α,α'-Dimethylglutarimide.—Twenty-five grams of methyl methacrylate was condensed with ethyl sodiocyanoacetate as described above and the alcoholic solution of the product was refluxed for ten hours with 40 g. of methyl iodide.[7] Five grams of water was added, then 70 g. of solid potassium hydroxide was introduced and dissolved by refluxing the solution. After refluxing for six hours, the alcohol was completely removed by evaporating to dryness under reduced pressure followed by the addition of 50 cc. of water and another evaporation to dryness. An aqueous solution of the residue was extracted with ether, acidified and again extracted with ether. Evaporation of the second ether extract yielded a crude acid which solidified on standing and was pressed on a porous plate to yield 11.5 g. of impure crystals. No solvent was found from which this acid could be recrystallized readily, but it is believed to have been the dicarboxylic acid formed by decarboxylation of the tricarboxylic acid during the alkaline hydrolysis, since it apparently evolved no carbon dioxide when heated to 230°. Without further purification it was converted to the imide by treatment with acetyl chloride and ammonia as described above. The α,α'-dimethylglutarimide was purified by sublimation and recrystallization to the reported melting point of 172–174°; yield, 4 g.

Anal. Calcd. for $C_7H_{11}O_2N$: N, 9.92. Found: N, 9.90.

2,6-Dichloro-3,5-lutidine.—The reaction of α,α'-dimethylglutarimide with three moles of phosphorus pentachloride occurred readily at room temperature as with the other imides described above except that some unreacted phosphorus pentachloride was left in the mixture at the end of the reaction. The product was recrystallized from dilute acetic acid to a constant melting point of 97–98°. Neither this compound nor 2,5,6-trichloro-3-picoline gave a precipitate with cold or hot alcoholic silver nitrate solution, thus excluding the possibility of side chain halogenation.

Anal. Calcd. for $C_7H_7NCl_2$: Cl, 40.28; N, 7.96. Found: Cl, 40.12; N, 8.09.

Summary

The reaction of phosphorus pentachloride with glutarimide has been shown to occur with the spontaneous evolution of hydrogen chloride to form 2,3,6-trichloropyridine which was subsequently hydrogenated to pyridine.

Similar reactions with α-methyl- and α,α'-dimethylglutarimide with phosphorus pentachloride yielded the corresponding pyridine homologs containing three and two atoms of chlorine, respectively. Based on the earlier reaction they were assigned structural formulas of 2,5,6-trichloro-3-picoline and 2,6-dichloro-3,5-lutidine.

AUSTIN, TEXAS RECEIVED OCTOBER 13, 1942

[CONTRIBUTION FROM THE HENRY PHIPPS INSTITUTE OF THE UNIVERSITY OF PENNSYLVANIA]

Proteins of Tuberculin

BY FLORENCE B. SEIBERT[1,2] AND J. WALTER NELSON

Introduction

The purification and isolation of the tuberculin protein has been difficult for many reasons. In the first place, two colloidal impurities, neither of which has proved to be responsible for eliciting or even contributing to the tuberculin skin reaction, are present in considerable quantities. These are nucleic acid and polysaccharide. Methods have, however, been found[3] for removing them practically completely from the protein with no significant loss in the potency of the protein. The remaining protein is far from being a molecularly homogeneous substance, as can be demonstrated by means of the ultracentrifuge, diffusion and electrophoresis. It is the purpose of this paper to discuss the evidence for the presence of at least two proteins with different properties in this protein mixture.

Experimental Technique.—The electrophoretic technique of Tiselius[4] has been extremely useful in demonstrating the presence of these proteins and in guiding the procedures for isolating them. All mobilities were determined in phosphate buffer, *p*H 7.6 to 7.7, $\mu = 0.1$, and a potential gradient of 9 to 10 volts per cm. Mobilities are recorded in units of cm.^{2} volt^{-1} sec.$^{-1}$.

