THE CHEMISTRY OF THE PROTEINS OF THE ACID-FAST BACILLI

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The protein components of bacteria are in most cases of great biological significance, and an understanding of their chemical compositions and properties is one of the pressing problems in bacteriological chemistry. There is reason to expect that such knowledge will be of value in throwing light on the organisms themselves and perhaps also on some of the biological reactions that they elicit. One aspect of the long study on the tubercle bacillus has been motivated by these considerations.

Naturally, most such investigations have dealt with the proteins of the human type of the tubercle bacillus, while studies on the proteins of the other types and of other members of the acid-fast group have been fewer and generally less intensive. As a result the advance in our knowledge has not been uniform, as will become evident from the following survey. No attempt will be made to review the extensive literature dealing with the clinical tests or epidemiological surveys that have been carried out with the "Purified Protein Derivative Tuberculin" or other preparations. We shall consider here only those studies that have dealt specifically with the differentiation of the proteins from different acid-fast bacilli, or those that have led to the chemical characterization of these proteins.

In the beginning of our accurate knowledge of tuberculosis, with the discovery of the causative bacillus in the disease, Koch's experiments in 1891 (35) led him to believe that the substance responsible for eliciting the specific toxic effect in the diseased animal was of protein nature; and Hammerschlag (23) was among

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the first to identify protein as one of the constituents of the tubercle bacillus. Weyl (93) in the same year, also isolated a protein and considered it to be a mucin, differing, however from other mucins in that on heating with dilute acid, a reducing substance was not split off.

About the same time Kühne (37), well known for his fractionation studies on proteins and their decomposition products, made exhaustive investigations on the constituents in Koch's tuberculin, but the "akroalbumose", a propeptone which he found and which was precipitable by acetic acid, could be identified also in the peptone used in the medium. In later studies, however, he made a tuberculin by growing tubercle bacilli upon a synthetic medium and was able to show the presence of a protein, which must have been a metabolic product of the bacillus. He used the alcohol precipitation method of Koch and isolated an albumose mixture which contained the whole of the active material. Hofmann (28) obtained six protein fractions from extracts of tubercle bacilli, two of which he called albumin and globulin.

Nucleoprotein Theory. Levene, however, in 1898 made the following statement (39): "The body substance of tubercle bacilli does not contain any proteids of albumin nature; that as the nucleus of cells it consists mostly of nucleoproteids; that one of the nucleoproteids differs from all the other nucleoproteids inasmuch as it is not precipitated by magnesium sulfate (and in this point resembles nucleo-histon), and does not give the [biuret] test; that it contains nuclein or nucleins as such." He obtained three proteins with coagulation points at 50–64°, 72–75°, and 94–95°. In later experiments he used a medium free of protein, in which the source of nitrogen was asparagine and the source of carbon was mannitol and glycerol.

In the same year Ruppel's (57) extensive researches appeared. At first he fractionated tuberculin by alcohol precipitation, then by precipitation with saturated sodium chloride, and finally the filtrate with acetic acid. The chief amount of material, however, remained in solution after this precipitation and could be obtained by dialyzing the solution, evaporating and again precipitating with alcohol. The final product did not precipitate from aqueous

solution with saturated sodium chloride, but was completely precipitated by ammonium sulfate and he therefore considered it to be mainly a deuteroalbumose.

By dialyzing the original tuberculin before fractional precipitation he could remove a heteroalbumose fraction. Furthermore, he studied innumerable methods of extracting the bacilli and finally used the method given by Koch of completely disintegrating the bacilli by mechanical grinding until no intact bacilli remained. By this method half of the bacilli by weight went into opalescent solution. The solution gave with acetic acid a considerable precipitate which was insoluble in excess of the acid, but soluble in dilute alkali. This precipitate contained four per cent phosphorus, gave a positive biuret test, but negative Millon and xanthoproteic reactions. From this product he obtained by Kossel's purification methods a product which fulfilled the specifications of a protamine. For example, it existed as a sulfate of a basic compound; it was phosphorus-free and precipitable by sodium picrate in neutral solution; it gave only the biuret reaction; and finally it precipitated proteins in ammoniacal solution. Ruppel named the product "Tuberkulosamin" and claimed it was bound in the tubercle bacillus to a nucleic acid, which he was also able to isolate, some of which existed in the free state. This acid contained 9.4 per cent phosphorus, and was named "Tuberculinsäure."

Klebs (34) and others were also of the opinion that there existed in the tubercle bacillus a nuclein and that it was responsible for the specific tuberculin activity. This hypothesis was further supported by Baldwin and Levene (40) on the basis of their results showing that trypsin more easily destroyed the tuberculin potency than did pepsin.

