THE CHEMISTRY OF TUBERCULIN

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Tuberculin to the bacteriologist is the concentrated bacteria-free liquid culture in which the tubercle bacilli have grown. To the physician it is a product of the tubercle bacillus which gives a specific reaction in an individual who has at some time been infected with the organism. To the chemist it signifies the particular ingredient of this culture medium which is responsible for the specific reaction, and this has come to mean protein (34); hence this fraction will receive most consideration in this article.

There are, however, other constituents of tuberculin which are of considerable interest to the chemist, in spite of the fact that they have not so far proved to be of great biological significance. For example, one finds, in addition to the protein, considerable quantities of polysaccharide and nucleic acid in a culture filtrate of the tubercle bacillus grown on a synthetic medium such as Long's (18). In fact, these two colloidal constituents exist in such significant amounts and in such combination with the protein that they become the chief impurities which are most difficult to remove from the protein during its purification. For this reason considerable research has been done upon these fractions and consequently much information is available concerning their chemical properties.

I. CARBOHYDRATE OF TUBERCULIN

The carbohydrate in tuberculin is in the form of polysaccharide. It exists chiefly as molecules sufficiently large that they do not pass through thick collodion membranes; however, some do pass the finest guncotton membranes used (37). Evidence has been presented to indicate that much of the polysaccharide

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is adsorbed to the tuberculin protein molecules, which it accompanies through many precipitations and from which most of it can be released by bringing the protein repeatedly to its isoelectric point. As much as 50 per cent of the colloidal content of raw tuberculin may consist of polysaccharide.

A. Composition of tuberculin polysaccharides

Polysaccharide isolated from the culture medium has been shown on hydrolysis by Renfrew (28), Dorset and Henley (7), and Heidelberger and Menzel (12) to consist of *d*-arabinose, *d*-mannose, and *d*-galactose. Watson (48) has detected also a small amount of glucosamine.

The polysaccharide can be determined quantitatively (26) after hydrolysis, which involves preliminary digestion with 3 per cent sulfuric acid for 7 hr., by means of the Shaffer–Hartmann microcuprous method or the Hagedorn–Jensen method. The latter method, however, yields more accurate results, since it will detect pentose as well as glucose, and consequently gives 95.3 per cent reducing substances, calculated as glucose, in pure tuberculin polysaccharide, whereas only 43.9 per cent glucose is found by means of the Shaffer–Hartmann method.

A simpler method, which does not involve a long period of preliminary digestion, is the carbazole reaction (6); this has been used almost exclusively by the author in recent years (33) with an adaptation to the Evelyn colorimeter.

As will be shown later, it is extremely difficult to remove all traces of nucleic acid and protein from the polysaccharide, but it seems reasonable, in view of the data at hand, to consider the nitrogenous constituents as impurities, at least of a certain portion of the polysaccharide.

B. Physicochemical properties of the polysaccharides

When studied in electrophoresis by means of the Tiselius technic (47), the tuberculin polysaccharide from the human-type tubercle bacillus was found to possess no, or very little, mobility; consequently it could be separated easily from the protein in raw tuberculin by allowing the latter and other impurities to migrate away from it (40). In this way a small amount of a product was isolated which proved by analysis to be 90 per cent polysaccharide. This product, when studied in the ultracentrifuge, was found to have the same sedimentation constant, S_{20} about 1.8S (one Svedberg unit, $S1 = 1 \times 10^{-13}$ C.G.S. units), as a polysaccharide which had been chemically isolated from raw tuberculin and which contained only 0.09 per cent nitrogen. There was no drift in the value of the sedimentation constant with change in concentration. The diffusion constant, D_{20}, was 11.0×10^{-7}; the specific volume was 0.619 at 20°C.; and a molecular weight of 9000 was calculated, with a molar frictional ratio, f/f_0, equal to 1.5. These polysaccharides showed a remarkable degree of homogeneity, in contrast to most polysaccharides hitherto studied, especially those of other bacteria.

Later Tennent and Watson (46) isolated by means of electrophoresis polysaccharides from the culture filtrates of the human-, avian-, and bovine-type tubercle bacilli and of the leprosy bacillus and *B. phlei*, and studied their molecular-kinetic properties and serological specificities. The molecular weights of

the polysaccharides from the human-type tubercle bacillus and the *B. phlei* filtrates were found to be 7300, that from the avian-type tubercle bacillus filtrate was 7200, and that from the leprosy bacillus filtrate was 2500. The dissymmetry numbers and the ratios of major to minor axis indicated that none of the polysaccharides was greatly elongated, and that they probably have a closed rigid structure.

A simple scheme for separating the polysaccharide from the protein and nucleic acid on a large scale by means of electrophoresis has been outlined by Seibert and Watson (41). By this method two types of polysaccharide were isolated from a large quantity of heated tuberculin filtrate. One polysaccharide was colorless, contained only about 0.2 per cent nitrogen, and did not migrate in the electrical field. The other, present in much larger quantity, had a low mobility, and the nitrogenous impurity could not be removed to less than 0.85 per cent nitrogen, even by electrophoresis for 114 hr. in phosphate buffer at pH 7.3, $\mu = 0.02$, and a potential gradient of about 3.7 volts per centimeter.

These two types of polysaccharides were studied comprehensively by Watson (48), who found the immobile form of polysaccharide which contained less nitrogen to be less easily hydrolyzed and to have a lower content of reducing sugar, of nucleic acid, and of glucosamine, a higher neutral equivalent, a higher positive rotation, a lower molecular weight, and a more spherical form than the one with low mobility. He concluded that these two polysaccharides corresponded to many of the fractions with similar properties which were isolated by Heidelberger and Menzel (12) from the bodies of the tubercle bacilli.

C. Biological properties of the polysaccharides

All of these polysaccharides specifically precipitated the serum of animals immunized against the tubercle bacillus, as has been shown by Laidlaw and Dudley (15), Mueller (25), Renfrew (28), Masucci, McAlpine, and Glenn (21), Seibert, Pedersen, and Tiselius (40), and Tennent and Watson (46). The last two investigators concluded that the polysaccharides were genus-specific rather than species-specific. Heidelberger and Menzel (12) and Karjala and Heidelberger (14) had also previously been unable to demonstrate that the human or avian tubercle bacillus polysaccharides were species-specific.

Enders (9) claimed that he was able to produce uterine strip contraction as well as typical lethal anaphylactic shock with the polysaccharide isolated by Mueller (25) in guinea pigs actively sensitized with dead tubercle bacilli or passively sensitized with the serum of immunized rabbits, but since his preparation contained 0.3 per cent nitrogen it can be questioned whether the polysaccharide itself was responsible for the reactions. The polysaccharide has appeared to resemble a haptene rather than an antigen, since antibody production did not result from repeated injection of the polysaccharide or polysaccharide-containing fractions, while a high precipitin titer could be obtained when it was added to immune sera (12, 32).