Evidence for Several Proteins in Tuberculin.—Two or more proteins can be demonstrated to be present in all tuberculins, whether or not the original culture has been heated. Their relative proportions, as can be demonstrated by the electrophoretic diagram, depend upon the source and manner of preparing the tuberculin.

For example, Fig. 1 shows three typical types

(1) Aided by a grant from the Committee on Medical Research of the National Tuberculosis Association.

(2) The data in this paper were used in an address given before the American Chemical Society at Buffalo, New York, on September 10, 1942, on the occasion of the awarding of the Francis P. Garvan gold medal.

(3) F. B. Seibert and J. T. Glenn, *Am. Rev. Tuberc.*, **44**, 9 (1941).

(4) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

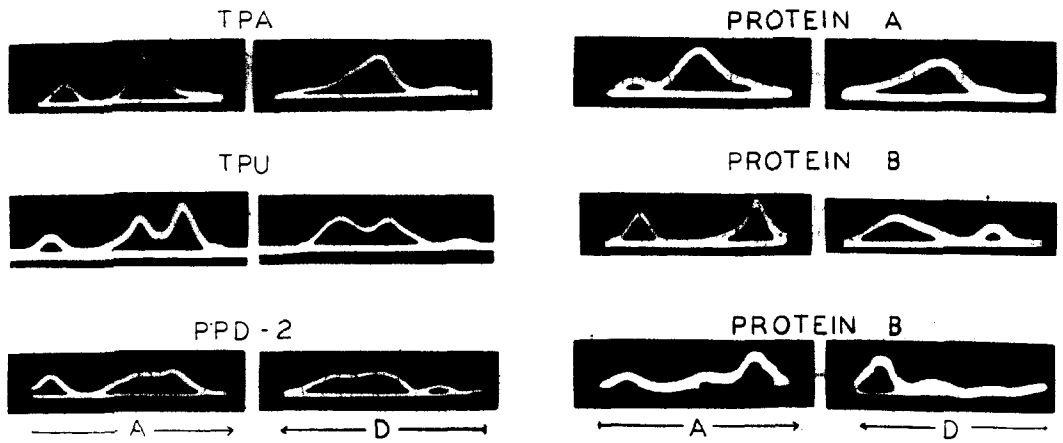

Fig. 1.—Electrophoretic diagrams of three types of tuberculin protein mixtures on the left; of purified proteins A and B on the right.

of partially purified tuberculin protein. TPA represents a fraction obtained[5] from unheated tubercle bacillus filtrate by repeated precipitation with half saturation ammonium sulfate, and obviously contains at least two proteins, the slower one predominating. TPU was made[6] from a similar unheated culture filtrate but in this case a precipitate at *p*H 4.7 was removed and then the supernatant was reprecipitated with half saturation ammonium sulfate. Two proteins were evident in this case, with mobilities similar to those in the TPA, but the faster protein predominated. PPD-2 was made[3] from a culture which had been heated in the Arnold sterilizer for two hours and then filtered free of the bacilli and repeatedly precipitated by half saturation with ammonium sulfate. In this case, both protein components in more nearly equal proportion were present. Table I gives the approximate mobilities observed for these preparations as well as those found for the purified fractions. The slower protein will be designated as A, and the faster one as B. On the right side of Fig. 1 are shown the A and B proteins isolated in relatively pure form, with only a very small amount of the other protein present. These purifications are being continued and improved at the present time and will be described in detail in a future communication. It is becoming clear that additional proteins can be isolated.

TABLE I

The mobilities of the predominating components are in blackface type.

Product	Mobilities of proteins in cm.^{2} volt^{-1} sec.$^{-1}$ × 10^{-5}	
TPA	**−3.7**	−5.9
Protein A	**−3.5**	
TPU	−3.9	**−6.7**
Protein B		**−6.9**
PPD-2	**−3.3**	**−5.8**
Protein B	−4.2 (trace)	**−7.3**

(5) F. B. Seibert and B. Munday, *Am. Rev. Tuberc.*, **23**, 23 (1931).
(6) F. B. Seibert, *J. Biol. Chem.*, **76**, 345 (1928).

