THE PROTEINS IN UNHEATED CULTURE FILTRATES OF HUMAN TUBERCLE BACILLI

I. Fractionation and Determination of Physical-Chemical Properties*‡

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INTRODUCTION

Different preparations of P.P.D. (purified protein derivative) (1) and of O.T. (old tuberculin) (2) tuberculins are known to differ in their skin activity. Since the latter property is associated wholly with the protein component of tuberculins (3), a partial explanation of this phenomenon is that the protein contents of lots of O.T. may be significantly different, but this can hardly account for the variation found in preparations of P.P.D. (3). The presence of two or more proteins with unequal skin activities is one possible explanation, as they might be found in varying amounts in different preparations; or, as has been suggested, degradation of the protein during preparation might account for the observed facts (3).

In an attempt to get a complete picture of the proteins in tuberculin, we have fractionated with ammonium sulfate unheated culture filtrates of both a virulent strain of human tubercle bacillus and a strain of low virulence, and studied the protein fractions both serologically and by physical-chemical methods.

That two or more proteins exist in tubercle bacilli or in tuberculins has been indicated by other workers (4), but complete separation and proof of antigenic individuality have not been obtained. By means of electrophoresis Seibert and Nelson (5) have demonstrated in unheated culture filtrates of tubercle bacilli protein-containing materials of at least two different mobilities. Two of the fractions separated thus had some immunological specificity but cross-reactions were obtained. Furthermore, one fraction was found to be much less antigenic than the other, and it failed to sensitize the skin of rabbits. Thus it was not clear that the one was not a degraded form of the other.

* The physical-chemical work is more fully described in the thesis of one of the authors submitted to the Faculty of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree in June, 1944.

This phase of the investigation was carried out in the laboratories of Dr. J. W. Williams, and we wish to express our appreciation of his help.

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We have found, principally from the results of sedimentation velocity experiments, that there are two distinct proteins with differing sedimentation constants in tuberculin, one of which is the same as that obtained previously by Seibert *et al.* (6) by ammonium sulfate fractionation. Our data indicate a slightly higher molecular weight for this protein than the 32,000 determined by Seibert *et al.* The serological work has confirmed the individuality of the two proteins of different sedimentation constant and has, in addition, established the presence of a third, serologically distinguishable factor not detected in the physical-chemical experiments. Additional tests, including active skin sensitization in guinea pigs and passive skin sensitization of normal guinea pigs with serum from the animals actively sensitized, have further demonstrated the biological specificity of the two proteins and shown that both have similar degrees of antigenicity.

The present paper describes the fractionation of the unheated culture filtrates and the physical and chemical investigation of the products. The serological studies and a description of the skin activities of the fractions will be presented in the second paper of this series (7).

EXPERIMENTAL

The cultures were grown on Henley's synthetic medium (8), the virulent strain (DT)[1] for 21 weeks and the slightly virulent one (TB–1)[2] for 9 weeks. Thereafter the cultures of the two strains were treated in exactly the same way. Cellular material was removed by filtration through Mandler filters. The filtrates were immediately concentrated and washed by ultrafiltration (1) with phosphate buffer (0.005 M, pH 7.4 to 7.5) at 5°C. Throughout the process merthiolate was maintained at a concentration of 1/10,000 to prevent bacteria from growing, and both the ultrafiltration and the subsequent fractionation were carried out at 0–5°C. to avoid denaturing the protein and also to prevent contamination. To ascertain whether any significant constituents were being lost by ultrafiltration, parts of the ultrafiltrates were in turn concentrated and washed in the alundum cups impregnated with heavier cellulose acetate films. All preparations were lyophilized immediately after they had been washed by ultrafiltration.

The fractionation scheme is given in Fig. 1 and the amount obtained and chemical composition of each fraction are in Tables I and II. Corresponding fractions of the two strains have the same symbol; "av" is affixed to designate those from the slightly virulent tubercle bacillus, and "v" to indicate those from the virulent strain.

All precipitations were carried out according to the following scheme. A solution containing approximately 1 per cent protein was dialyzed in several changes of the precipitating solution, usually an ammonium sulfate solution 0.25, 0.5, or 0.75 saturated at 5°C., either at the normal pH or neutralized with sodium hydroxide to pH 7.2 as indicated in Fig. 1. After about 24 hours the precipitate in the dialysis bag was centrifuged. The supernatant liquid

[1] The culture DT was obtained through the courtesy of Dr. Florence B. Seibert. Its virulence was checked in guinea pigs just before the culture filtrates were prepared from it.

[2] TB–1 is the number of the stock culture of the Department of Agricultural Bacteriology of The University of Wisconsin.