Non-Nuclein Nature of Active Principle Based on Reaction to Enzymes. Löwenstein and Pick (44), however, claimed that the tuberculin product was destroyed by digestion with pepsin as well as with trypsin and had, therefore, the characteristics of a polypeptide. Danièlopolu (14, 15), Pfeiffer, Trunk and Leyacker (54), Mueller (53) and others also found that pepsin as well as trypsin destroys the active substance. Seibert (62) studied the

effect of pepsin, trypsin and erepsin upon a purified, water-soluble, non-coagulable tuberculin protein and followed, simultaneously, the breakdown of the protein molecule by chemical analyses and the loss in activity. It was concluded that when the whole protein molecule is attacked, as by pepsin in acid or trypsin in alkaline solution, activity is lost, whereas when the peptide bonds in the proteose molecule are attacked, as by erepsin, no loss in activity occurs. In view of the recent isolations of highly purified, crystalline enzymes, it would be of value to restudy this problem.

This contention of Ruppel concerning the nucleoprotein nature of the active tuberculin fraction was further refuted by Toenniessen (88). His preparation, which he called "Tebeprotin," was made by heating the bacilli in dilute mineral acid, extracting with alkali, filtering through a Berkefeld candle and then precipitating and reprecipitating with acetic acid. It contained 12.3 per cent nitrogen, no phosphorus, no purine bases, and gave a negative Molisch reaction but positive biuret and Millon reactions; 0.1 mg. of "Tebeprotin" had a tuberculin potency equivalent to 10 mgm. of Old Tuberculin.

Other Theories as to the Nature of the Active Principle. In addition to the opinions cited above, the debate as to the nature of the active substance in tuberculin was carried on by the following investigators. Much (52) believed it was associated with lipoid. Bieling (5) claimed it was not a single pure substance, but something which readily adsorbs to numerous other substances, including protein, protein derivatives, kaolin, aluminum hydroxide, silicic acid, etc. Jastrowitz and Weinberg (29) and Sandor (59) were of the same opinion. Later Boquet (8) thought it accompanied something of proteose nature, and so also did Kallós and Hoffmann (33). Some of these views were due largely to the experiments on dialysis of tuberculin.

Dialysis of the Active Principle. On the subject as to whether or not the active principle dialyzes the views have been numerous. Zieler (95), Danièlopolu (13), Löwenstein and Pick (44), Zinsser (96), LeGuyon and Albert-Weil (38), and Maschmann and Küster (46), among others, claimed that the active principle did

dialyze through membranes of viscose, collodion, vegetable parchment or fish-bladder. On the other hand, Ruppel (57), Marie and Tiffeneau (45), Selter and Tancrè (82), Seibert and collaborators (61, 65, 66), Tytler (89), and Kallós and Hoffmann (33) maintained that the active principle could be held back on dialysis and that it could be fractionated and even concentrated in this manner, provided a suitable membrane was employed. In actual fact, pressure-dialysis, or ultrafiltration is one of the important steps recently used on a commercial scale in securing the active principle in concentrated form before its final purification.

Obviously membranes of different porosities were used by the different investigators and the matter resolves itself, not so much into the question as to whether the active principle of tuberculin does or does not dialyze, as into the question of how small a molecule can possess the tuberculin activity and also as to whether this activity can accompany molecules of different sizes. That the activity does accompany molecules of different sizes is the prevailing opinion at the present time, and much evidence has accumulated to show that this is so. This will be discussed later.

Association of the Active Principle with Normal Protein Fractions. In addition to the literature so far cited, mention should also be made of the following studies, all of which show that tuberculin activity accompanies fractions which could be considered to be more or less normal proteins. Half-saturation with ammonium sulfate, as well as complete saturation, precipitated active protein fractions (63) from tuberculin filtrates obtained by growing tubercle bacilli on synthetic medium. It was found, furthermore, by Seibert (64, 67) that a small amount of a protein with high tuberculin activity could be crystallized at an optimum pH of 4.9, by Hopkins' method for crystallizing egg albumin. Very fine needles formed and they usually grouped themselves into burrs. In one experiment, in which the protein was redissolved and recrystallized fourteen times, the potency was increased rather than decreased.

Masucci and McAlpine (47) made a preparation, called MA-100, which was obtained after precipitation eight times by half-

saturation with ammonium sulfate, once with five volumes of 95 per cent alcohol at pH 4.7, four times at pH 4.7, and finally hydrolysis with barium hydroxide. Later a very simple method for obtaining the active protein from tuberculin filtrates, in powder form, in which the potency and analyses were easily duplicated, was given by Seibert and Munday (70). This product was made by concentrating large quantities of filtrate from tubercle bacilli grown on synthetic medium by ultrafiltration and then precipitating once with trichloracetic acid and removing the trichloracetic acid with ether. This product, called TPT, was used extensively in many researches.