Figure 2 demonstrates that a diagram similar to that for human tuberculin protein PPD-2 is obtained with bovine tuberculin PPD which had been made in exactly the same manner. The mobilities in the two cases were practically the same. A preparation made from avian tuberculin by the same method showed, on the other hand, a single broad curve with an intermediate mobility.

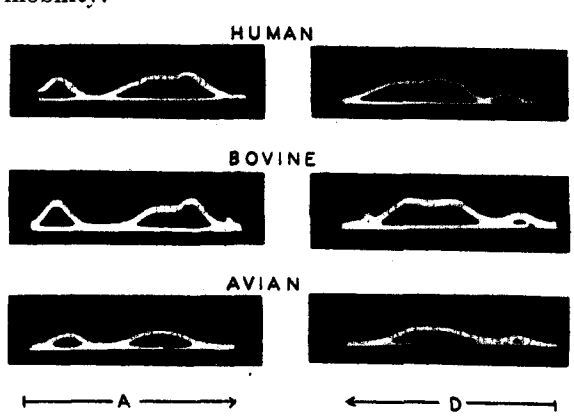

Fig. 2.—Electrophoretic diagrams of purified protein derivative preparations from human, bovine, and avian tubercle bacilli.

Properties of Proteins A and B

Charge and Size of Protein Molecules.—It is obvious that the two proteins A and B differ in chemical composition, as evidenced by the different electrical charges. They furthermore differ

in molecular size. This fact has been demonstrated directly as well as indirectly.

If the sedimentation and electrophoretic diagrams of preparation TPA are compared (Fig. 3), it is obvious that the predominating component sediments at the more rapid rate and is, therefore, the larger molecule. From the electrophoretic diagram it is clear that the predominating component has the slower mobility. Therefore, it would seem that the larger molecule migrates with the slower mobility.

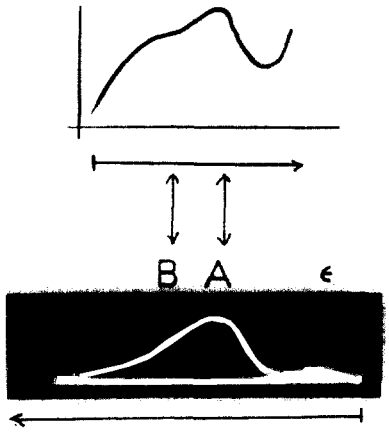

Fig. 3.—Upper curve, sedimentation diagram of TPA. Lower curve, electrophoretic diagram of TPA. The corresponding A and B proteins in the two diagrams are indicated by vertical arrows.

This conclusion is supported by the facts that a protein molecule with a molecular weight of about 32,000 was isolated[7] from TPA and that another molecule which had a mobility of -6.9×10^{-5} was found to have a molecular weight of 16,000.

Denatured Protein Molecules.—During these studies it became apparent that not only could the two molecules, with molecular weights of 32,000 and 16,000, be found in tuberculin solutions, but also many others of many different sizes. It is probably this fact that makes the resolution of the different proteins by means of electrophoresis so difficult. Molecules as small as 6000 to 9000 molecular weight have been isolated[7,8] as well as huge ones. For example, the preparation TPA-1 (see Fig. 4), showed evidence in the ultracentrifuge of two molecules with sedimentation constants of 3.0 and 1.5×10^{-13}. On electrodialysis, in which the hydrogen ion concentration slowly reached *p*H 4.6, a

(7) F. B. Seibert, Kai O. Pedersen and A. Tiselius, *J. Exptl. Med.*, **68**, 413 (1938).

(8) D. Watson, Thesis, University of Wisconsin, 1941.