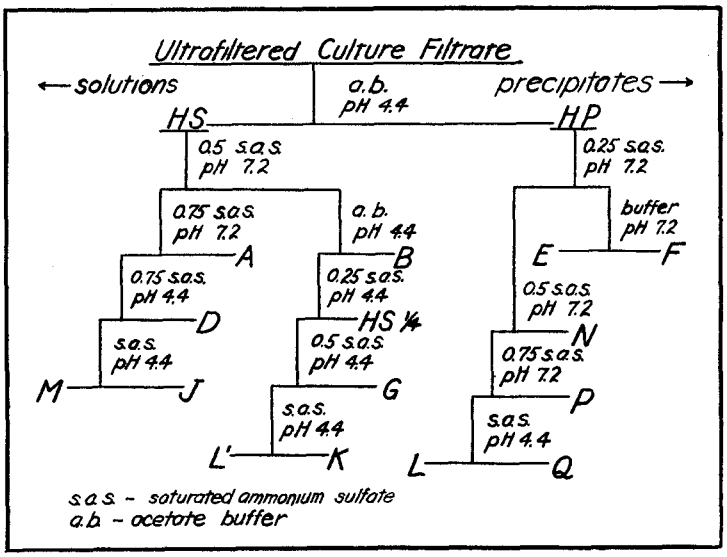

FIG. 1. Fractionation scheme for unheated culture filtrates.

TABLE I

Composition and Yield of Tuberculin Fractions from Strain DT

Fraction	Protein	Polysaccharide	Nucleic acid*	Total yield of protein‡
	per cent§	*per cent*	*per cent*	*mg.*
HS	32	68		3,380
HP	69	11	20	775
B	86	14	0	16
HS¼	94	6	0	119
G	87	12	0.5	115
K	69	28	3	20
L'	10	89	1	3
A	71	29		332
A reprecipitated	91	8	1	99
D	72	27	1	202
J	27	55	18	106
M	5.5	94	0.5	95
E	87	5	8	112
N	94	2	4	154
P	78	8	14	180
Q	23	25	52	22
L	17	1	82	12

* Nucleic acid values of less than 15 per cent are not accurate due to interference of protein and polysaccharide.

‡ The original volume of the culture medium was 12 liters.

§ Per cent of the sum of the three substance for which analyses were made.

was poured off and dialyzed with the next precipitant. The precipitate was washed at least once with the precipitating solution and recentrifuged. It was then dissolved in 0.02 M phosphate buffer of pH 7.0 to 7.2, and dialyzed in frequent changes of this buffer until all ammonium sulfate was removed. Determinations of polysaccharide, nitrogen, and nucleic acid were made on this solution.

Following Seibert (9), the nucleic acid contents of the fractions were estimated by the diphenylamine reaction of Dische, and the polysaccharide contents by the carbazole reaction. We are indebted to Dr. Florence B. Seibert for a sample of Hammarsten's pure sodium thymonucleate and also for a sample of purified tuberculin polysaccharide, which were used as

TABLE II

Composition and Yield of Tuberculin Fractions from Strain TB-I_{av}

Fraction	Protein	Polysaccharide	Nucleic acid	Total yield of protein*
	per cent	*per cent*	*per cent*	*mg.*
HS	29	71		1,034
HP	92	5	3	232
B	86	14	0	53
HS¼	94	6	0	65
G	84	16	0	43
K	69	30	1	6
L'‡	85	15		1.5
A	75	24	0.5	232
A reprecipitated	88	12	1	57
D	73	27	0.5	43
J	41	59	0.4	47
M§	7	93	0	54
E	83	12	4	9
N	93	6	0.7	15
P	85	11	4	26
Q	85	9	6	11
L‡	54	46		1.6

* The original volume of the culture medium was 10 liters.

‡ Total amount too small to determine nucleic acid.

§ Dialysis bag broke and some material was lost. Total amount, therefore, in error but percentages correct.

standards in the determinations. Two or more concentrations of each standard were run concurrently with each set of determinations to serve as a check on reagents and methods. Nevertheless, for fractions containing appreciable amounts of both nucleic acid and polysaccharide such colorimetric determinations are not accurate. The nucleic acid gives a characteristic color with carbazole (10) which is quite different from the brownish pink given by the tuberculin polysaccharide. A polysaccharide analysis using the carbazole reagent on pure nucleic acid showed that at the wave length of light used in the analysis, the nucleic acid absorbed twice as strongly as did the polysaccharide. The polysaccharide analyses given in Tables I and II are, therefore, corrected for nucleic acid by subtracting twice the nucleic acid value from the polysaccharide value. The nucleic acid values themselves are questionable, however, on samples containing small amounts of nucleic acid and large amounts of either protein or polysaccharide. It was found that if the nucleic acid nitrogen were less than 15

per cent of the total nitrogen, the color developed by the reagent was greenish blue instead of violet, and the relationship between the amount of sample and the logarithm of the fraction of light absorbed was no longer linear. The ratio of the amount of light absorbed using a 660 mμ filter to that with a 620 mμ filter for the fractions low in nucleic acid was compared with the same ratio for pure nucleic acid, and incorrect ratios were obtained for all containing less than 15 per cent nucleic acid. Protein contents were estimated from nitrogen determinations (11) by using the value 16.3 for the percentage of nitrogen in tuberculin proteins (6), and by correcting for nucleic acid nitrogen.

