The tritylation method is preferred over the tosylation and iodination method since it is a single step reaction and fewer variables are involved.

Experimental

Materials

Hydrolyzed Cellulose Acetate.—Cotton linters were acetylated as previously described[5] with acetic anhydride and an amount of sulfuric acid equal to 7% of the weight of the cellulose. At the completion of the esterification sufficient acetic acid:water (2:1) was added to provide 7–8% water in the system. The hydrolysis solution was maintained at 100° F. and samples were removed from time to time. The cellulose acetate was precipitated and washed in distilled water.

Some changes were made in the acetylation conditions when variations were made in the amount of catalyst used. The liquid–solid ratio in the esterification bath was maintained at 9.2 to 1, and the amount of water in the hydrolysis bath at 7–8%.

Other hydrolysis series were initiated with commercial cellulose acetate to obtain higher water content during hydrolysis. As an example, 400 g. of cellulose acetate, 40.4% acetyl, was dissolved in 2400 ml. of 70% acetic acid at 100°F. A solution of 9.6 ml. of concentrated sulfuric acid (7% by weight, based on the cellulose content of the cellulose acetate) in 400 ml. of 70% acetic acid was added rapidly with stirring and the solution maintained at 100° F. during hydrolysis. Samples containing less than 1.5 acetyl groups per glucose unit were very water susceptible and were precipitated and washed in methanol.

Trityl Chloride.—This product was prepared by the Friedel and Crafts reaction.[6] Additional purification was effected by recrystallization from cyclohexane; m. p., 111° from the cooling curve of the melt.

Tritylation.—Ten-gram samples of cellulose acetate, dried at 105°, were dissolved in 50 ml. of anhydrous pyridine (water content not over 0.1% by the Karl Fischer method) and an amount of trityl chloride added according to the hydroxyl content of the cellulose acetate. The mixture was heated to 70–75° and the trityl chloride brought into solution by occasional stirring or inverting of the container. After maintaining at that temperature for twenty-four hours in an electric oven, the solutions were diluted with acetone, precipitated and washed in methanol. Reprecipitation of representative samples from acetone into methanol showed trityl content unchanged, hence this step was not included in routine procedures.

Trityl analyses were carried out according to the method of Hearon.[2a]

(6) "Organic Syntheses," **23**, 102 (1943).

Summary

1. The primary hydroxyl contents of hydrolyzed cellulose acetates of different acetyl contents have been determined by reaction with trityl chloride.

2. The percentage of primary hydroxyl is influenced by the amount of water in the hydrolysis bath. An increase in water concentration gives an increase in percentage of primary hydroxyl.

3. The percentage of primary hydroxyl is not affected by the temperature, or by the amount and nature of the catalyst during the hydrolysis.

4. The amount of sulfuric acid catalyst in the *esterification* influences the percentage of primary hydroxyl in the early part of the hydrolysis.

ROCHESTER 4, N. Y. RECEIVED OCTOBER 3, 1949

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

Comparison of the Filtrate Fractions and of Certain Bacillary Extracts of the H 37, H 37 RV and H 37 RA Strains of Tubercle Bacilli[1]

BY FLORENCE B. SEIBERT, CRETYL CRUMB AND MABEL V. SEIBERT

The electrophoretic technique furnishes a simple means of determining the proportions of different components in any tuberculin. This is conspicuously shown in the case of samples of culture filtrates from tubercle bacilli grown on synthetic medium, filtered free of bacilli through a Seitz filter, and concentrated by ultrafiltration in the cold. Fractions with similar mobilities have already been isolated and studied separately and their chemical properties ascertained.[2] For example, the accompanying picture (Fig. 1) shows these components, which have been studied under identical electrophoretic conditions of *p*H 7.6, μ 0.1, in phosphate buffer, superimposed over each other so that the relative distances traveled from the start are indicative of their relative mobilities. They have been characterized further in order of slowest to fastest components, as polysaccharides I and II, A protein, B and C proteins with approximately the same mobility, a number of nucleo-proteins, and finally free nucleic acid itself.

(1) Presented before the Division of Biological Chemistry of the American Chemical Society, Atlantic City, September 1949.

(2) F. B. Seibert, *Am. Rev. Tuberc.*, **59**, 86 (1949).

Experimental

Comparison of Components in Different Batches of Filtrates from the Same Strain or from Different Strains.—It is possible to see the relative proportions of the components in the filtrates from the same strain made under identical conditions at different times, as well as in the filtrates made from different strains. It was interesting to find a very close similarity in the filtrates made from the same strain at different times but not in the filtrates from certain other strains; see Fig. 2. For example, it is clear, even without any isolation studies or mathematical evaluation of the curves, that the DT strain yields far more protein in proportion to polysaccharide than does the H 37 strain, which yields a relatively high percentage of polysaccharide. Furthermore these results were reproducible.