The work of Sabin, Joyner, and Smithburn (29) showed that the polysaccharides of the tubercle bacillus exerted a chemotactic effect on neutrophilic

polymorphonuclear leucocytes. These cells were immediately accumulated at the local site of injection, and soon after intraperitoneal injection young polymorphonuclear leucocytes appeared in the blood stream. No general toxicity resulted, a result which has been confirmed by the author with the polysaccharides from tuberculin.

Cournand and Lester (5) stated that a polysaccharide fraction isolated from the tubercle bacillus by Heidelberger and Menzel (12), when injected intracutaneously into tuberculous human beings, elicited both a distinct early and a delayed type of reaction. Since their polysaccharide was known to contain nitrogen, which might represent contaminating tuberculin protein, they treated their preparation with trypsin and still were able to obtain the early type, whereas the delayed type of reaction similar to that obtained by means of the tuberculin protein was destroyed. This they concluded was evidence that the polysaccharide itself was responsible for causing the early type of reaction.

McCarter and Watson (22) reinvestigated this conclusion and found that the undenatured form of tuberculin protein caused reactions reaching their maximum at 6 hr., as well as the typical delayed type of tuberculin reaction, and that the tuberculin protein when digested with trypsin, even to the extent that it was no longer precipitable by trichloroacetic acid, still possessed considerable ability to cause the early type of reaction. Moreover, weak doses of unhydrolyzed protein injected into sensitive individuals, or strong doses into less sensitive individuals, gave reactions which were definite at 6 hr. and faded out in 48 hr. The loss in activity, under the effect of trypsin, of the protein contained in the polysaccharide was much slower than it was in the isolated protein. This suggested that the combination of the two affords a certain degree of protection to the protein. Moreover, it is evident from the work of Linterstrøm-Lang *et al.* (17) that native proteins are less readily attacked by proteolytic enzymes than denatured proteins.

The strongest evidence of McCarter and Watson that the protein in the polysaccharide was the cause of the early-type reaction lay in the results obtained in an experiment on mild acid hydrolysis. The concentration of acid and conditions of hydrolysis were determined which were sufficient to hydrolyze the polysaccharide completely, as followed by a determination of the per cent of reducing sugar found with increasing time of hydrolysis. It was then noted that under these conditions, even though the polysaccharide was completely hydrolyzed, its ability to cause an early type of reaction was never completely destroyed, just as was true in the case of the pure protein when similarly treated. These results make it probable that the polysaccharide *per se* is not responsible for a significant specific local skin reaction.

II. NUCLEIC ACID OF TUBERCULIN

Nucleic acid is the other colloidal constituent which is found usually accompanying the protein. Its concentration in various protein fractions may vary from zero to 30 or 40 per cent, depending upon the source and the mode of preparing the protein. For example, the preparations that are made from tubercu-

lin obtained from heated cultures usually contain more nucleic acid than those protein fractions precipitated directly from unheated culture filtrates, and there is evidence that at least a portion of the protein and nucleic acid in these heated preparations may exist as nucleoprotein, which travels as a single component in electrophoresis at all hydrogen-ion concentrations.

Much of the nucleic acid is bound in a looser combination to the protein, since at a pH of 5.0 or at a more alkaline pH the two dissociate, whereas at more acid reactions they travel as a single component (33). It was pointed out that, in view of the fact that the dissociation constants of the imino group of histidine in protein and also of the secondary phosphoric acid group in nucleic acid are both in this region, it is probable that the link between the protein and the nucleic acid may occur through these two groups. Complexes between nucleic acid and seralbumin have been described by Stenhagen and Teorell (44), and between nucleic acid and ovalbumin by Longsworth and MacInnes (20).

Recognition of this type of combination led to a practical method for removing nucleic acid from the protein during the purification of the latter. For example, in the preparation of a large quantity of purified tuberculin protein, which was known as *Purified Protein Derivative* (36) and was to be used as a standard in tuberculin testing, the final purification was made by repeated precipitation with ammonium sulfate at a pH of 7.0, and the resulting product contained only 1.2 per cent of nucleic acid.

A. Composition and quantitative determination

All of the nucleic acid found in tuberculin appears to be of the desoxyribose type and can be measured quantitatively by means of the diphenylamine reaction (40), whether it is free or in combination with protein. A modification of the original method given by Dische (6), utilizing the Evelyn colorimeter, has been outlined by Seibert (33).

B. Physicochemical properties

By analysis it was found (40) that the component in tuberculin migrating with the greatest mobility in electrophoresis was nucleic acid. When free it traveled at practically the same rate as the thymus nucleic acid isolated by Hammarsten (11). In the case of both nucleic acids the main gradient exhibited a sharp and distinct boundary when the migration proceeded into buffer, whereas there was considerable spreading when the migration was into the solution. Moreover, very small gradients with lower mobility continually separated from both nucleic acids during their migration. These were considered to be small amounts of protein which gradually separated from the nucleic acid, since analyses had indicated there was approximately 4.7 per cent of protein present in the tuberculin nucleic acid, and 1.5 per cent in the Hammarsten preparation. It was interesting that with the separation of these components from the latter preparation the mobility[2] of the nucleic acid in phosphate buffer at pH 7.3 and $\mu = 0.02$ gradually increased from -17.5 to -23.5 to -26.1×10^{-5}. This effect of the

[2] All electrophoretic mobilities are recorded in this paper in units of $cm.^2 \ volt^{-1} \ sec^{-1}$.

presence of protein in lowering the mobility of nucleic acid has been observed to be true also in the case of the tuberculin nucleic acid. The fastest component of tuberculin preparations has been found to have, with varying concentrations of protein and nucleic acid and under the same conditions of pH, ionic concentrations of buffer, and potential gradient, a wide range of mobility, but always lower than that found for the purest nucleic acid so far studied (-23.6×10^{-5} on the descending side at pH 7.3, $\mu = 0.02$, and a potential gradient of 6.5 volts per centimeter) (41). This same effect has been noted by Stenhagen and Teorell (44) in mixtures of seralbumin and thymonucleic acid.

The viscosity of the nucleic acid isolated from tuberculin is very much less than that of the thymonucleic acid of Hammarsten, indicating that its molecular weight may also be much less than the 500,000 to 1,000,000 determined by Signer, Caspersson, and Hammarsten (42) for the latter preparation.

The present war has interrupted further studies of the tuberculin nucleic acid which were planned by Professor A. Tiselius of Uppsala.

C. Biological properties of the nucleic acid

No obvious reaction is elicited in tuberculous animals by intracutaneous or intraperitoneal injection of the nucleic acid. It has not proved to be antigenic or to precipitate antisera to the tubercle bacillus. So far, therefore, no important biological function has been found for the nucleic acid. Indirectly, through its avidity for the tuberculin protein, there may be ascribed to it a rôle as modifier of the properties of the protein.