Gough (21) obtained an active preparation from tuberculin filtrate by first removing a precipitate at about pH 5.0 and then adsorbing the active substance on benzoic acid, from which it was freed with acetone. Boquet and Sandor (7) precipitated an active fraction by means of phosphotungstic and sulfuric acids and then freed it by means of a saturated solution of baryta. Gözsy and Vásárhelyi (22) tried to increase the degree of purification of a preparation already purified, by means of chromatographic adsorption, but were not able to do so and this fact they claimed argued for the purity of the original material. However, Seibert, Pedersen and Tiselius (78) had shown that fractions obtained by chromatographic adsorption and elution at pH 8, gave asymmetrical curves when studied in the ultracentrifuge, indicating more or less molecular heterogeneity of the isolated fractions. It is probable that the same phenomenon of interaction of components was in effect as was shown later by Seibert and Watson (81) to be so prominent in their attempts to purify the polysaccharides of tuberculin. For example, it was not possible to remove the nitrogenous impurities (protein and nucleic acid) to less than about one per cent nitrogen from one of their polysaccharide fractions by exposure to an electrophoretic potential gradient of 3.7 volts per cm. (about 400 v. and 17 ma.) for 114 hours. On the other hand, another polysaccharide fraction was readily purified to a content of 0.2 per cent nitrogen in half the time under identical conditions.

Active Protein from Bacillary Extracts. Numerous prepara-

tions, considered to be protein, have been isolated from extracts of tubercle bacilli. Coghill (11) obtained an active water-soluble protein from bacilli previously defatted by repeated extraction with cold ether, and also a less active fraction by extracting the residue with alkali. The proteins were precipitated with acetic acid. Dienes, *et al.* (16) used aqueous and weak alkaline extracts of tubercle bacilli and precipitated the proteins with sodium sulfate or acid and heat. Later Dienes (17) fractionally precipitated the bacterial extracts at different hydrogen ion concentrations obtained by adding increments of hydrochloric acid. He thought he could detect by means of the precipitin reaction that the proteins obtained from the weak alkaline extracts were different from those obtained from the aqueous extracts. Tytler (89) extracted ground dried bacilli with acetone and then with water at 37° for 2 to 6 weeks. The protein was then precipitated from the aqueous extract with acetic acid at pH 3.8 to 4.0, redissolved at pH 5.5 to 6.0, reprecipitated with ammonium sulfate, dialyzed and dried under high vacuum from the frozen state.

An attempt to cause less denaturation of the bacilli during the defatting process was undertaken by Gough (21). He used Hardy and Gardiner's (25) method of extracting an ice-cold suspension of living tubercle bacilli, grown on synthetic medium, with a large excess of absolute alcohol at $-10°$, and then replacing at $-3°$ the solvent with successive changes of mixtures of alcohol and ether containing increasing proportions of ether up to anhydrous ether. The idea that this procedure does leave proteins unharmed, however, has been questioned by McFarlane (50), since he found fundamental changes in the physical state of serum proteins dried in this manner.

Heidelberger and Menzel (26) also attempted to isolate the proteins of the bacillary bodies in undenatured form. They used living bacilli which had been frozen and dried in vacuum, and killed them by immersion in cold acetate buffer at pH 4.0 for 30 days. After this the cells were extracted four times in cold redistilled acetone, three times with purified anhydrous ether, dried *in vacuo*, ground for ten days, reëxtracted two more times with cold ether, and dried. The cell residues were then suc-

cessively extracted with buffers ranging from pH 4.0 to 11.0, and then finally in 0.1, 0.2 and 0.5 normal sodium hydroxide at room temperature. Each fraction was then precipitated with acetic acid to maximum flocculation and repurified several times. Among the many fractions obtained they identified two antigenic components, and finally, by subfractionating each fraction, they found at least three components.

Search for Albumin and Globulin. Attempts to identify typical albumin and globulin fractions in the tuberculin filtrate or in the bacillary extracts have also been made. As early as 1894 Hofmann (28) classified two of his six fractions from bacillary extracts as albumin and globulin, the former being soluble in water and the latter in weak acid. Seibert's (67) method of crystallizing a protein from the tuberculin filtrate would naturally classify it as an albumin, and furthermore, no significant protein fraction was ever found in the unheated culture filtrates which would precipitate out on dialysis against water. Coghill (11) looked for the two types of protein in his bacillary extracts, and found a water-soluble fraction, precipitable with acetic acid, which he classed as an albumin. But he could find no fraction which corresponded to globulin, since a 5 per cent sodium chloride extract yielded a protein fraction from which nothing would precipitate on dialysis against water.