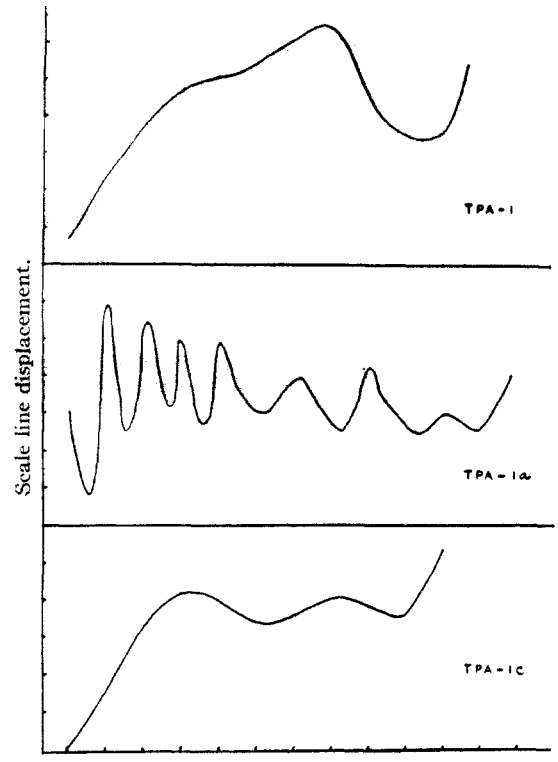

Fig. 4.—Ultracentrifuge diagrams.

fraction, TPA-Ia, settled out. When it was redissolved at neutrality and studied in the ultracentrifuge, molecules with sedimentation constants of 10.4, 15.7, 23.5, 30.4, 36.8 and 46.7 were found. The supernatant, TPA-Ic, corresponded in composition to the original material. These huge molecules had obviously formed from the original smaller ones. In the same way large molecules with very high sedimentation constants were often found in the gel forming on the ultrafilters during concentration. Some of these fractions, such as PPD-b2 previously studied,[7] which has a tendency to gel, have very low diffusion constants in comparison with what they should have if they were spherical molecules. In other words, their molar frictional ratios are extremely high, indicating extensive elongation. Watson[8] emphasized the idea that with denaturation of the tuberculin protein molecule there may first occur an unfolding from the globular form, as postulated by Pauling,[9] and then a break in the opened chain. In this way, the 6000 to 9000 and 16,000 molecules could be formed by splitting of the 32,000 ones, and the huge ones by polymerization.

(9) L. Pauling, THIS JOURNAL, **62**, 2643 (1940)

Solubility data would support the idea that the 16,000 molecules may be a denatured form of the 32,000 ones, since the smaller molecules are insoluble at *p*H 4.9 and lower, whereas the larger ones are soluble at all *p*H's down even to 2.75. Attempts are being made, however, to secure sufficient of the larger size molecule to permit of an attempt to transform it to a smaller one.

Although the less soluble and smaller molecules are found in greatest proportion in heated culture filtrates while the larger ones are found in unheated filtrates, it is probable that heat alone will not prove to be the cause of the transformation, as shown by the following experiment. A preparation PPD-2 (Fig. 1) containing approximately equivalent amounts of the two types of protein, as shown by the electrophoretic diagram, was subjected to prolonged evaporation at neutrality during eight hours on a boiling waterbath. After this treatment the electrophoretic diagram showed the same relative proportion of the two proteins with practically the same mobility and the potency was not materially reduced.

TABLE II

RELATIVE POTENCY OF PRECIPITATED AND SOLUBLE FRACTIONS OF TUBERCULIN PROTEIN

Prepn. number	Average size skin reactions in 6 to 8 tuberculous guinea pigs in millimeters at 48 hours with 0.0005 mg. of Precipitate at *p*H 4.3	Supernatant
1	11 × 11 × 2.7	17 × 16 × 3.4
2	15 × 14 × 2.8	17 × 16 × 3.0
3	16 × 16 × 2.8	17 × 16 × 3.3
4	13 × 14 × 3.0	17 × 16 × 3.5
5	16 × 17 × 3.2	21 × 21 × 3.8

Potency of the Proteins A and B.—The smaller protein molecule (B), which has been shown to be insoluble at *p*H 4.3 to 5.0, has also been found to be less potent as a tuberculin for eliciting the skin reaction in tuberculous animals. Table II illustrates this fact. Furthermore, evidence from experiments made on fractions separated in electrophoresis shows that the fraction with the greater mobility, which is B protein, has the lower potency (Table III).