The fractionation of the concentrated and washed culture filtrates was greatly complicated by the presence of polysaccharide, often amounting to 70 per cent of the total material in solution, and of nucleic acid in the "v" preparations. The nucleic acid was almost entirely separated by adjusting the solution to pH 4.4, whereupon a heterogeneous protein-nucleate and any denatured protein precipitated. The precipitate, HP in the fractionating scheme, was further fractionated, as it contained considerable protein. Fractions E, N, and P were obtained by successive precipitations with 0.25, 0.5, and 0.75 saturated (at 5°C.) ammonium sulfate brought to pH 7.2 with NaOH, at which pH more protein than nucleic acid is precipitated by ammonium sulfate (9). A final precipitation at pH 4.4 with saturated ammonium sulfate brought down the remaining protein leaving only a few milligrams in solution. Part of the 0.25 HP fraction (F) would no longer dissolve in phosphate buffer after removal of the ammonium sulfate. Although it dissolved in a borate buffer of pH 9, it precipitated after a few days and was discarded.

Most of the protein remained in the solution (HS) after the initial precipitation at pH 4.4, together with nearly all the polysaccharide. Precipitation at pH 7.2 with 0.5 saturated ammonium sulfate brought down a protein fraction containing 35 per cent polysaccharide. To lower the polysaccharide concentration, the further precipitations were carried out at pH 4.4, near the isoelectric point of the tuberculin proteins. The assumption was that at this pH the solubility of the protein would be a minimum, while that of a neutral polysaccharide would be unchanged. The fraction was dissolved in phosphate buffer, adjusted to pH 4.4 in acetate buffer by dialysis, and precipitated with 0.25 saturated ammonium sulfate. A small amount which precipitated in the acetate buffer was collected by centrifugation as fraction B. The fractions from both strains were found to contain about 10 to 15 per cent polysaccharide. A second precipitation of the protein at a concentration of about 0.3 per cent yielded a fraction in each case containing only 6 per cent polysaccharide at a sacrifice of about one-third of the protein. Further decrease in the polysaccharide content was impractical because of the large loss of protein.

The supernatant solutions from these precipitations and, separately, that from the 0.5 saturated ammonium sulfate precipitation, were further fractionated as indicated in Fig. 1. The final solution remaining contained fraction M, almost pure polysaccharide. The A fractions, containing a relatively large amount of material in both preparations, were reprecipitated from dilute (about 0.3 per cent) solution and fractions containing 90 per cent protein were obtained.

All the fractions in which there was sufficient material were sedimented in the Svedberg oil-driven ultracentrifuge at 60,000 R.P.M. in a 0.02 M phosphate buffer, 0.2 M in sodium chloride, at a pH of 7.0. Diagrams of the scale line displacement, which is proportional to the concentration gradient, *versus* distance in the cell are given in Figs. 2 *a*, *b*, and *c* for some of the fractions together with the sedimentation constants calculated therefrom. The constants for the pure fractions are s°_{20} values, having been corrected to 20°C. and to a process taking place in pure water. This last correction, which amounts to 3 or 4 per cent, is meaningless for mixtures as it involves the partial specific volume of the solute, and so only the density correction has been made for the impure fractions and their constants are thus given as s_{20} values. Since little information is obtained from one experimental curve, several for each

individual experiment are given. These show the changes in concentration gradients in the cell with time. The two proteins found were detected by a combination of this information with that obtained from the chemical analyses, as shown in the following description of the fractions.

Fraction HS¼.—Ten sedimentation experiments were made on five different preparations of fraction HS¼, the purest protein fraction, at concentrations of 0.25 to 1.0 per cent. Curves from representative experiments are shown in Fig. 2 *a*. No change of sedimentation constant with concentration was observed. All the peaks were symmetrical and this fact, together with the low polysaccharide content, indicates that the fraction is probably a fairly homogeneous protein. The mean of the sedimentation constants for all experiments was 3.4 S with a precision of about 5 per cent.

Four diffusion experiments were made with two preparations of $HS¼_T$ at concentrations from 0.25 to 0.5 per cent, using a Lamm-Polson micro diffusion cell, and the Lamm scale method for observing the rate of diffusion (12). The low concentrations used made the experimental error in individual values of the diffusion constant rather large. The mean value of D^0_{20}, 7.3×10^{-7} cm.^{2} per second, has a precision of about 10 per cent. The experimental curves showed little or no deviation from the normal curve: thus no gross inhomogeneity with respect to diffusion constant existed in the preparation. This test for homogeneity is not sensitive to variation in diffusion constant of the order of 100 per cent (13).

The apparent partial specific volume of HS¼ was calculated from the results of very careful density measurements.[3] Three values, at concentrations of 0.276, 0.360, and 0.502 per cent, were obtained. The mean value, 0.738, had a probable error of less than 1 per cent.