III. PROTEIN OF TUBERCULIN

When the polysaccharide and nucleic acid are removed almost completely from tuberculin by mild methods, as indicated above, there remains a very complex protein solution. It can be demonstrated that in this solution there are proteins which differ as to solubility, coagulability, diffusibility, sedimentability, electrophoretic mobility, antigenicity, and potency.

A. Types and properties of proteins present in tuberculin

It has become evident that raw tuberculin contains at least two proteins, with somewhat definite properties, which may be considered as undenatured proteins. They have been named proteins A and B.

Both of these proteins have been found to be soluble at all hydrogen-ion concentrations, and they have been distinguished so far chiefly by the fact that in phosphate buffer at pH 7.7 and $\mu = 0.1$ the A protein has a lower mobility (-3.3 to -4.4×10^{-5}) than the B (-6.3 to -6.7×10^{-5}) in electrophoresis. Much more at the present time can be said about the A protein than about the B, since the latter has only recently been recognized.

The pH–mobility curves of the A and B proteins have been studied in a solution containing a mixture of the two (see figure 1). It is significant that the chief difference found between these proteins was that the B protein contains more polar groups whose dissociation constant is in the neighborhood of pH 5.0–

6.0 and may, therefore, be imidazole groups. It is highly probable that it is one of these two soluble proteins which was found to crystallize (30).

Other properties which have been noted for the A protein are that it is coagulable by heat, does not diffuse through thick guncotton or cellophane membranes, and has a molecular weight of about 32,000 (40). For specific data see table 1. It exists in largest quantity in tuberculin preparations which have not been

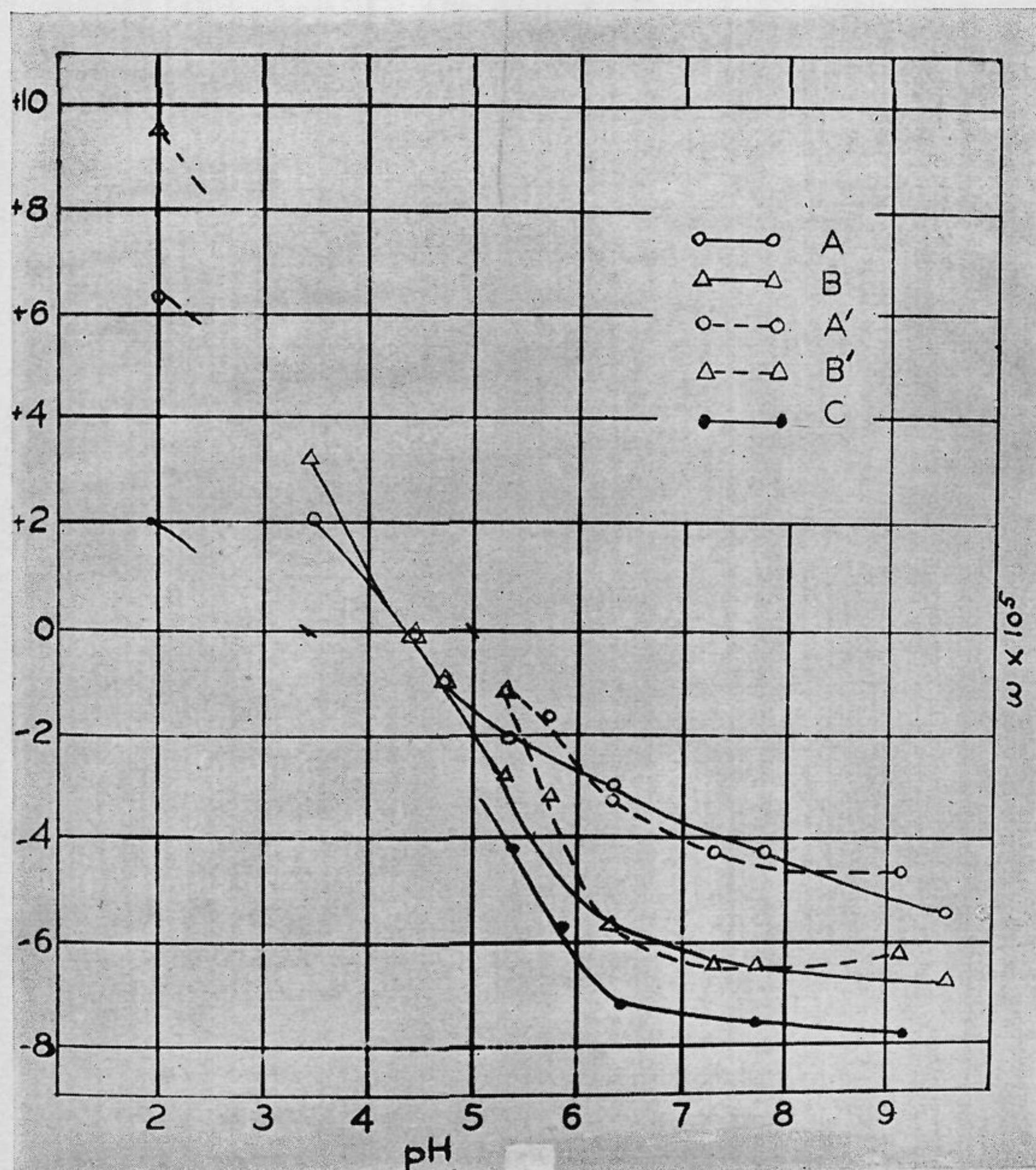

FIG. 1. pH–mobility curves. A and B were soluble proteins of TPU-84. A′ and B′ were proteins precipitated from PPD-S at pH 4.7. C was protein TPT-18E(A).

heated at all, or heated only mildly, and is considered to be a highly antigenic form of tuberculin protein. The properties of the B protein have not yet been studied, since it has only recently been recognized.

Recent experiments are showing that at pH 4 to 5 there can be precipitated, especially from heated tuberculins, fractions which show two protein components with approximately the same electrophoretic mobilities as proteins A and B.

These have been called A′ and B′[3] and may be considered as denatured forms of the A and B proteins which have lost their solubilities in the region of the isoelectric point.

Moreover, these two proteins, A′ and B′, differ from the A and B proteins in a shift to slightly more alkaline isoelectric points, as can be seen in the pH–mobility curves in figure 1. This corresponds to the findings with other denatured proteins (1, 27). With the exception of this shift in the isoelectric point, the curves do not differ greatly in character from those of the undenatured proteins. Slight differences can not be considered significant, since errors may be introduced in the determination of mobilities in mixtures where components are not well resolved, as was the case in these experiments.