Gough (20) obtained protein fractions from his bacillary extracts which precipitated at half and complete saturation with ammonium sulfate, as globulins and albumins are supposed to do, and he called them such, even though the globulin did not precipitate from aqueous solution on prolonged dialysis against water. The two fractions had distinct properties, indicating that a separation of different proteins had really been made, even though the tuberculin activity of both of them was about equal. The albumin fraction contained more carbohydrate in definite association, more phosphorus, and retained its precipitating power after treatment with 0.5 per cent sodium hydroxide, in contrast to the globulin fraction.

Protein Nature of the Active Principle. The study of the proteins produced and released by the tubercle bacillus has been

chiefly, as noted above, the story of a search for the active principle of tuberculin. In practically all of the studies cited, special attention has been given to the activity of the preparation and when a protein fraction appeared to be inactive or less active, interest in it was usually lost. It is obvious that the activity accompanies fractions which can be isolated in many different ways; and the number of preparations mentioned in this paper is only a fraction of the list of tuberculin products offered for therapeutic and diagnostic use (4). Excellent reviews are given by Wells and Long (92), by Long (42), and by Calmette (9).

One is much impressed with the high degree of stability of the active principle even under the drastic treatment often used, and it is not easy to find in the realm of known biologically potent soluble proteins one with such a remarkably high degree of stability. In this respect investigators in this field are fortunate. However, more careful studies of this protein do not show that it is really so constant in regard to its physical properties, since fractions with innumerable different combinations of properties can be obtained from the same solution, simply by varying the method of isolation.

All of these facts indicate that the biological potency must be inherent in some small, relatively stable part of the protein molecule, and many changes can be effected in the molecule as a whole, before there is recognizable loss in the biological activity. With such an explanation all the different views can be brought into harmony.

That the activity is associated with protein or some part of the protein molecule can no longer be doubted when one considers the evidence. To summarize, the facts are as follows:

1. Coagulable protein appears in the culture medium at about the same time as tuberculin activity.
2. Protein precipitants carry down the activity along with the protein.
3. When the protein fractions are purified the content of nitrogen parallels the potency.
4. Pepsin and trypsin destroy the potency simultaneously with the breakdown of the protein molecule.

5. The potency accompanies the crystallized protein and even has been shown to increase during 14 recrystallizations.
6. When protein precipitable by trichloracetic acid is found in tuberculin ultrafiltrates, there is tuberculin activity also in these filtrates. Otherwise, there is never more than a trace of potency.
7. The potency accompanies protein molecules of different sizes which have been shown to be practically homogeneous in sedimentation and diffusion. These will be discussed later.
8. The active principle migrates with the protein in electrophoresis.

Molecular Size of Active Principle. That there may be potent protein molecules of different sizes, or that there may be potent molecules of different sizes which represent consecutive breakdown stages in the protein molecule was the logical suggestion from the apparently conflicting opinions in the literature. Experiments with fractional ultrafiltration through graded membranes (69) also indicated that this was true. An attempt to investigate this suggestion was made in 1933 (73) with the physico-chemical technics then generally available, and it seemed as though the potency could accompany molecules ranging in size from about 2000 to 34,000. However, a much more accurate and comprehensive study of this problem was made in Professor Svedberg's laboratory, Upsala, Sweden, by Seibert, Pedersen and Tiselius (78), using the precise ultracentrifuge, diffusion and electrophoresis technics there available. These studies led to the conclusion that practically homogeneous fractions with molecular weights of 16,000 and 32,000 could be isolated from tuberculin, and both of them were highly potent. A molecule even as small as 9000 had some potency. Recently X-ray diffraction patterns have been obtained on some of the homogeneous proteins (85).

Previously it had been shown (71) that the molecule with 16,000 molecular weight did not stimulate the production of antibodies (precipitins), nor elicit a typical Arthus reaction when repeatedly injected into normal rabbits, whereas the molecule of 32,000

molecular weight was highly antigenic. The smaller molecule had been isolated from heated tuberculin, like Old Tuberculin, and the larger one came from unheated culture filtrate. Moreover, the small molecule became antigenic after it was adsorbed to aluminum hydroxide and charcoal (76) indicating that it was acting like a haptene. This phenomenon seemed to indicate that we were dealing not with an irreversible breakdown of an antigenic whole protein molecule to a proteose, with loss in antigenicity, but possibly with a situation such as was assumed earlier (78); namely, that the potency might be inherent in a small unit molecule, similar to one of Svedberg's protein units of 17,000 molecular weight and that when two or more of these aggregated, the larger molecule would become antigenic. There would be no difference in the nitrogen content of the two products.

However, recent researches (80, 91) have shown that tuberculin protein molecules even smaller than 17,000 molecular weight have considerable antigenicity. Since one of these, which has been studied by electrophoresis, has been shown to be somewhat heterogeneous and to consist of at least two components, the relationship of molecular size and antigenicity is still not entirely clear.