By use of the values of partial specific volume ($\bar{V}$), diffusion constant (D^0_{20}), and sedimentation constant (s^0_{20}) the frictional ratio, f/f_0, was calculated:

$$f/f_0 = \frac{10^{-8}(1 - \bar{V}\rho)^{1/3}}{(D^2 s \bar{V})^{1/3}}$$

This ratio compares the frictional resistance of the molecules to sedimentation and diffusion with that of spherical molecules with the same molecular weight. From it the ratio of the minor semi-axis, b, to the major semi-axis, a, of the molecule was calculated, using Perrin's expression

$$f/f_0 = \frac{\left(1 - \frac{b^2}{a^2}\right)^{1/2}}{\left(\frac{b}{a}\right)^{2/3} \log \frac{1 + \left(1 - \frac{b^2}{a^2}\right)^{1/2}}{\frac{b}{a}}}$$

[3] Details of the determination of partial specific volume have been published (18, page 17). The method used was similar to that described by Tennent and Vilbrandt (16).

This applies exactly to a prolate ellipsoid of revolution, which is a sufficiently accurate approximation to the shape of a protein molecule in solution.

The molecular weight of HS¼, from the equation

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

together with the values of f/f_0 and a/b, is given in Table III. HS¼ appears to be a globular, hence probably undenatured protein. It is also evident, both from the values of the constants and from the method of preparation, that the HS¼ protein corresponds to a protein reported by Seibert *et al.* in 1938 (6). The values of s^o_{20} and D^o_{20} given for her preparation TPA-30b are 3.3 S and 8.2×10^{-7} respectively. These together with a value for the partial specific volume of 0.7 give a molecular weight of 32,000, compared with 44,000

TABLE III

Molecular Weights of Tuberculin Components

Material	s^o_{20} (S)	$D^o_{20} \times 10^7$ cm.²/sec.	$\bar{V}$	Molecular weight	f/f_0	a/b
$HS¼_v$	3.4	7.3	0.738	44,000	1.25	5
$HS¼_{av}$*	3.4		0.738	44,000		
Polysaccharide M_v	1.7	7.6	0.619‡	18,000	1.71	13
Polysaccharide M_{av}	2.1	7.0	0.619‡	23,000	1.71	13

* There was not enough material available to carry out diffusion experiments on $HS¼_{av}$.
‡ Value of Seibert *et al.* (6).

for HS¼. The difference between the two molecular weights lies in the difference in the partial specific volume. The probable error in our value of $\bar{V}$ is less than 1 per cent and the probable error in the molecular weight of HS¼ is only about 10 per cent.

Fraction B (Fig. 2 *a*) appeared to be HS¼ which had undergone a change in solubility, precipitating at pH 4.4. Its sedimentation constant was 3.2 S, but the curves were not as regular as those for HS¼ and indicated a spread of molecular sizes or shapes. The change in solubility occurred rather rapidly in solutions of HS¼ at room temperature, much more slowly at 5°C. Presumably it came about very rapidly when the tuberculin was heated; when a culture filtrate heated for 2 hours was fractionated according to the scheme in Fig. 1, no precipitate appeared when the solution was dialyzed in 0.25 saturated ammonium sulfate.

It should be mentioned in this connection that measurements of the diffusion constant of HS¼ were carried out at 25°C. In the future it would be better to conduct diffusion experi-

ments at a lower temperature, near 0°C., to minimize the danger of denaturing the protein during an experiment. However, the time required for a diffusion experiment was much less than that for the first appearance of a precipitate at room temperature, so that the change in the measured diffusion constant due to denaturation was less than the experimental error inherent in the method.

HS Fractions Containing the 2 S Protein.—When the sedimentation curves for the other fractions were examined, it became evident that a clear cut separation of HS¼ from the rest of the protein had not been achieved in all these fractions. In most of the predominantly protein fractions derived from the acetate-soluble portion from the initial precipitation (A, D, and G, Fig. 2 *b*) the peaks were unsymmetrical and gave evidence of one component with a sedimentation constant of about 3.4 S, obviously HS¼, and another with a constant of about 2 S. In no case were the peaks sufficiently resolved so that accurate constants for both components could be calculated from one set of curves, but in several cases, fractions A_v and A_{av} (Fig. 2 *b*), for example, the faster protein predominated in the "v" preparation, determining the value of the constant, and the slower in the "av". Also in two different preparations of G_v, the faster component was in excess in one sample and the slower in the other, making it possible to calculate constants of 3.4 and 2.2 S respectively (Fig. 2 *b*). The latter value was also found in G_{av}. This sample gave remarkably symmetrical peaks on sedimentation, although it contained 16 per cent polysaccharide. This, together with the fact that all fractions in which the slower protein predominated, no matter what the polysaccharide content, showed sedimentation constants in the neighborhood of 2 S, leads us to believe that the sedimentation constant of the slower protein is very near that of the polysaccharide.