These A′ and B′ proteins were obtained by precipitating the preparation PPD-S (36) at pH 4.7, redissolving the precipitate in phosphate buffer at pH

TABLE 1

Molecular properties of tuberculin proteins

PROTEIN FRACTION	PRECIPITATES AT pH	SEDIMENTATION CONSTANT S_{20} (S units)	SPECIFIC VOLUME	DIFFUSION CONSTANT $D_{20} \times 10^7$	MOLECULAR WEIGHT	MOLAR FRICTIONAL RATIO f/f_0	ELECTROPHORETIC MOBILITY* $\mu \times 10^5$
A	Soluble	3.3	0.700	8.2	32,000	1.2	3.3 to 4.4
A'	4.3 to 4.7 (?)						3.5 to 4.6
B	Soluble						6.3 to 6.7
B'	4.3 to 5.0	1.2	0.739	6.7	16,000	1.9	6.5 to 7.3
C	4.3 to 5.0	Heterogeneous		Heterogeneous			7.6 to 8.7
D	4.3 to 5.0	Heterogeneous		Heterogeneous			5.1 to 5.6

* In phosphate buffer, at pH 7.7, $\mu = 0.1$, and a potential gradient of 9 to 10 volts per centimeter.

7.7, and reprecipitating nine times at pH 4.7 with acetic acid, using the glass electrode.

Since it was only recently, mainly through these experiments, that the apparent existence of the denatured form of the A protein—namely, the A′ protein—was recognized, its further properties have not yet been determined.

The B′ protein has been recognized for some time and therefore studied to some extent. It was found not to be coagulable by heat and to have a molecular weight of about 16,000 (40); its molar frictional ratio of 1.9 indicates that it is elongated, with about an 18-to-1 ratio for the major to the minor axis. Probably this indicates uncoiling of the native molecule, which occurs in the early stages of denaturation. This fraction has been shown to be non-antigenic but potent as a tuberculin.

In addition to these native and denatured proteins a whole series of molecules

[3] The B' protein in this paper corresponds to the B protein noted in a previous paper (39).

with intermediate or higher mobilities and lowered tuberculin potency may be found, especially in heated tuberculin. They also precipitate at pH 4 to 5 and consequently make the separation of B′ protein difficult.

The data assembled indicate that some of these molecules, which have been called D proteins, may represent extensively denatured proteins which may have been formed by the polymerization of small degraded units of the proteins, resulting in chains of many different lengths and consequently many different molecular sizes. Ultracentrifuge evidence for the existence of such molecules was presented in a previous paper, dealing with fraction TPA-1a (39). The electrophoretic diagram of this fraction at pH 7.7 and $\mu = 0.1$ showed the presence of a chief component with a mobility -5.1×10^{-5}, intermediate between A and B proteins, and two very small ones with mobilities -3.7×10^{-5} and -7.6×10^{-5}. Another recently isolated fraction, Q(E), contained a single electrochemical component with a mobility of -5.6×10^{-5}, and has been reported by Mrs. Bevilacqua in Dr. J. W. Williams' laboratory to be extremely inhomogeneous in sedimentation and diffusion.

TABLE 2

Molecular properties of some tuberculin protein fractions

TUBERCULIN	SEDIMENTATION CONSTANT S_{20} (S units)	DIFFUSION CONSTANT $D_{20} \times 10^7$	MOLECULAR WEIGHT	SVEDBERG DISSYMMETRY NUMBER f/f_0	RATIO OF MAJOR TO MINOR AXIS a/b
69, avian	1.34	3.38	36,800	2.85	40.0
69-2, avian	1.20	8.21	13,600	1.64	11.8
67, human	0.62	6.71	8,500	2.33	29.6
67-2, human	0.99	14.64	6,300	1.19	4.1

Pedersen (27) noticed a similar polydisperse sedimentation but electrophoretic homogeneity of denatured serum albumin. If one accepts the idea of polymerization of degraded units, the fact of electrophoretic homogeneity can be explained on the principle established by Abramson (1),—namely, that regardless of the size, particles of similar material travel with the same velocity.

Watson (22, 48) has given impressive evidence that precipitation at pH 4 to 5 does precipitate the more elongated or denatured forms of the tuberculin protein. Table 2, containing data from his studies, shows that the fractions which were precipitated at pH 4.3 from both an avian-69 and a human-67 tubercle bacillus-type protein contained larger molecules than the soluble fractions, 69-2 and 67-2, remaining in the filtrates. The noteworthy fact, however, is that in both cases these larger molecules were also much more elongated than the soluble protein fractions, indicating more extensive unleafing of the molecule and even polymerization. This fact was true even when the molecules were considerably degraded in size by heat, as in the case of the 67 fractions listed.

In view of these facts, it must be clear that a molecule, like 69 in table 2 with a weight of about 36,800, probably due to polymerization by elongation, may have

very different properties from those of a globular molecule such as the A protein mentioned above which has a similar molecular weight, 32,000.

Still other protein molecules which are in combination with nucleic acid may be present in tuberculin solutions. These all have higher mobilities at pH 7.7 and $\mu = 0.1$ in phosphate buffer: -7 to -8×10^{-5} or more, depending on the amount of nucleic acid in combination. They have been called C proteins. It is not yet clear whether the nucleic acid combines only with the native A or B protein, or with the denatured forms A′ and B′. It is, however, highly probable that combination does occur with the B and B′ types of proteins, since they are the ones which give evidence of the presence of extra imidazole groups and it has been demonstrated (33) that the combination between nucleic acid and at least some of the protein is through this grouping.

A fraction, TPT-18E(A), containing 5.9 per cent nucleic acid, was also a single homogeneous electrochemical component (see first fraction in figure 3) but was reported by Mrs. Bevilacqua to be very inhomogeneous in sedimentation and diffusion (see C protein in table 1). Its pH–mobility curve (C in figure 1) shows that if the enhanced mobility was due to combined nucleic acid, this nucleic acid was bound in a sufficiently strong union that it did not separate above pH 5, as had been found with a protein–nucleic acid preparation previously studied (33). It may, therefore, be considered a true nucleoprotein.

B. Denaturation of the protein of tuberculin

It has been pointed out above that denatured forms of the A and B proteins have been identified. They were no longer soluble at all pH values but were precipitated at pH 4 to 5, and the mobility curves indicated a shift in the isoelectric points. Evidence also exists that with this denaturation there is an uncoiling of the molecules and even degradation. Under certain conditions, then, these denatured molecules may combine with molecules like themselves, forming long random chains and fibrous bundles in which the net charge may be the same as that of the original A′ and B′ molecules, or with each other, in which case the net charge would lie between that of the A′ and B′ molecules, as is evident in the case of the D molecules (see table 1). Mixtures of all such molecules would explain the broad electrophoretic peaks observed in solutions of heated tuberculin.