Theory of a Reversibly Dissociable Component System. It is obvious, however, that the system of tuberculin protein molecules under consideration is extremely complex. The results so far available might suggest a reversibly dissociable component system such as postulated by Hardy (24), Sørensen (83), and Block (6) for some of the well-known proteins. According to these investigators the proteins which one isolates from whole serum as definite chemical entities by simple chemical treatment with neutral salts, do not exist as such in the serum. Whole serum protein is electrically inert, according to Hardy (24) and Tiselius (87). It may be considered as a system of reversible components so combined in its natural environment as to act as a single substance, but which can be readily dissociated into fractions whose composition and properties depend upon the degree of dilution and the reagents used. Sørensen (83) stated that "Within each complex all the atoms or atom groups are inter-

linked by main valencies, whereas the various complexes of components are reversibly interlinked by means of residual valences." Thus if one of the components contained a high proportion of a certain amino acid, a fraction could be isolated which would contain a very high percentage of this particular amino acid, depending upon the method used for isolation and the amount of rearrangement of the co-precipitation systems. For example, Block (6) obtained by fractionating the whole serum with neutral salts, different proteins which varied from 4 to 39 per cent in their content of lysine. A good review of these hypotheses and the available evidence for them can be found in Schmidt's book (60).

It is true that innumerable tuberculin protein fractions can be isolated which vary in the proportions of protein, carbohydrate and nucleic acid components and also in their potency, toxicity and antigenicity. Moreover, fractions with rather definite proportions of these components and constant biological properties can be repeatedly produced if the same method is used, at least from the same lot of raw tuberculin. It is clear also that the biological properties mentioned are not dependent upon the presence of carbohydrate or nucleic acid, except insofar as a question of contamination or dilution may be concerned, since highly potent protein fractions demonstrated to be almost free of these constituents have been isolated (79). Thus we cannot consider the active principle to be a true nucleoprotein or a mucoprotein even though the potency may be present in such molecules. It would seem that the potency of any fraction isolated depends upon the inclusion within its complex of a particular protein component that contains the specific group responsible for the tuberculin potency. It is likely that this component is present in considerable proportion, since it is difficult to isolate completely inactive carbohydrate, nucleic acid or protein fractions from the tuberculin, and relatively easy to secure highly active tuberculin preparations with small differences in potency.

The problem of isolating and identifying the specific component in the tuberculin protein-complex, therefore, becomes even much more complicated than in the case of serum, because of the

presence of the nucleic acid and polysaccharide components in addition to the proteins, of which it is still not certain how many exist.

Amino Acid Analysis of the Proteins. The question as to the existence of such a reversibly dissociable system in tuberculin protein fractions could possibly be answered by making comparative amino acid analyses on the purified protein preparations. An attempt was made to do this by Seibert and Munday (74) in 1933, and there proved to be very much more total basic nitrogen in the small molecule (SOTT) than in the large molecule (TPA). However, not too much significance can be placed on these results since satisfactory micro-methods were not then available to show that the SOTT contained much more nucleic acid than the TPA, and this may have accounted for the high basic nitrogen. Moreover, the presence of polysaccharide seriously affects the accuracy of the results. The difficulty in an adequate study of this problem is the securing for analysis of sufficient quantities of the fractions really freed of nucleic acid and polysaccharide and molecularly homogeneous, such as those isolated and proved to have molecular weights of 16,000 and 32,000.

Analyses of the amino acid content of the proteins isolated from extracts of tubercle bacilli have been made by Tamura (86), Johnson and Brown (31), Johnson and Coghill (32), Campbell (10) and Popper and Warkany (55). Tamura emphasized the high content of phenylalanine, and Johnson and Coghill emphasized the high content of hexone bases and low content of cystine. Popper and Warkany found 1.1 per cent typtophane and 1.4 per cent tyrosine and pointed out that these constituents varied from the quantities usually found in animal and plant tissues.

Denaturation of the Tuberculin Protein. The exact composition of the active tuberculin protein complex becomes further complicated by the question of denaturation. In addition to the supposition that the potency of a fraction may depend upon the proportionate amounts of active protein, inactive protein, polysaccharide and nucleic acid present, there is also the possibility that some denaturation of the active protein may have taken

place. The degree of this denaturation may vary with different preparations according to their treatment.