Immunological Specificity of the Two Proteins.—The larger molecule (A) was also shown[7] previously to be the more antigenic. Nevertheless, both molecules have some antigenicity and evidence is accumulating to show a definite immunological specificity for them. While neither protein has so far been secured in pure form in sufficient quantity to obtain clear-cut immunological data, fractions predominating in one or the other protein, as determined by the electrophoretic diagrams, have yielded antisera which displayed a definite specificity for the predominating component and a low specificity for the smaller component.

Rabbits sensitized with the fraction predominating in the A protein showed marked skin sensitization to this fraction and even to Old

TABLE III

RELATIVE POTENCY OF FASTER AND SLOWER PROTEIN FRACTIONS SEPARATED IN ELECTROPHORESIS

Original tuberculin	Separated fractions	Dosage, mg.	Average size skin reaction in millimeters	Number of tuberculous guinea pigs tested
TPU-PN (1)	Faster protein	0.005	12 × 12 × 1.3	5
	Slower protein	.005	15 × 17 × 1.9	
TPU-PN (2)	Original solution	.0005	22 × 21 × 3.0	4
	Faster protein	.0005	20 × 17 × 2.6	
TPU-PN (2)	Original solution	.005	27 × 21 × 3.8	6
	Faster protein	.005	18 × 18 × 2.8	
PPD-72-2	Faster protein	.005	14 × 15 × 2.9	7
	Remaining fraction	.005	16 × 16 × 2.8	
			Reaction from intraperitoneal injection	
TPU-PN (1)	Original material	2.0	Died	1
		1.5	Lived	1
		1.0	Lived	1
	Faster protein	2.0	Lived	2
		1.5	Lived	3
	Slower protein	2.0	Died	1
		1.5	Died	1
		1.0	Died	1
TPU-PN (2)	Original material	0.5	Died	1
	Faster protein	0.5	Lived	1