Comparison of Components in Filtrates of H 37, H 37 Rv and H 37 Ra.—A study similar to the one above was made of the H 37 strain, as well as of the virulent (H 37 Rv) and avirulent (H 37 Ra) dissociates of this strain, isolated by W. Steenken at the Laboratory of the Trudeau Sanatorium. We express our thanks to him for furnishing us with these dissociants.

About 100-liter bottles each of Long synthetic medium were planted with the three strains of tubercle bacilli and incubated eight weeks. Then the bacilli were filtered off

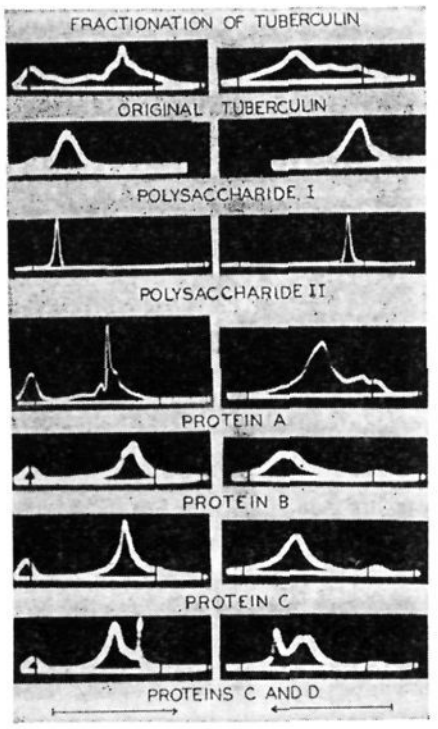

Fig. 1.—Fractionation of tuberculin.

alive, first through paper and then through the Seitz filter. The filtrates were concentrated by ultrafiltration in the cold room at 1 to 3° to 100 to 150 ml.

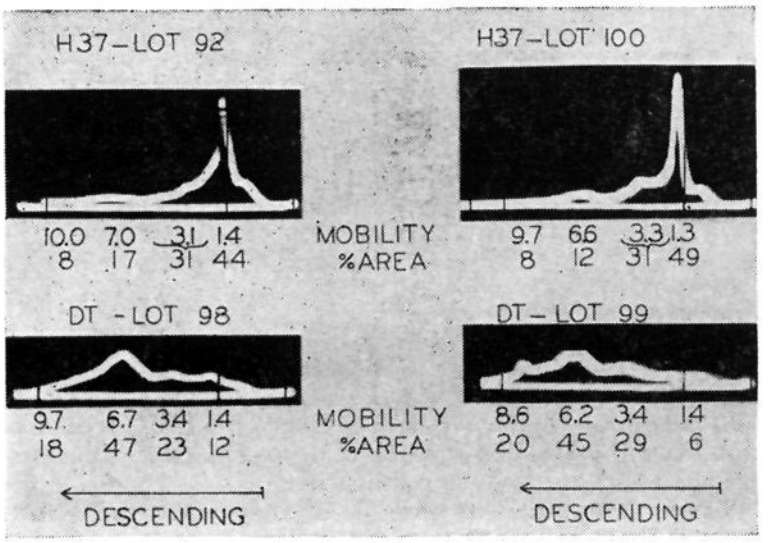

Fig. 2.—Components in tuberculins dependent upon batch and strain.

The H 37 Rv bacilli showed very different growth characteristics from the H 37 Ra. For example, the growth was thin, and continuously spread over the surface of the medium, even creeping up the side of the bottle. The liquid was much more opalescent and had a lighter amber color than in the case of the H 37 Ra. The latter culture grew in more discrete, dense clumps, and the odor was sharper.

Figure 3 shows the electrophoretic diagrams of samples of the unheated concentrated filtrates of these three strains. It can be seen that the H 37 Rv strain yielded proportionally less of the slow as well as the fast proteins but considerably more of the polysaccharide with the sharp peak, presumably polysaccharide II, than did the H 37 Ra. The H 37 diagram appears to be a composite of the other two, as would be expected. The mobilities of the corresponding peaks (for convenience shown directly beneath the respective peaks) are well within the experimental error to be expected in cases of such heterogeneous mixtures, and the percentage areas calculated for the integrated peaks (shown directly beneath the mobilities) bear out the relative comparison of component concentrations