This picture of uncoiling of the protein, followed by splitting and then polymerization, fits well with modern concepts of protein denaturation in general. Bull (3) has stated, "All of the results I have reported dealing with denaturation indicate, as nearly as I can judge, that the reaction consists essentially in a rupture of certain linkages in the compact spherical native protein molecule with the production of a very asymmetric polar molecule. The degree of extension of this molecule is probably to a certain extent dependent on the pH of the solution; at some distance from the isoelectric point the presence of many groups of like electrostatic charge would tend to produce an extended structure, while in the neighborhood of the isoelectric point, the structure would probably be more compact." Astbury, Dickinson, and Bailey (2) showed by means of x-rays that the denaturation of egg albumin and seed globulins proceeded first by disappearance

of the shorter spacing and then further breakdown and aggregation into fibrous bundles. Mirsky and Pauling (24) explained denaturation as primarily a rupture of the labile hydrogen bonds, with a resulting uncoiling of the molecule.

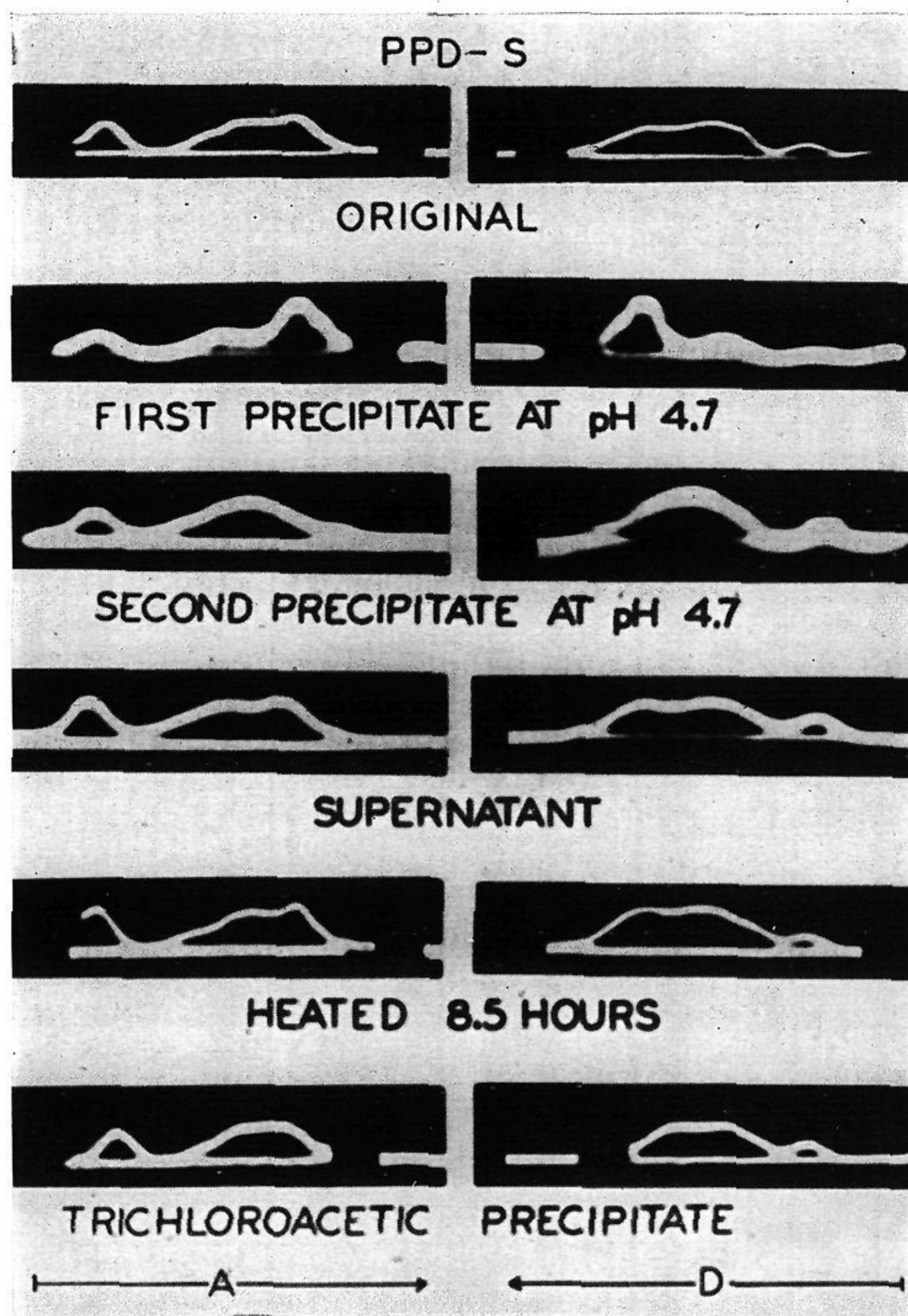

FIG. 2. Electrophoretic diagrams of heated tuberculin protein PPD-S treated in various ways.

It is not yet clear whether the B protein is derived from the A by the unmasking of imidazole groups, or whether it is a naturally occurring protein of different chemical composition. No published evidence for the transformation of one into the other so far exists.

Attempts to cause such a transformation have so far yielded negative results. For example, figure 2 gives first the electrophoretic diagram of PPD-S, a fraction

made from heated tuberculin (36), and shows the presence of two types of protein with mobilities about -3.5×10^{-5} and -5.7×10^{-5} in phosphate buffer at pH 7.7 and $\mu = 0.1$. The fraction which precipitates from this solution at pH 4.7 (second curve) contains mainly a faster fraction. On standing, the supernatant from this precipitation again yielded a small precipitate at pH 4.7, but this contained chiefly a slower component (third curve). The final supernatant contained the two original components, both of which were soluble at all hydrogen-ion concentrations and were probably the native proteins A and B.

If drastic heating were able to transform one protein into the other, one of the components of such heated material (SH, fifth curve) should predominate, but this is not the case. In this experiment PPD-S was evaporated continuously in a boiling water bath for 8.5 hr., with addition of Long's synthetic medium at frequent intervals to maintain the original volume.

Furthermore, precipitation of some of the supernatant with trichloroacetic acid yielded a fraction, shown in the sixth curve, again not very dissimilar from the original material. The detection of small changes, which could only be achieved by quantitative measurements of the areas under the curves was, of course, not advisable in mixtures of proteins so poorly resolved.

While it has so far been difficult to demonstrate a change from the A type to the B type protein in the laboratory, it is the common experience that insoluble or denatured protein readily forms from the soluble type during purification. This fact was clearly demonstrated during the recrystallization of the tuberculin protein (30), and was the reason why crystallization was considered to be an impractical procedure. The factors which lead to the production of the denatured A′ and B′ type proteins at one time and to the D type of protein, with intermediate mobility, at another time from the same original fraction are not yet clear. For example, during the first fractionation of TPU-84, the fraction which precipitated at pH 4.7 to 5 had a mobility of -6.5×10^{-5} in phosphate buffer at pH 7.7 and $\mu = 0.1$ (see figure 4 and table 4), whereas in a second fractionation carried out in the same way, a fraction with mobility -5.5×10^{-5} resulted.