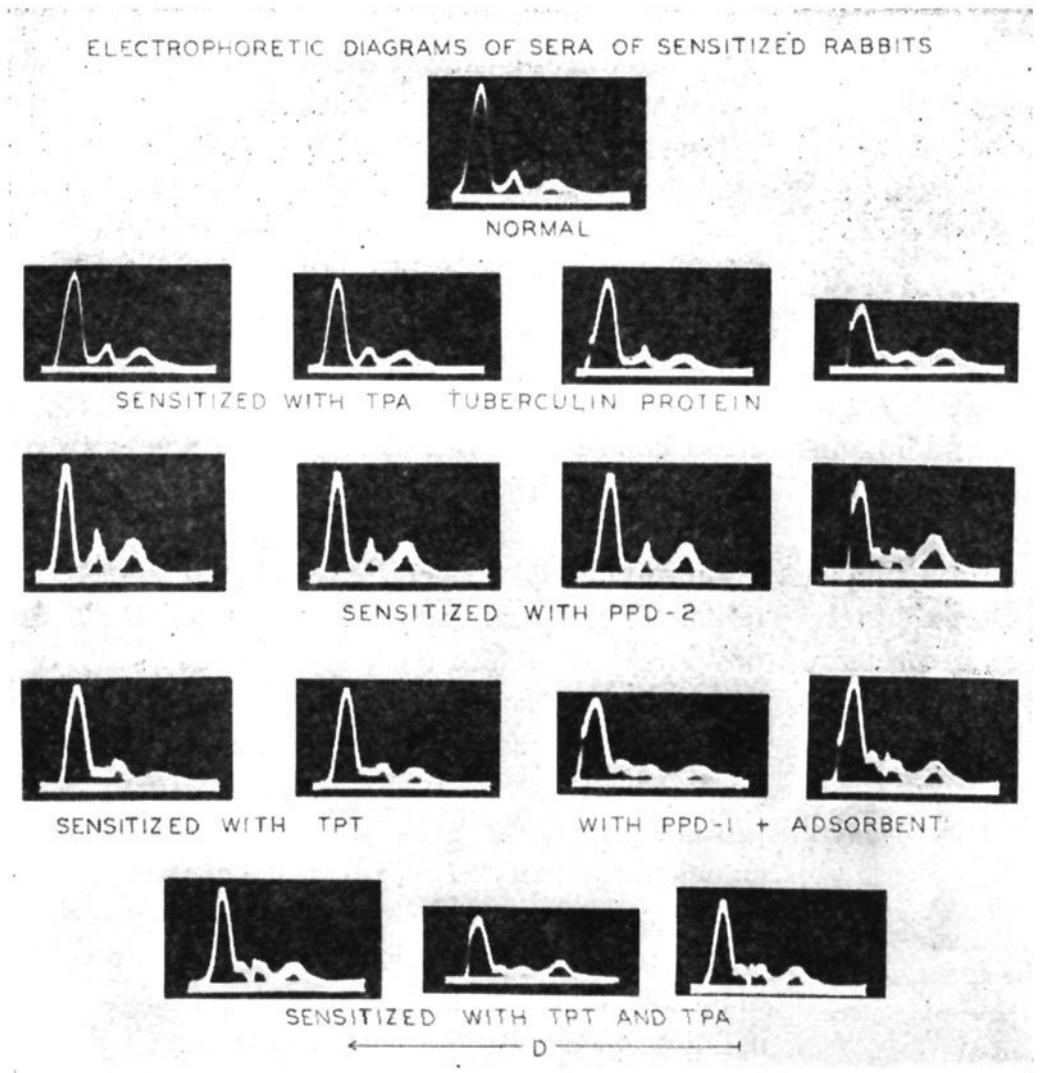

Fig. 5.—Electrophoretic diagrams of sera of rabbits sensitized with tuberculin protein preparations predominating in A or B proteins.

Tuberculin. Those sensitized with the fraction predominating in B protein showed none or slight sensitization.

TABLE IV

CROSS PRECIPITIN REACTIONS WITH A AND B PROTEINS

Antisera made by immunization with	Relative amounts of A and B	Precipitin titers with TPA	Precipitin titers with PPD-1
TPA	A Large	100,000	0
	B Small	400,000	2,000
		400,000	10,000
		100,000	0
PPD-1	A Small	200	400
	B Large		
PPD-1 adsorbed to $Al(OH)_3$	A Small	40,000	400,000
	B Large	100,000	400,000
PPD-2	A Moderate	30,000	10,000
	B Moderate	20,000	

TABLE V

QUANTITATIVE CROSS PRECIPITATION OF ANTIBODIES TO A AND B PROTEINS

Antigen	Milligrams of nitrogen in precipitate, per ml. antisera to TPA	Milligrams of nitrogen in precipitate, per ml. antisera to PPD-1
TPA	0.149	0.063
PPD-1	.007	.035

Table IV gives the evidence for specificity obtained from precipitin titers and Table V shows in a quantitative manner that the specific antigen gives the larger amount of precipitate with the antisera.

A similar specificity appears in studies of the antibodies by means of the Tiselius electrophoresis technique. In Table VI and Fig. 5 it is seen that antisera made by immunization with TPA, which predominates in the A protein, show as a rule a higher percentage of gamma globulin than is found in normal sera. On the other hand, the percentage of alpha globulin is, in general, higher if the animal has been immunized with fractions predominating in B protein. In a number of cases there are increases in both alpha and gamma globulins, and this is to be expected, considering the state of purity of the products.