Fraction K_v (Fig. 2 *b*) and fractions J_v and J_{av} are important in that they are the only 2 S fractions which the serological work showed to contain no HS¼. Sedimentation experiments on J_v or J_{av} did not seem to be warranted because of the high polysaccharide contents of these fractions. No physical-chemical or serological work was done with the K_{av} preparation as there was so little material available. While K_v contained almost 30 per cent polysaccharide in addition to a small amount of nucleic acid, it gives the best indication of the sedimentation constant of the 2 S protein. The fact that the curves are slightly asymmetric, with spreading on the faster side, may indicate that the polysaccharide sediments slightly faster than the protein, but this spread may also be due to the presence of nucleic acid. The constant of 1.9 is due principally to the 2 S protein, and is probably not far from the value that would be found for pure 2 S protein containing no polysaccharide.

Polysaccharide.—Fraction M, the polysaccharide, was the only fraction, other than HS¼, that could be called a pure substance. It contained some nitrogen, amounting to about 6 per cent protein, if one assumes all the nitrogen to be in a protein impurity, but its sedimentation curves were as regular as

those of HS¼, yielding a value for s^o_{20} of 1.7 S for M_v and 2.1 S for M_{av} (Fig. 2 *c*). It is not known at present whether the difference in the two values is real or merely indicates a need for more experimental data. Since the polysaccharide appeared to be homogeneous in its sedimentation behavior, and the amount of protein impurity was small, diffusion experiments were carried out, which, together with the partial specific volume of 0.619 determined by Seibert *et al.* (6), gave sufficient data to calculate the molecular weight and shape. These data are given in Table III. Because of the relatively low diffusion constant the molecular weight of the polysaccharide is greater than most values reported previously for tuberculin polysaccharides (14, 15).

HP Fractions.—A close similarity had been found in the sedimentation curves of corresponding fractions of the "v" and the "av" preparations obtained from the acetate-soluble fraction, HS, of the initial precipitation. The chief difference lay in the larger proportion of HS¼ found in the various fractions from HS_v. A correspondence was not found, however, among the fractions derived from the two precipitates, HP_v and HP_{av}. Fraction E_v exhibited fairly symmetrical curves with a constant of about 4.6 S, while E_{av} had scarcely any peak (Fig. 2 *c*). The curves for the latter spread badly, and the constant calculated from the maxima of the successive broad peaks was between 2 and 3 S. Fractions N_v and P_v, predominantly protein, but containing 5 and 15 per cent nucleic acid respectively, both had constants of 5.1 S, and produced curves which approached those typical of nucleic acids (16), having a long slope on the left-hand side (towards the top of the cell) and a sharp drop on the right-hand side (Fig. 2 *c*). Fraction Q_v, 50 per cent nucleic acid, showed this behavior also, but here it was much more pronounced (Fig. 2 *c*). The material responsible for the constant of 5 S could not be detected in E_{av}, N_{av}, or P_{av}. Since the serological work showed the HP fractions to contain only those proteins found in the HS fractions, none of which had constants larger than 3.5 S, the presence of this material seemed to be correlated with the relatively larger amount of nucleic acid in HP_v, 20 per cent, contrasted with the 3 per cent present in HP_{av}. This in turn was possibly connected with the length of autolysis of the two cultures, 21 weeks for the "v" and 9 weeks for the "av". It should be mentioned that the material responsible for the constant of 5 S was not an artifact arising from the method of fractionation, as peaks with this constant were observed in H_v, the concentrated culture filtrate itself, and in HP_v, the fraction that precipitated from acetic acid buffer at pH 4.4 (Fig. 2 *a*).

In the sedimentation of fractions E_v and N_{av} (Fig. 2 *c*) a second, very fast peak could be seen. In E_v, at a concentration of 1.2 per cent, its constant was about 15 S, while at a concentration of 0.3 per cent its value was about 20 S. In N_{av} the peak was extremely well defined, and had a constant of 19.3 S. Whether the material responsible for these peaks was native to the tuberculin or was formed during the course of fractionation cannot be said.