Table 1 is a compilation of data on a selected number of fractions studied at various times, and illustrates that the potency is not dependent upon the amount of nucleic acid or polysaccharide present, since large or small quantities of these impurities may be found in preparations with potency one-half or twice as great. There is also no definite correlation with the antigenicity

TABLE 1

A comparison showing the lack of correlation between nucleic acid or polysaccharide content of certain preparations and their potency or antigenicity

PREPARATION	NUCLEIC ACID	POLYSACCHARIDE	RELATIVE TUBERCULIN POTENCY	RELATIVE ANTIGENICITY
	per cent	*per cent*		
TPA-30 K	0	2.0	*	Good
Rx	5.1	7.2	x	
98	27.7	32.6	x	
63	3.0	4.4	2 x	Moderate
19	21.8	24.0	2 x	Poor
65	0.8	4.8	4 x	Moderate
R5	21.2	20.1	4 x	Moderate

* The potency is not recorded, since it was greater than 2 x (x stands for the lowest activity recorded above) at 24 hours but less at 48 hours, indicating a more typical anaphylactic type of reaction. Preparation TPA-30 K (considered to be highly antigenic) was isolated from an unheated tuberculin filtrate, while all the others were obtained from heated fractions.

of the fractions. It would seem that these biological properties are more dependent upon the type of protein components present, and possibly the extent of their denaturation.

In the case of one "Purified Protein Derivative Tuberculin," a fractionation could be made (78) into three protein components of quite different properties. For example, one (Fraction b3) was highly potent, a poor antigen, fairly homogeneous in ultracentrifugal sedimentation and in diffusion, with a molecular weight of about 16,000, and a frictional ratio of 1.9, indicating some dissymmetry of the molecule.

There was present also a small amount of molecules about half this size, (Fraction b3b), which were less active and apparently homogeneous in the ultracentrifuge. The third component (Fraction b2) occurred in considerable quantity, behaved like very heterogeneous, thread-like or hydrated molecules in sedimentation and diffusion, and was less active. This fraction dissolved with difficulty in buffer at pH 8.0, swelling at first and then showing indication of gelling. There is reason to believe that this reaction indicates the presence of denatured protein molecules. Thus varying proportions of these three components would give products with varying potencies and physical properties.

A similar denaturation occasionally occurs during concentration of tuberculin on ultrafilters, as evidenced by the formation of a gel on the membrane. This gel has been shown (78) to consist of particles of many sizes and even of huge dimensions, with sedimentation constants as high as $S_{20} = 46$. It is usually less soluble and sometimes insoluble. Furthermore, these gel fractions are always less potent.

It is quite probable, therefore, that at least one form of denaturation of the tuberculin protein may occur first through a splitting or opening of the unit molecule with considerable loss in potency and exposure of extra polar groups, through which a polymerization of these elongated molecules may take place to form long thread-like structures. Since the titration curves (78) indicate that the extra exposed groups may be ones which dissociate at about a pH 5.0, it is possible that the imino group of histidine may be important in determining the potency of tuberculin. The investigation into the question as to which groups of the molecule are important in determining the tuberculin activity has of necessity waited upon the availability of preparations with molecules of relative homogeneity.

"Purified Protein Derivative Tuberculin." As stated earlier, the study of the tubercle bacillus proteins has always been a search for the active principle and methods for isolating it in pure, stable and highly potent form for use in the diagnosis of tuberculosis. From this standpoint, the search can be considered to have culminated at the present time in the production of "Purified

Protein Derivative Tuberculin," which has been used extensively in different parts of the world and which has been accepted as a standard tuberculin. Its preparation by means of trichloracetic acid, its properties, and some clinical results obtained with it were given in 1934 (75). It was a powder, whose potency has remained stable for at least five years and which could readily be made into quantitative solutions. It was relatively non-antigenic in comparison with the unheated protein.

Further study by means of spectral absorption (84) and electrophoresis (78) revealed the fact that this preparation still contained considerable nucleic acid and polysaccharide and that the amount varied with different preparations, according to how much was present in the original Old Tuberculin from which it was isolated. It was found also (79) that the nucleic acid migrated in electrophoresis along with the protein as a single component at pH numbers lower than 5.0, but readily separated and travelled with a much greater velocity at more alkaline levels. This suggested that a product with a lower content of nucleic acid might be obtained by precipitating the protein at pH 7.0. Therefore, saturated ammonium sulfate which had previously been neutralized with disodium phosphate was used as the precipitant.

Furthermore, other modifications were later introduced into the process, with the object of making the procedure less drastic and thus of decreasing the amount of denaturation of the protein. For example, the culture was heated in the Arnold sterilizer but the prolonged concentration on the steam-bath was eliminated, and the rest of the procedure was carried out at 5°. The final product was dried from the frozen state instead of by means of ether. It proved to be twice as potent as previous preparations. A very large quantity of the new "Purified Protein Derivative" was made by this method for use as a new official standard for tuberculins. Its preparation was reported by Seibert and Glenn (80). Satisfactory "Purified Protein Derivative" preparations for use in diagnosis made by these methods or slight modifications of them have been reported by Jensen, *et al.* (30) in Denmark, by Doig, *et al.* (18) in England, by Wong and Chu (94) in China, by Vásárhelyi and Gözsy (90) in Hungary, and by Leyva (41) in the Philippines.