In several cases the sera were precipitated with the specific antigen, the precipitate removed, as was done by Tiselius and Kabat[10] with the pneumococcus antisera, and the supernatant again run in electrophoresis. Table VII gives the results. In these cases the actual milligrams per ml. of serum were calculated for the original and for the precipitated sera. Since a total volume change may occur during dialysis of the sera when equilibrating against the buffer, the total concentrations of the precipitated sera were corrected to that of the original sera, and in this way it is easier to note a significant decrease in the quantity of any component. The corrected figures are therefore recorded in Table VII instead of the actual figures obtained. The respective decreases in components are recorded as differences in the appropriate columns.

It will be noted that, in general, the largest decreases were found in the slowest components (gamma and beta), when the rabbit had been immunized with the fraction predominating in the A protein, and the largest decreases were found in the faster components (albumin and

(10) A. Tiselius and E. A. Kabat, *J. Exptl. Med.*, **69**, 119 (1939).

TABLE VI

ELECTROPHORETIC ANALYSES OF VARIOUS IMMUNE SERA

Rabbit number	Product used for sensitization	Total protein concentration	Per cent. of total protein as				Relative amounts of A and B proteins
			Albumin	Globulins α	Globulins β	Globulins γ	
35 rabbits	Normal range	5.6	75.0	2.4	10.8	11.9	
		4.4–6.9	68.4–81.5	0–4.7	7.9–15.1	7.9–15.5	
2083	TPA	6.5	58.0	7.9	14.1	20.1	A Large
140	TPA	6.5	70.6	5.2	10.2	13.9	B Small
143	TPA	7.5	67.7	3.4	10.5	18.6	
A6=3	TPA	6.8	66.9	1.8	13.1	18.3	
162	PPD-2	6.0	58.9	2.1	13.9	25.1	A Moderate
164	PPD-2	6.8	62.7	3.7	11.2	22.5	B Moderate
170	PPD-2	5.7	59.3	5.7	12.7	22.6	
689	PPD-2	7.0	52.9	8.6	9.4	29.1	
157	PPD-1 + charcoal	6.3	60.6	11.2	12.4	15.7	A Small
148	PPD-1 + $Al(OH)_3$	5.7	63.2	9.6	12.8	14.4	B Large
82	TPT	5.8	67.7	8.6	12.2	11.5	A Small
84	TPT	7.2	70.7	6.9	10.4	12.0	B Large
3	TPT + TPA	5.8	59.8	8.5	14.6	17.1	
7	TPT + TPA	5.4	61.0	6.7	11.6	20.7	
196	TPT + TPA	5.0	64.0	8.7	12.6	14.8	

alpha) when the immunization was caused by the component predominating in B protein. Slight decrease in both of these components was found in the serum of a rabbit sensitized with B. C. G.

The correlation of these results with the amount of actual specific precipitate obtained was good in some and not in other cases, and more results must be obtained before any conclusion can be drawn as to the reliability of this method of study. It is not yet known, for example, what is the nature of the specific precipitate, its solubility in the solvent used for washing, etc.

These studies indicate that possibly two antibodies to the tuberculin proteins A and B may be present in the serum, one travelling in electrophoresis with the slowest components, and the other with the fastest components.

Identification of Antibodies in Isolated Serum Fractions.—Since all of the sera gave precipitins with the tuberculin proteins it was interesting to isolate the slowest and the fastest components and make an attempt to determine with which the antibodies were associated.

The following separations were made. Antisera from three rabbits sensitized with PPD-2, and having a high gamma content, were pooled and precipitated with 18% sodium sulfate, the precipitate redissolved and reprecipitated with 15% sodium sulfate and again redissolved and dialyzed against buffer, according to the method of Kekwick and Record[11] for obtaining gamma globulin. The electrophoretic diagram showed this fraction to be chiefly gamma globulin with only a small amount of beta globulin. The supernatant was saturated with sodium sulfate and the precipitate dissolved and dialyzed against buffer. This contained the remaining proteins and only a trace of the gamma fraction, as demonstrated by its electrophoretic diagram. This gamma fraction, as well as the whole immune serum, precipitated the protein antigen, agglutinated live tubercle bacilli, and inhibited the growth of tubercle bacilli *in vitro*, whereas the remaining fraction did not. Similar evidence that antibody can be found in the gamma fraction was obtained with this fraction when it was isolated by means of electrophoresis, instead of by precipitation.