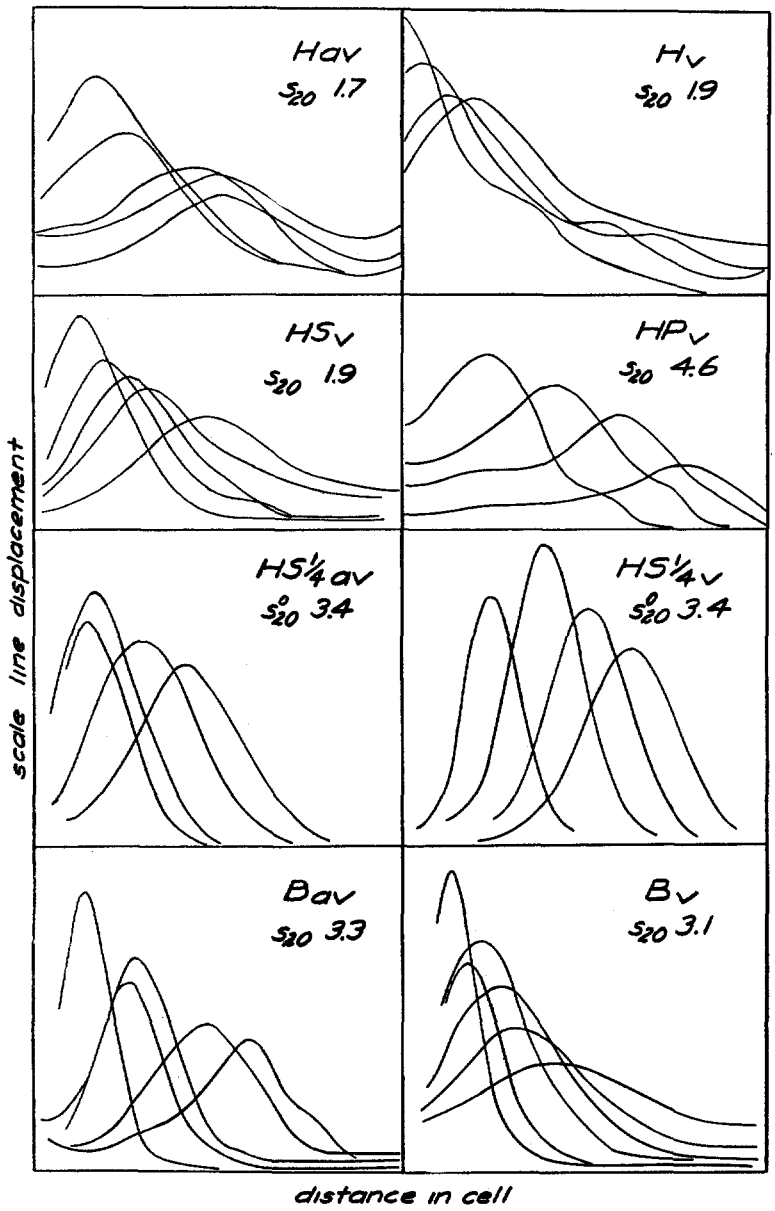

FIG. 2 *a*. Sedimentation curves of preparations from unheated culture filtrates.

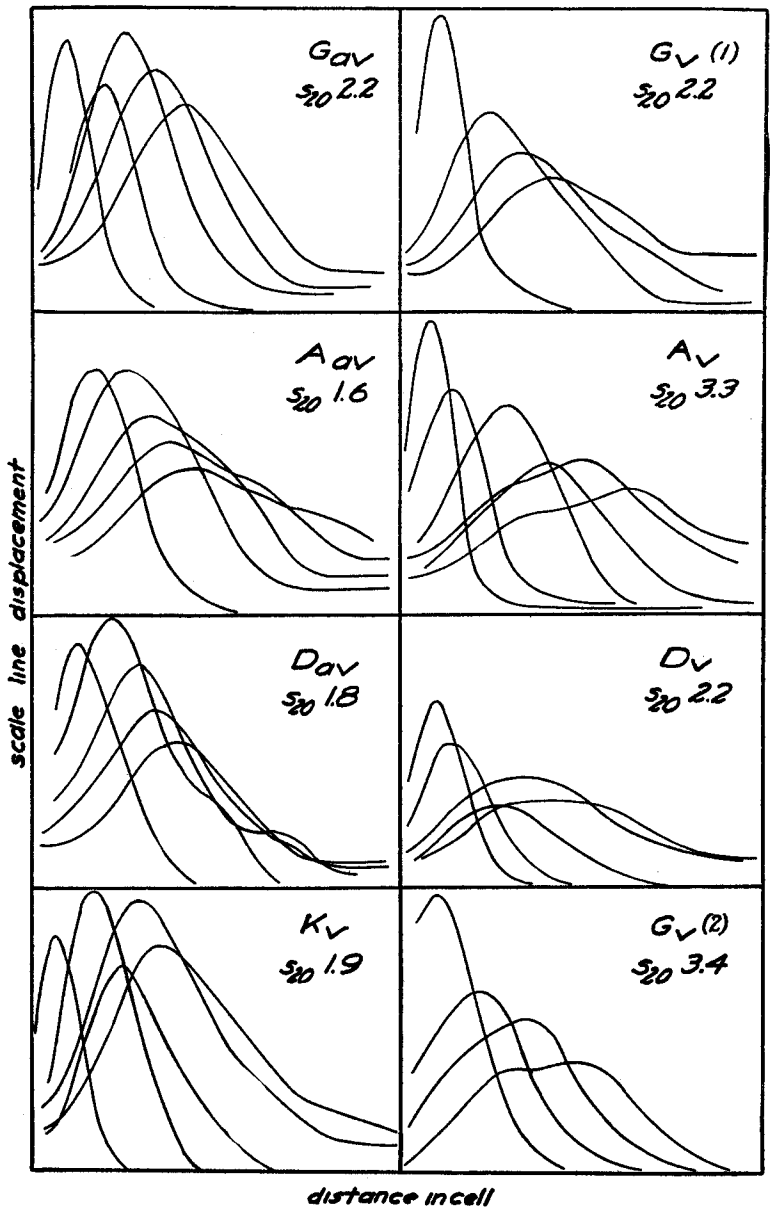

FIG. 2 *b*. Sedimentation curves of fractions from unheated culture filtrates.