CHEMICAL AND IMMUNOLOGICAL STUDIES ON PROTEINS OF OTHER ACID-FAST BACILLI

In comparison with the human type tubercle bacillus, no other group of acid-fast bacilli has received as much study. In fact, very little experimental material is available. Tamura (86) compared the analyses of the amino acids in *Mycobacterium tuberculosis* (human type) with those in *M. lacticola perrugosum* and found lower contents of *l*-phenylalanine and valine but higher arginine and *l*-proline in the latter.

One of the most interesting studies was that of Coghill and Bird (12) on the protein of *M. phlei* (Moeller). They found marked differences in the properties of this protein and that isolated similarly from the (H37) human strain of tubercle bacillus. For example, with the protein of *M. phlei*, no precipitate occurred even in concentrated solutions with acetic acid, and only about one-third as much nitrogen appeared in the aqueous extract or in the 0.5 per cent sodium hydroxide extract as with the protein of the human tubercle bacillus. However, 14 times as much of the water-soluble nitrogen in the *M. phlei* fraction was protein nitrogen as in the case of the tubercle bacillus. Thus, definite differences were demonstrated in the proteins of these two bacilli as well as in their nucleic acids. The nucleic acid of the human type tubercle bacillus contained thymine, whereas that of *M. phlei* contained instead uracil and methyl-cytosine.

Renfrew (56) found that less water-soluble and less alkali-soluble protein as well as less polysaccharide existed in the avian tubercle bacillus than in the human strain.

Since immunological methods are as a rule very sensitive, some serological studies have been made in the hope of differentiating the proteins of the acid-fast bacilli. Serologically by means of the agglutination test, Koch (35) had shown that there is a relationship between the human tubercle bacillus and a large number of other acid-fast organisms, including some from cold-blooded animals, and that this relationship did not exist with other pathogenic bacteria. Recent work has shown that there is probably a serological group-specificity due to the polysaccharides of the acid-fast bacilli, whereas a type-specificity is apparently due to the proteins in these bacilli. For example, the proteins prepared

from cultures of a human, bovine and avian type tubercle bacillus and a timothy hay bacillus by precipitation with ammonium sulfate or trichloracetic acid (68) could be differentiated by means of the precipitin reaction. The specific antigen always gave the highest titer, indicating a definite difference in the chemical composition or structure of these proteins. The fact that there were some very definite cross-reactions indicates that there are probably certain chemical groups common to the proteins in the different acid-fast bacilli. The human and bovine type proteins were the most difficult to distinguish, although even here there was some difference. Later studies were made by Seibert (77) on the potency, precipitin reaction, and acid-base combining capacity of proteins made identically by the trichloracetic acid precipitation method (TPT) from culture filtrates of five different strains of human tubercle bacilli in comparison with the proteins from a bovine, an avian, a timothy hay and two leprosy strains. The potencies of the five human strain proteins differed only slightly in some cases. Serologically they were identical. The acid-base combining capacity curves showed a much closer correspondence between the five proteins from human type tubercle bacilli than between them and the proteins made from the other types of acid-fast bacilli. In view of more recent work, these last experiments should be repeated on fractions more pure with respect to nucleic acid contamination, since the acid-base combining capacity of the latter acid would markedly influence the shape of the curve. Henderson (27) utilized some of the same methods for differentiating 27 strains of acid-fast bacilli, among which were 16 isolated from patients with leprosy, and he was able to make definite groupings, based upon the cross-reactions.

The precipitin test was also used successfully by McCarter, Kanne and Hastings (49) for distinguishing between the proteins of the human, bovine, avian, and two saprophytic acid-fast bacteria isolated from the cow and hog. Protein from Johne's bacillus was also distinguishable from all of these except the avian type. Similar differentiation of the different acid-fast bacilli was made by Menzel and Heidelberger (51). They isolated protein fractions from the living cells of *M. phlei*, and the

avian, and bovine tubercle bacillus by the same method which they had previously used on the human type. Corresponding fractions were distinguishable from each other by means of the quantitative precipitin reaction, but the human and bovine proteins were very closely related.

Potency tests also showed some difference in the proteins from different types of tubercle bacilli. Seibert and Morley (72) precipitated by means of trichloracetic acid the proteins from the tuberculins of three different human strains, one of which was an extremely avirulent type, and they could not distinguish between them in potency when tested in tuberculous guinea pigs. They did, however, find a difference between the potencies of the respective proteins made similarly from bovine, human, and avian tubercle bacillus filtrates, with decreasing potency for tuberculous guinea pigs in the order mentioned. This has recently been confirmed. Purified protein derivatives were made by the recently modified technic referred to above, from bovine, human and avian tubercle bacillus filtrates; the protein from the bovine type proved to be much more potent than that from the human type, and the latter was more potent than the protein from the avian type when tested intracutaneously in human beings. Moreover, the skin reaction with the avian protein was different. The area was soft and diffuse and usually did not reach the degree of induration or severity characteristic of the typical tuberculin reaction, except when the reactions were large.