The conclusion that the rise in the gamma fraction in these immune rabbits was due at least in part to antibody is strengthened by the fact that when an increased gamma fraction from the pleural fluid of a tuberculosis patient was isolated by precipitation, or when the gamma fraction from the serum of a patient with sarcoidosis was isolated by electrophoresis, or when whole normal rabbit serum was studied, no evidence of precipitins or agglutinins was found. Furthermore stimulation, rather than inhibition of the growth of tubercle bacilli *in vitro*, was found with these fractions.

Acknowledgment.—Part of the work was made possible through a gift from George H. Jackson, in memory of his wife, Amy Jackson.

(11) R. A. Kekwick and B. R. Record, *Brit. J. Exptl. Path.*, **22**, 29 (1940).

TABLE VII

EFFECT OF SPECIFIC PRECIPITATION AS SHOWN IN ELECTROPHORESIS

Rabbit number	Product used for sensitization	Antigen used for precipitation	Milligrams per ml. of serum				
			Total	Albumin	Globulins α	Globulins β	Globulins γ
35 rabbits	Normal range		56.0	42.0	1.3	6.1	6.7
			44.0-69.0	38.3-45.6	0-2.6	4.4-8.4	4.4-8.7
2083	TPA		66.0	38.2	5.3	9.0	13.7
		TPA	66.0	38.7	5.3	8.9	13.2
						0.1	0.5
140	TPA		63.1	42.6	4.1	7.3	9.1
		TPA	63.1	43.3	4.0	7.3	8.5
					0.1		0.6
170	PPD-2		65.2	37.0	2.8	9.8	15.6
		PPD-2	65.2	38.4	3.1	9.3	14.4
						0.5	1.2
164	PPD-2		52.4	31.7	2.6	7.5	10.6
		PPD-2	52.4	32.0	3.1	7.2	9.7
						0.3	0.9
162	PPD-2		69.0	40.0	5.9	6.0	17.4
		PPD-2	69.0	39.2	7.3	6.5	15.9
				0.8			1.5
A6=3	PPD-2		60.7	35.5	1.6	10.2	13.3
		PPD-2	60.7	36.7	1.7	10.6	11.7
							1.6
84	TPT		71.6	50.6	4.9	7.4	8.6
		TPA	71.6	48.9	5.7	8.3	8.7
				1.7			
3	TPT + TPA		58.3	34.9	5.0	8.5	10.0
		TPA	58.3	35.2	4.2	8.7	10.25
					0.8		
196	TPT + TPA		50.0	32.0	4.3	6.3	7.4
		TPA	50.0	30.1	4.9	6.9	8.2
				1.9			
141	BCG		50.8	32.4	3.0	7.3	8.1
		TPA	50.8	32.8	2.7	7.6	7.7
					0.3		0.4

All determinations were made in phosphate buffer. $\mu = 0.1$, pH 7.7 on serum diluted 1:4.

Summary

Tuberculin contains at least two protein components which can be distinguished by their different mobilities in electrophoresis. The slower one appears to be the larger molecule, with a molecular weight of about 32,000, and the faster one appears to have a molecular weight of 16,000. The larger one is more potent as a tuberculin and more antigenic, but both possess an immunological specificity.

Immunization of rabbits with the larger molecule produced sensitization to the protein and even to Old Tuberculin and elicited the presence of antibodies in the gamma component of their sera. This was shown by means of electrophoresis and by the demonstration that antibodies existed in the isolated gamma component.

There is some electrophoretic evidence to indicate that the smaller protein molecule may elicit antibodies which appear with the faster serum components, alpha globulin or even albumin.

PHILADELPHIA, PA. RECEIVED SEPTEMBER 14, 1942