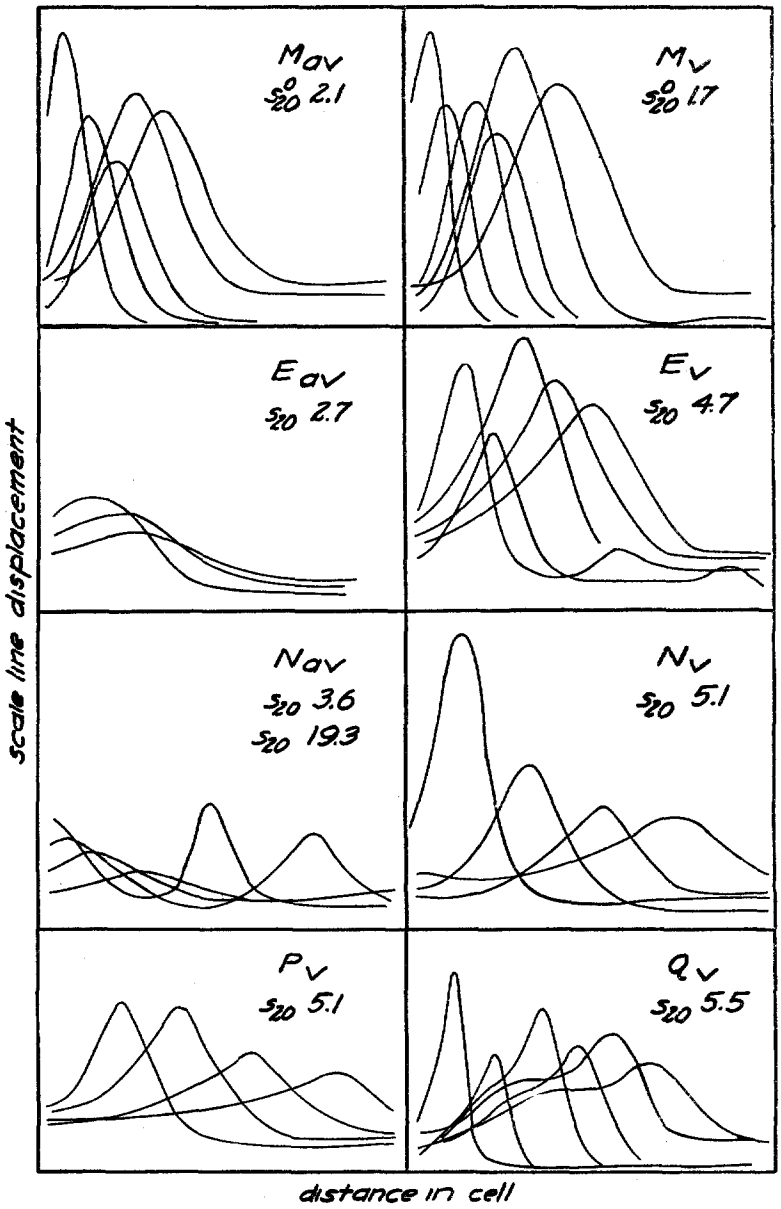

FIG. 2 *c*. Sedimentation curves of fractions from unheated culture filtrates.

DISCUSSION

We have obtained in the fractionation and sedimentation of unheated tubercle bacillus culture filtrates reliable evidence of only two proteins. The first, HS¼, can be described by its physical constants, since it was obtained in a relatively pure form. The second protein, present in almost all the other fractions from HS, was obtained free from HS¼ but in all cases contaminated with polysaccharide. It is probable that its sedimentation constant is close to 2 S. The small amount of material found in fractions E_v and N_{av} with a sedimentation constant near 20 S is suspected of being an artifact. The substance giving the peaks with a constant near 5 S in the fractions from HP_v appears from the immunological work to be a mixture of the proteins found in the HS fractions, so that it is probably not a distinct molecular species.

The nature of the peak with sedimentation constant varying from about 4.6 S in H_v, HP_v, and E_v, to 5.5 S in Q_v is not known. It appears to depend on the presence of nucleic acid and does not occur in the HP_{av} fractions. A peak with a constant of 4.9 S was observed by Seibert *et al.* (6) in sedimentation experiments with fractions of unheated culture filtrate which had been obtained by electrophoresis so that a mixture of protein and nucleic acid, nearly free of polysaccharide, was obtained. They observed no such peak in the sedimentation of the whole culture filtrate, but this may have been due to the fact that the nucleic acid content of the whole culture filtrate was quite low, 0.5 per cent, as compared to about 5 per cent in our H_v, in which the peak, though small, was observable. There seems to be an increase in sedimentation constant with increasing nucleic acid content in going from E_v to Q_v, and there is a marked change in shape of the sedimentation curve, which approaches, in Q_v, the characteristic curves for nucleic acids. Much light might be thrown on this question if nearly pure nucleic acid, say fraction L, were mixed with different proportions of the protein fractions A and HS¼, and the behavior of the mixtures in sedimentation studied. Lack of sufficient material, among other things, prevents the carrying out of this experiment. Until further work is done with known mixtures of undegraded nucleic acid and proteins, we can observe only that a relatively small amount of nucleic acid seems to have a very large effect on the sedimentation constant of the protein with which it is sedimented.