Further evidence that these fractions, which are insoluble at their isoelectric points, are denatured molecules lies in the fact that they always show a decreased tuberculin potency, as will be discussed in the section on biological reactions.

The apparent great lability of the undenatured tuberculin protein may account for the difficulty so far experienced in attempts to isolate a reasonable quantity of this fraction. In seeking other possible explanations for this difficulty one cannot overlook the additional possibility of association and dissociation among the different proteins of tuberculin as the concentrations vary during the purification processes, in a manner similar to that described for serum proteins by McFarlane (23) and extensively studied by Pedersen (45).

The possibility of denaturation of tuberculin protein as a result of great dilution must be considered because of its great practical importance. Cohn and Edsall (4) warn against high dilution of protein solutions, since the proteins may become denatured and separate into insoluble precipitates or the molecular weight

may be greatly diminished. While chemical data are so far lacking to establish such a denaturation effect in the case of the tuberculin protein, it is highly probable that it may occur. It is recognized, for example, that the high dilutions of tuberculin protein (1 ml. = 0.2 γ) used for the skin test in detecting tuberculosis must be made fresh on the day of test in order to avoid loss in potency.

C. Separation of the protein molecules

It is obvious from the results obtained that those protein molecules which can be precipitated at pH 4 to 5 are the denatured proteins which probably have been formed from native protein. The separation of the native molecules from these molecules, which exist in various stages of denaturation with consequent different chemical properties, is very difficult.

An attempt was made to separate the proteins in a trichloroacetic acid precipitate (TPT-18) of unheated tuberculin protein (38) containing about 6 per cent of nucleic acid by fractionation at various hydrogen-ion concentrations according to solubilities. Figure 3 shows the electrophoretic diagrams, at pH 7.7 and μ = 0.1 in phosphate buffer, of some of the fractions obtained. In all cases the electrophoresis lasted 1 hr. By noting the distance traveled by each component from the δ or ϵ boundary one can see that the sloping heterogeneous curve of the original material has been resolved into its different components. Table 3 gives the mobilities and principal solubilities of the fractions shown in the curves.

Separation of the proteins in an unheated tuberculin may prove to be a more practical problem, since it is obvious from the electrophoretic diagrams (figure 4) that the components can be more satisfactorily resolved than in the case of the denatured fraction. Figure 4 shows that varying proportions of the two types of soluble proteins, A and B, can be found in different preparations. In the case of the TPU-84 unheated tuberculin, when the fraction insoluble at pH 4.7 (first curve in figure 4) was removed, there remained two distinct soluble proteins (second curve). When this soluble supernatant was precipitated by one-fourth saturation with ammonium sulfate, the two types of proteins, A and B, precipitated in equivalent amounts (third curve), whereas half saturation with ammonium sulfate gave a product whose picture was similar to the original. Attempts are now being made to separate these two proteins. The last two curves show fractions in which the A protein predominated. All of the curves in figure 4 represent electrophoresis for 1 hr. in phosphate buffer at pH 7.7 and μ = 0.1; the actual mobilities are recorded in table 4.

D. Biological reactions of the protein of tuberculin

Tuberculin protein is highly potent in causing a local skin reaction in human beings who have been infected with the tubercle bacillus. For this reason it has been very useful as a diagnostic agent for helping in the detection of tuberculosis. As a result of infection with the organism, an animal or human being develops a hypersensitivity to the protein fraction. The hypersensitivity developed to the protein is often of high degree and may persist throughout life.

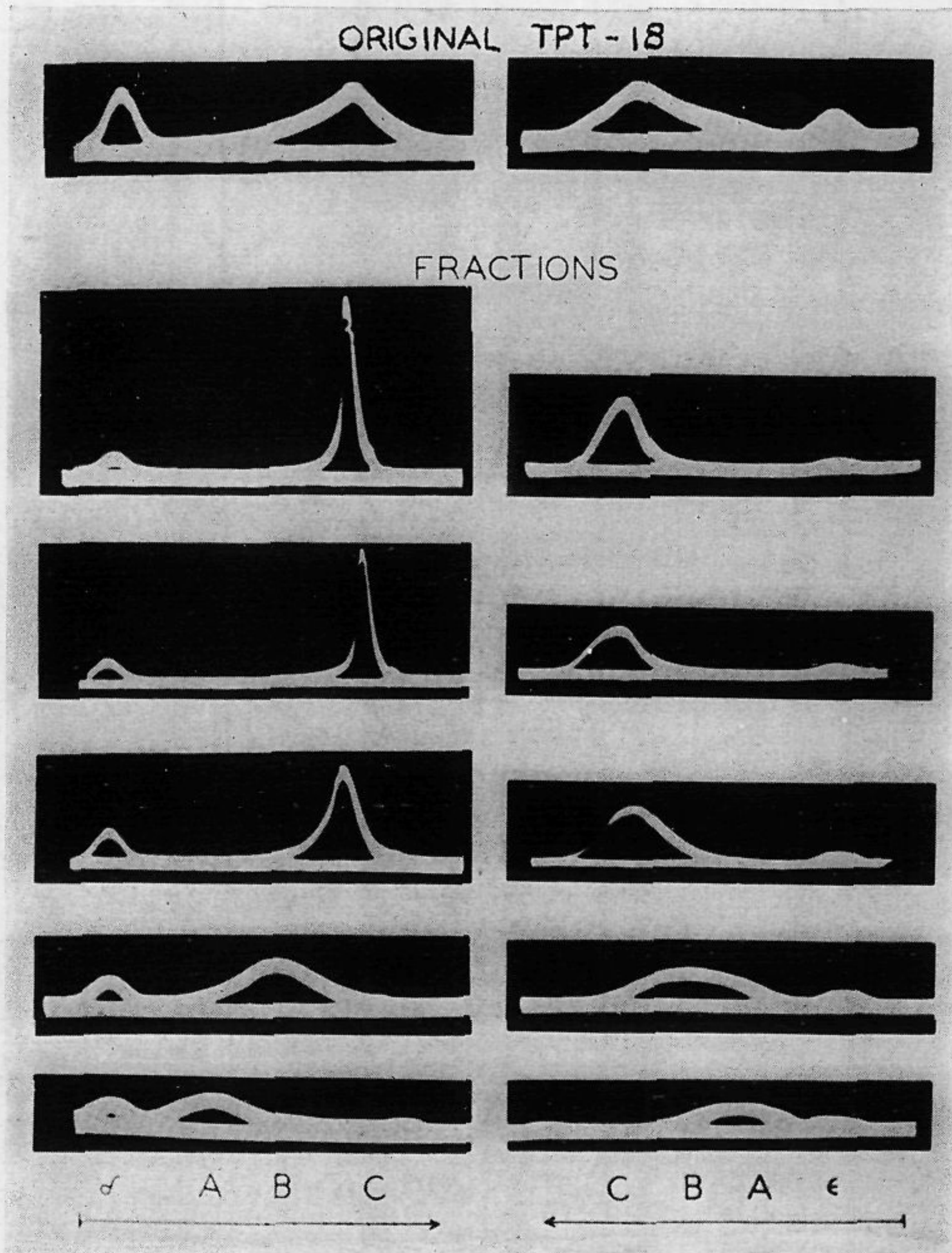

FIG. 3. Electrophoretic diagrams of tuberculin protein, TPT-18, and fractions thereof