Some interesting results obtained with the avian purified protein derivative in several series of intracutaneous tests on students have been reported by McCarter, Getz and Stiehm (48). In their study a considerable number of students, especially those coming from farms, reacted to the avian but not to the human purified protein derivative. No student with active tuberculosis failed to react to the human purified protein derivative. Protein from the smegma bacillus filtrate gave results somewhat like those with the avian bacillus protein, and that from the bovine bacillus filtrate reacted like the purified protein from the human bacillus filtrate.

A purified protein derivative prepared from a culture filtrate of

the timothy grass bacillus gave reactions (43) in subjects who required comparatively large doses (0.005 mgm.) of the analogous protein from the human bacillus to cause a reaction. Smaller doses, equivalent to the usual first dose (0.00002 mgm.), gave no reactions.

Similar intracutaneous cross-reactions had been found by Fenger, *et al.* (19) to be caused by the proteins from various acid-fast bacilli. Their fractions had been made from unheated culture filtrates and were purified by means of ammonium sulfate (MA-100). The amounts required to produce reactions varied with the source of the preparation and were as follows: 0.0001 mgm. of bovine, 0.0005 mgm. of human, 0.001 mgm. of avian, and 0.01 mgm. of *M. phlei.* Thus, they also found that the bovine was stronger and the avian weaker than the human type protein.

Recently Aronson, Parr and Saylor (1) have reported intracutaneous cross-reactions in subjects who were highly sensitive to the human purified protein derivative, with 0.00002 mgm. doses of protein fractions (TPTs) of *Mycobacterium phlei, M. smegmatis, M. ranae, M. marinum, M. avium, M. butyricum, M. leprae* and *M. thamnopheos.* There was also evidence of regional variation in the sensitizations. For example, in the Philadelphia and Arizona areas, there were 18.2 and 17.1 per cent reactors to the *M. phlei* protein fraction, in contrast to 0.4 per cent in the South Dakota area. In the same areas, 98, 82 and 54 per cent respectively of those tested reacted to the human purified protein derivative tuberculin. These reactions cannot be considered non-specific, since about 93 per cent of children vaccinated (2) intracutaneously with BCG vaccine became sensitive and reacted even after two or three years to this purified protein derivative tuberculin.

CONCLUSION

Among the proteins of the acid-fast bacilli, none has received as much study as that from the human type tubercle bacillus. Most of these studies have been the result of a search for the specific active principle of tuberculin. The conclusion that this active substance is protein, has been supported by comprehensive

and convincing evidence; and the apparently divergent views previously held that it was associated with mucoprotein, nucleoprotein, proteose, etc. are explained by the existence of a complex interrelationship between the protein, nucleic acid, and polysaccharide molecules in tuberculin. Recent physico-chemical technics have demonstrated a fact frequently suspected, namely that tuberculin proteins of various molecular sizes (10,000 to 32,000) are potent. Thus, the disagreement in the literature as to whether or not the active material is dialyzable has been due to the fact that different investigators were working with potent molecules of different sizes.

Apparently, the biological potency must be inherent in some small, relatively stable part of the protein molecule, and many changes can be effected in the molecule as a whole before there is recognizable loss in the biological activity. Tuberculin in its original form, as produced by the tubercle bacillus may, therefore, be considered a reversibly dissociable component system, which consists of active protein, inactive protein, polysaccharide and nucleic acid. The relative proportions of these various components and properties (potency, specificity, toxicity, antigenicity) in any fraction would depend upon the degree of dilution and the reagents used for isolating the fraction. With this knowledge, it is apparently possible to isolate molecularly homogeneous substances which would repay careful study along the following lines: (a) their precise amino-acid composition, (b) their reactions to specific and highly purified enzymes, and (c) their specific chemical groups responsible for the biological properties which have made tuberculin an interesting and important biological product. Heretofore analyses of this character have been of doubtful significance because of the impurity and heterogeneity of the products studied.

It has been suggested that the mechanism involved in the denaturation of the protein involves a splitting in the unit molecule and then a polymerization of the split, elongated molecules into long thread-like structures capable of forming gels.

From the practical standpoint the chemical studies have led to the isolation of a purified and highly potent protein in large quantity for use as a new official standard for tuberculins. This

product has been designated "Purified Protein Derivative Tuberculin."

The proteins from several other acid-fast bacilli have been isolated by similar methods, and have been shown mainly by immunological methods to differ from the protein of the human type tubercle bacillus. Recent work indicates that a serological group-specificity exists in the polysaccharides, while a type-specificity is due to the proteins of these bacilli.

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