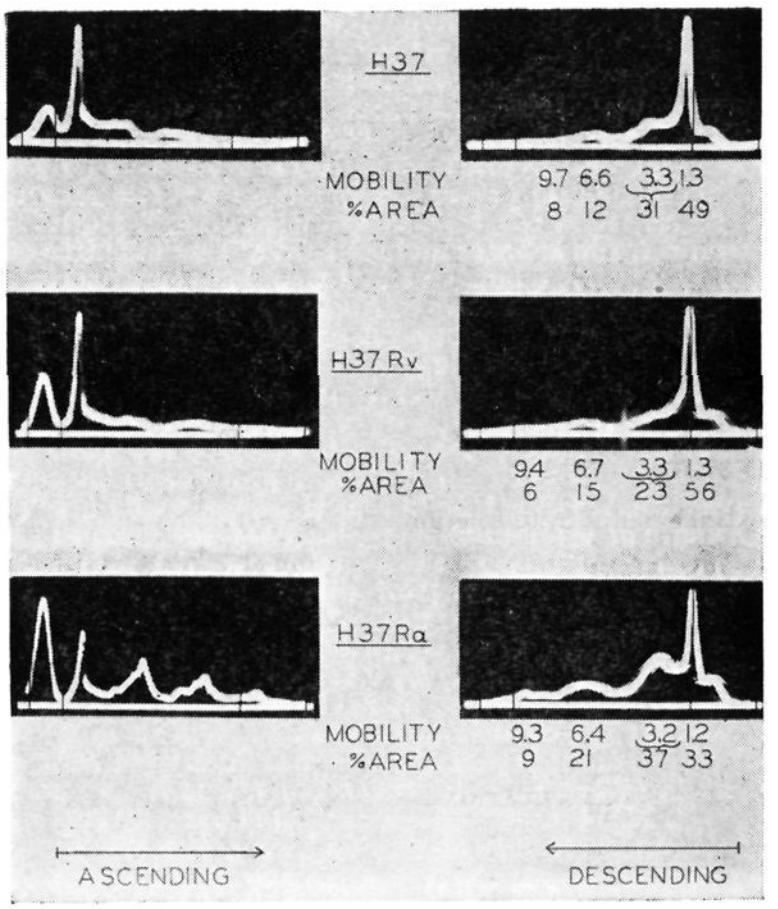

Fig. 3.—Comparison of H 37, H 37 Rv and H 37 Ra concentrated unheated filtrates.

expressed above. Table I gives a summary of the yields, nitrogen contents and relative percentages of the electrophoretically distinguishable components, in relation to their mobilities. When the grams of protein isolated are calculated from the total yields of solids and their nitrogen contents, as shown in Table II, it becomes evident that more protein was actually obtained from the H 37 Rv than from the H 37 Ra strain since the total yield of the latter was less. The chief difference between the two strains lay in the great excess of polysaccharide, represented mainly by the slowest electrophoretic component, yielded by the H 37 Rv strain.

Comparison of Fraction Extracted from the Bacillary Bodies.—An attempt was made to determine whether differences might exist in the bacillary bodies themselves of the H 37, H 37 Rv and H 37 Ra strains. Accordingly an extraction of the bacilli by heating with water was made as follows.

To the live and unwashed bacilli remaining in the bottles after the original liquid was drained off for the above studies, distilled water was added and the suspensions were autoclaved for a half-hour at 15 pounds pressure. After this the liquids were deep greenish-amber in color. They were filtered free of the bacilli, first through paper and then through the Seitz filter, and concentrated by means of ultrafiltration in the cold.

A comparison of the electrophoretic diagrams of these heated aqueous bacillary extracts (Fig. 4) revealed significant differences between the strains. For example, the extract of the H 37 Rv bacilli showed the presence of more polysaccharide II relatively, as seen in Table I, and actually, as seen in Table II, than did the H 37 Ra strain. The actual amounts of the slow protein with mobilities around 3.1 to 3.9 were the same in both cases, but more of the faster proteins, with mobilities 5.8 to 6.0, was found in the H 37 Rv than in the H 37 Ra strain. The proportion of mostly free nucleic acid with mobility 13.3 to 15.6 appeared lower in the H 37 Rv strain, but the actual yields were the same with the two strains.

Fractionation of the H 37 Concentrated Filtrate.—In a previous study[2] it was shown that fractionation during which the *p*H, concentration of components, alcohol con-

TABLE I

ANALYSES OF UNHEATED AND HEATED COMPONENTS OF H 37, H 37 Rv AND H 37 Ra FILTRATES

Tuberculin from strain	Total solids yield, g.	Nitrogen, %	Electrophoretic components, % of total as components with mobilities (descending diagrams) (cm.^{2} volt^{-1} sec.$^{-1}$) of 1.2–1.6	2.1–2.8	3.1–3.9	4.0–4.9	5.4–5.8	6.0–6.7	7.7–8.1	9.1–9.7	10.8	13.3–15.6
Unheated filtrates												
H 37	17.3	5.7	49	11	11	8		12		8		
H 37 Rv	16.0	5.9	56	16		7		15		6		
H 37 Ra	10.8	7.3	33		37			21		7		2
Aqueous heated extracts												
H 37	12.3	9.7	35		17		17		5			26
H 37 Rv	7.0	10.4	32		17		29		4			18
H 37 Ra	5.2	9.5	26		24			23				26
Unheated filtrate fractions												
H 37–I		15.1			4			73		11	12	
II		7.3	16	11	62			11				
III		3.2	45	35	20							
IV		5.3	47	26	10	6		11				
4		0.02	100									