TABLE 3

Mobilities of fractions separated from a trichloroacetic acid precipitate

FRACTION	SOLUBLE AT pH	INSOLUBLE AT pH	MOBILITIES $\mu \times 10^5$
Original (TPT-18)			−8.0 (chief component)
E(A)*	11.0	5.8	−8.3
E(less soluble)	11.0		−8.1
D(C)	7.6	5.8	−7.6
C1 + D1	4.3	Not at all	−6.4
C1	2.8	Not at all	−5.2

* As noted earlier in the text, this fraction was very heterogeneous on sedimentation and diffusion.

A protein fraction which has been separated from tuberculin practically free of polysaccharide and nucleic acid has a potency such that 0.02 γ given intracu-

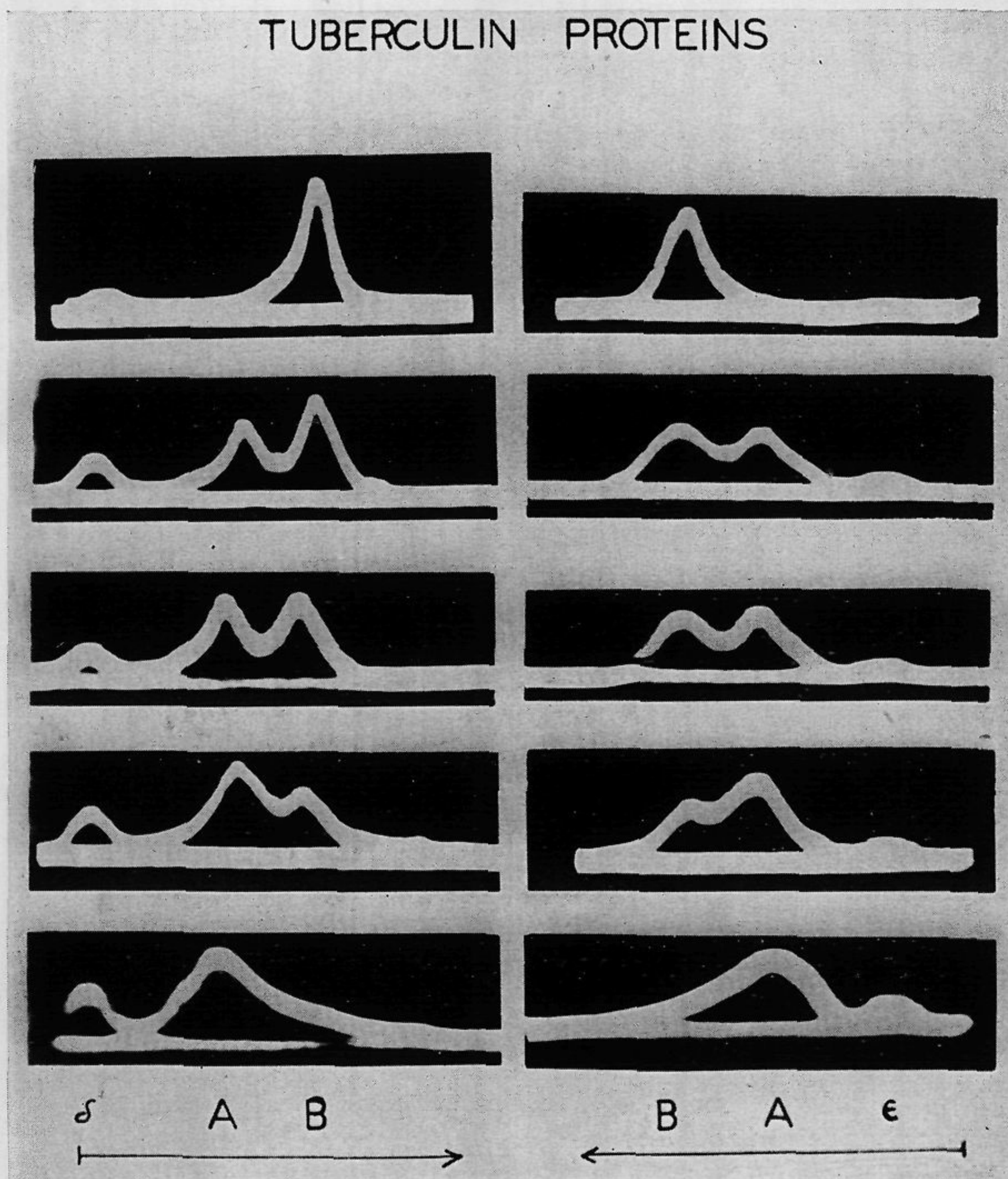

Fig. 4. Electrophoretic diagrams showing varying proportions of proteins A and B in unheated tuberculin fractions.

TABLE 4

Mobilities of fractions separated from unheated tuberculins

FRACTION	MOBILITIES $\mu \times 10^5$	
TPU-84: Precipitate at pH 4.7	−6.5	
TPU-84: Supernatant	−6.7	−3.9
TPU-84: Precipitate after one-fourth saturation with ammonium sulfate	−6.6	−3.8
M 9b: Precipitate after half saturation with ammonium sulfate	−6.3	−4.1
#85C3: Precipitate after half saturation with ammonium sulfate		−3.5

taneously in a volume of 0.1 ml. will give after 24 to 48 hr. a definite area of swelling in an infected person. This dose accordingly has been chosen as a standard first test dose (35, 36). In case no reaction occurs a second test with 5 γ is given, in order to detect a lower degree of sensitivity. No reaction whatever occurs if there has been no infection.

Guinea pigs are less sensitive and require 0.5 to 5 γ to cause an appreciable local reaction. When given intraperitoneally 1 to 1.5 mg. is lethal in 24 to 48 hr. for tuberculous guinea pigs but as much as 100 to 150 mg. is required to kill a normal guinea pig.