As to the two proteins for which there is both physical-chemical and serological evidence, one might raise the question, Is one a degradation product of the other? Previous work (3) on the sedimentation and diffusion behavior of proteins isolated from culture filtrates which had been heated 2 and 3 hours and also from an O.T. preparation indicated that the sedimentation constants of the proteins became smaller as the time of heating of the filtrates from which they were obtained increased. Sedimentation experiments on an O.T. pre-

pared from a portion of the H_T culture filtrate, and precipitated four times with 0.5 saturated ammonium sulfate, have shown no indication of material with sedimentation constant higher than 1 S. Indeed no constant can be calculated as the maximum speed of the centrifuge, 60,000 R.P.M., does not supply sufficient driving force to sediment the material at a measurable rate. In none of the heated preparations have sedimentation constants as high as 2.0 or 3.4 S been found. Yet it does not seem possible, in view of the solubility characteristics of HS¼, that it is the precursor of the 2 S protein, the protein in the O.T., or of the proteins studied by McCarter and Watson (3). Our experience with the tendency of HS¼ to precipitate out at room temperature leads us to believe that it would be more likely to precipitate than to break up into smaller molecules when subjected to heat. The fact that no precipitate has been obtained with 0.25 saturated ammonium sulfate in an attempt to fractionate a 2-hour-heated portion of the H_T culture filtrate according to the scheme in Fig. 1 seems to bear this out, although in the serological work, some HS¼ has been found in the 2-hour-heated preparation. The final argument against this relationship is the fact that serologically the HS¼ and 2 S proteins are completely distinct. There is no overlapping in their activities as precipitants as is often the case with native and denatured proteins. It seems probable therefore, that the proteins studied by McCarter and Watson and those present in the O.T. made from H_T culture filtrates are derived by the action of heat on the 2 S protein. It is unfortunate that neither time nor sufficient material has permitted experiments on the effect of heat on fractions such as D, A, or G.

In a recent article Seibert (17) described certain tuberculin proteins referred to as A and B proteins, A being the larger molecule of the two, and B the smaller. It would be convenient to correlate these with the HS¼ and the 2 S proteins, respectively, described here, but this cannot yet be done. The A protein can be identified with HS¼ only if by A is meant TPA-30b described in 1938 (6). The B protein *may* be the same as the 2 S protein although the two definitely differ in certain biological properties; two sedimentation velocity experiments carried out by one of the authors (18) on a sample of this protein sent by Dr. Seibert, showed fairly good peaks, giving a sedimentation constant, s°_{20}, of about 1.8 to 2.1 S, which agrees with what we believe the sedimentation constant of the 2 S protein to be, but until serological experiments prove the two to be the same, or we can obtain some 2 S protein uncontaminated with polysaccharide, no definite statement that they are identical can be made. The reason we prefer to identify HS¼ with TPA-30b rather than with the more recent A protein of Seibert is the following: TPA-30b and HS¼ were isolated in much the same manner, they were both essentially pure protein material, and their sedimentation and diffusion behavior, determined under similar conditions, was the same within the experi-

mental error of the methods. The A protein, on the other hand, is known principally by its electrophoretic behavior, and no data have been published concerning its purity or its sedimentation and diffusion constants.

The third factor discovered in the serological work and called there the "third" antigen, does not appear in the physical-chemical investigations. It is an extremely strong antigen, so that although it is present in all the protein fractions from the virulent strain, except HS¼ and J, it is possibly present in amounts too small to be detected in the sedimentation experiments. This antigen is not present in any fraction from the less virulent strain.

Ammonium sulfate as a fractionating agent leaves much to be desired in the case of tuberculin. If the two proteins could be freed of polysaccharide and yet left unaltered, their separation might then be effected by this reagent. Electrophoresis can do this, but at the expense of time and material.

SUMMARY

Concentrated culture filtrates of two strains of human tubercle bacilli, a virulent and a slightly virulent one, have been fractionated to give fourteen fractions in each case. Chemical determinations and sedimentation velocity measurements have been carried out on those fractions for which significant results could be obtained. The evidence is that two distinct proteins are present, in addition to a polysaccharide and nucleic acid. The physical measurements have not demonstrated the presence of any other proteins. One of the proteins has been isolated in pure form, and found to have a molecular weight of 44,000 ± 5,000, based on measurements of partial specific volume, sedimentation velocity, and diffusion rate. This protein is believed to be the same as one previously isolated by Seibert *et al.* (6), who assigned it a molecular weight of 32,000. The other protein was not obtained sufficiently free from polysaccharide so that its molecular weight could be determined, but it is believed to have a sedimentation constant of about 2 S. Sedimentation and diffusion constants have been obtained for the polysaccharide, which appears to be a homogeneous molecular species with a molecular weight of about 20,000. The source in unheated tuberculin of the proteins obtained from heated preparations is discussed.

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