical shape.

Since the most probable molecular weight of PPD-b3 is about 16,000, or approximately the same as found for the majority of the molecules in the original medium, and since the original medium has in past researches been shown to be antigenic, it would seem more likely that the lack of antigenicity in PPD-b3 may be due to a distortion or change in configuration of the molecule rather than or as well as to a disaggregation into smaller units. Moreover, in view of the fact that adsorption to aluminum hydroxide or charcoal renders the non-antigenic molecule antigenic (27) it is probable that some change in physical property rather than the presence of a special constituent is responsible for the antigenic properties. When the molecule is broken down even as far as 9000, as in the case of PPD-b3b, some loss in tuberculin potency occurs.

**SUMMARY**

Studies have been made by means of sedimentation in the ultracentrifuge, and by diffusion and electrophoresis, to determine the molecular weights and homogeneity of the tuberculin protein and polysaccharide molecules as found in their natural state in the unchanged filtrates from culture media after growth of tubercle bacilli. These results have been compared with data obtained on fractions isolated by chemical procedures from them or from old tuberculin.

By means of electrophoresis in the Tiselius apparatus it was possible to separate the protein from the polysaccharide, as these two fractions occur naturally in the original culture medium filtrates of acid-fast bacilli.

The protein from the bovine strain of bacillus proved to be homogeneous in sedimentation ($s_{20} = 1.6$), diffusion ($D_{20} = 12.0$) and electrophoresis, with a molecular weight of about 10,000.
The tuberculin polysaccharide isolated in electrophoresis appeared to be practically the same in sedimentation and in precipitin reaction as the polysaccharide isolated by chemical procedure. The latter proved to be homogeneous in sedimentation ($s_0 = 1.6$) and diffusion ($D_{20} = 11.0$) with a molecular weight of about 9000.

A practically homogeneous protein was isolated from the culture filtrate of the human tubercle bacillus H 37 by fractional ammonium sulfate precipitation, with a molecular weight of 32,000 ($s_0 = 3.3$; $D_{20} = 8.2$). It was electrochemically homogeneous, with an isoelectric point at pH 4.3 and an isoionic point at pH 4.7. It could be dried or frozen with no loss in homogeneity. It was highly antigenic in the precipitin reaction and produced the anaphylactic type of local skin reaction in tuberculous guinea pigs, in contrast to the true tuberculin type of reaction caused by a purified PPD fraction. Furthermore death resulted in tuberculous guinea pigs from intracutaneous injection of exceptionally small amounts.

A protein with molecular weight of about 17,000 was isolated from the filtrate from cultures of the timothy bacillus.

The nucleic acid originally occurring in old tuberculin (OT) seems to be responsible for the high electrochemical mobility observed. From OT and the PPD made from it, potent but non-antigenic molecules of 16,000 and 9000 weight and with a low content of nucleic acid were isolated. With increase in size these deviated much from the normal compact spherical shape, and aggregation was evident from the tendency toward gel formation. The smallest molecule (9000) was homogeneous ($s_0 = 1.0$; $D_{20} = 10.0$) and had lost some tuberculin potency.

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**EXPLANATION OF PLATE 13**

Photographs of tuberculin protein crystals.

Fig. 1. Tuberculin protein crystals when freshly made (needles).

Fig. 2. Intermediate form (spherulite) of tuberculin protein crystals after storage in ice box.

Fig. 3. Hexagonal plates of tuberculin protein crystals after storage for a year.
(Seibert et al.: Tuberculin protein and polysaccharide molecules)