Thus it seems that the reaction is due primarily to sensitization but that there may also be a certain degree of primary toxicity involved when large doses are concerned. The suggestion has been repeatedly made (8, 37) that the toxic factor, causing death in tuberculous guinea pigs, may differ from the factor responsible for giving the local skin reaction.

That tuberculin protein is an excellent antigen is obvious from this high degree of sensitization in infected individuals and has also been demonstrated by the fact that it readily stimulates the production of antibodies such as precipitins (31) and causes anaphylaxis (16), uterine strip contraction and bronchial spasm (16), and the Arthus reaction (32). Some tuberculin protein fractions, however, appeared to be less or not at all antigenic (32) but still capable of producing the skin reaction, and these were chiefly isolated from heated preparations.

A careful study (40) of the type of protein that was present in the various preparations which varied in antigenicity and potency revealed the fact that the more native protein, and therefore the larger one, of molecular weight about 32,000, was the most antigenic and gave an early type of reaction reaching its maximum at 24 hr. and fading at 48 hr., whereas the smaller one, of molecular weight about 16,000, was non-antigenic and gave a delayed type of reaction, with maximum size at 48 hr. and less at 24 hr. More recently (39) these two proteins have been identified as the slow and the fast type of protein in electrophoresis. They would be classified, respectively, as the A and the B′ proteins described in this paper. Their relationship to the A′ and the B proteins remains to be ascertained.

It has been shown (39) that a certain amount of immunological specificity can be demonstrated between the slow and the fast proteins. Fractions which predominated in the slow one caused, when injected repeatedly into rabbits, a definite rise in the γ-globulin of the serum, and this γ-globulin on isolation was shown to contain specific antibodies. Fractions predominating in the fast protein caused a rise in the α-globulin fraction of serum. In these latter experiments the data showed (39) that the removal of a specific precipitate by means of the antigen caused a decrease in the proportion of α-globulin content of the remaining serum in some cases and of albumin in others. With the recent developments reported by Longsworth (19), i.e., better resolution of serum components in the longer electrophoresis cell and the separation from albumin of a previously unrecognized component, designated as α_1, in diethylbarbiturate buffer, it becomes probable that the antibody produced by injection of the fast type of tuberculin protein (B) may really be in an α_1-fraction rather than part of the albumin.

For similar reasons the antibody produced by injection of the slow type of tuberculin protein (A) may prove on better resolution to exist in a component with mobility between the β- and γ-components rather than in the γ-globulin, as stated.

At any rate, it is obvious that different antibodies are formed to the two types of tuberculin protein. This is a reasonable result when consideration is given to the fact that the two protein antigens (A and B) differ in their polar groups, as indicated by their pH–mobility curves (figure 1).

The significance of these differences for the skin reaction remains to be observed, and this observation awaits the isolation of the pure proteins in adequate quantity for chemical and biological tests on the same fractions. That this type of study may prove of value is suggested by the fact that some confusion already exists in regard to the skin reaction with the present purified fractions. For example, different protein fractions have been shown to have different quantitative potencies. The one, PPD (39), now used as the standard has twice or four times the potency of some previously isolated fractions. It also contains more of the A type of protein. In addition to being more potent per milligram, it also elicits reactions in more people than do other preparations (13, 22). It has been questioned by Furcolow, Hewell, Nelson, and Palmer (10) whether some of these reactions to the larger dose might not be non-specific. In addition to giving larger and more reactions this fraction also gives rise to earlier reactions, which appear as early as 6 hr., as shown by McCarter and Watson (22).

All of these facts argue for the greater antigenicity of the preparation, which has actually been demonstrated (22, 36) by the facts that it causes the production of antibody in animals and, moreover, is shown by electrophoresis to contain a moderate amount of both types of proteins, A and B. It is logical to think that sensitization caused in the infected animal has been by means of the native forms of the protein or proteins and that greater specificity in detecting this hypersensitivity might be obtained through the use of the least denatured proteins, providing this can be done without actually sensitizing the host by means of the test dose. It is obviously important to obtain each type of protein in its native as well as denatured form for the purpose of answering these questions.

Preparations shown to contain considerable amounts of denatured tuberculin proteins have invariably proved to be less potent. For example, the fractions TPT-18E(A) and Q(E), described earlier, when tested intracutaneously in sensitized human beings, gave very much smaller local reactions than did the standard tuberculin protein, even when four times the standard dose was used (see table 5). It was significant that this difference was less marked and even reversed when the larger ($5\,\gamma$) dose was used.

IV. SUMMARY

Tuberculin contains chiefly three colloidal constituents, protein, polysaccharide and nucleic acid, of which the protein is the most important because of its specific biological reactions in tuberculous animals and human beings.

The polysaccharide and nucleic acid have both been isolated in pure form and their physicochemical properties are described. Neither one gave any signifi-

cant biological reactions, except a specific precipitin reaction between the polysaccharide and sera from animals immunized with the tubercle bacillus.

The protein portion of tuberculin is very complex. Two soluble native proteins (A and B) have been identified, which can be distinguished by their different mobilities in electrophoresis, and which have been shown to contain different amounts of polar groups dissociating between pH 5 and 6, suggesting the presence of imidazole groups. Denatured forms of these two proteins (A′ and B′) have also been identified. They were insoluble at their isoelectric points and differed from the native proteins in their electrochemical properties. One of these proteins was found to have a molecular weight of 16,000, half that of one of the native proteins, and also to be more elongated in its physical structure.

TABLE 5

Tuberculin potency of denatured tuberculin proteins

SUBSTANCE	DOSE	NUMBER TESTED	NUMBER POSITIVE	NUMBER MISSED	AVERAGE SIZE REACTION
	γ				*mm.*
Standard	0.02	69	56	1	29 x 24 x 2.4
TPT-18E(A)	0.08	69	44	12	17 x 15 x 1.9
Standard	5.00	17	14	0	17 x 14 x 1.8
TPT-18E(A)	20.00	22	15	4	19 x 17 x 2.0
Standard	0.02	57	41	0	34 x 28 x 2.7
Q(E)	0.08	57	39	2	15 x 14 x 1.7
Standard	5.00	16	14	0	20 x 17 x 2.0
Q(E)	20.00	16	14	0	17 x 15 x 1.9

Other more severely denatured proteins (D), which show extensive elongation and even great polydispersity in the ultracentrifuge, have also been found, chiefly in heated tuberculins. They frequently showed electrochemical homogeneity, with mobility intermediate between the mobilities of the two types of native protein.

Nucleoproteins with mobility much higher than that of the protein have also been studied.

Tuberculin potency can be ascribed to the native proteins and to those fractions in the early stages of denaturation. Antigenicity is a property belonging chiefly to the native forms. Some immunological specificity has been noted for the two different main types of protein. The more denatured the proteins are chemically, the lower is their potency for eliciting the tuberculin skin reaction.

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