TABLE II

CALCULATION FOR NON-PROTEIN CONSTITUENTS ACCOMPANYING ELECTROPHORETIC COMPONENTS

	Total solids, g.	Total protein based on nitrogen, g.	Total grams based on percentage composition in different electrophoretic components with mobilities (descending diagrams) of 1.2–1.6	2.1–2.8	3.1–3.9	4.0–4.9	5.4–5.8	6.0–6.7	7.7–8.1	9.1–9.7	10.8	13.3–15.6	Total g. in all but 1st component	App. non-protein, g.
Unheated filtrates														
H 37	17.3	6.16	8.5	1.9	1.9	1.4		2.1		1.38			8.63	2.47
H 37 Rv	16.0	5.90	9.0	2.6		1.1		2.4		0.96			7.06	1.16
H 37 Ra	10.8	4.92	3.6		4.0			2.3		0.76		0.22	7.28	2.36
Heated filtrates														
H 37	12.3	7.46	4.3		2.1		2.1		0.62			3.2	8.02	0.56
H 37 Rv	7.0	4.55	2.2		1.2		2.0		0.28			1.3	4.78	0.23
H 37 Ra	5.2	3.09	1.4		1.2			1.2				1.4	3.80	0.71

centration and temperature were controlled, yielded two different polysaccharides, I and II, protein fractions of different mobilities, nucleoproteins and nucleic acid. Of all these fractions the most complex and obviously the most impure were the proteins of low mobility. A still further fractionation, especially of these proteins, has been made in this study on the H 37 (Lot 100) unheated concentrated filtrate (see Fig. 5).

The fractionation procedure was as follows.

The filtrate (27.3 liters) from 204-liter bottles of H 37 grown for eight weeks on Long synthetic medium at *p*H 7.0 (6.4 at the end of the growth) was a deep amber color and opalescent but not contaminated. The bacilli were filtered off alive through paper and then through the Seitz filter, and the filtrate taken to the cold room at 1 to 3° for all further fractionations. It was concentrated on guncotton ultrafilters to 164 ml. This filtrate was examined in electrophoresis as shown in Fig. 5.

The concentrated solution was adjusted to *p*H 4.1 with acetic acid and the resulting precipitate I centrifuged off, and washed twice with acetate buffer of the same *p*H. This protein C (Fig. 5, Fraction I) had a mobility of 6.6, similar to the C protein fractions repeatedly isolated previously.[2]

The supernatant was very opalescent. It was adjusted to *p*H 6.5 and filtered through the Seitz filter to remove a very fine and insignificant precipitate. After filtration sufficient 95% alcohol was added to give successive concentrations of 30, 40 and finally 50% alcohol, until a heavy white precipitate (Polysaccharide II, Fraction V) resulted. This was centrifuged off and washed twice with 50% alcohol. When studied in electrophoresis, it had a mobility of 1.3. For further information concerning its properties see a previous publication.[3]

The supernatant was filtered through paper and a test showed that if the solution were adjusted to *p*H 4.0, no precipitate which would correspond to protein B in the previous fractionations,[2] resulted.

Therefore, it was decided to precipitate everything possible with 70% alcohol at *p*H 6.5. A heavy precipitate resulted and it was centrifuged off and redissolved in water. A test on this solution showed no precipitate when adjusted to *p*H 4.0. On the addition of sufficient saturated neutral ammonium sulfate to give half-saturation, a heavy white precipitate resulted, which is Fraction II in Fig. 5. It was centrifuged off, suspended in phosphate buffer *p*H 7.7, μ 0.1, dialyzed free of sulfate, and then clarified by filtration through the Seitz filter.

The supernatant from this half saturated precipitate was filtered, dialyzed free of ammonium sulfate, and concentrated by ultrafiltration to a small volume of clear yellowish fluid. This was called Fraction III and its electrophoretic diagram is shown in Fig. 5.

The entire supernatant from 70% alcohol precipitate also was dialyzed in sacs until free of alcohol and then concentrated by ultrafiltration to a small volume, refiltered and studied in electrophoresis. It was light yellow in color. This was Fraction IV in Fig. 5.

Examination of these electrophoretic curves reveals the

(3) F. B. Seibert, M. Stacey and P. W. Kent, *Biochimica et Biophysica Acta*, **3**, 632 (1949).

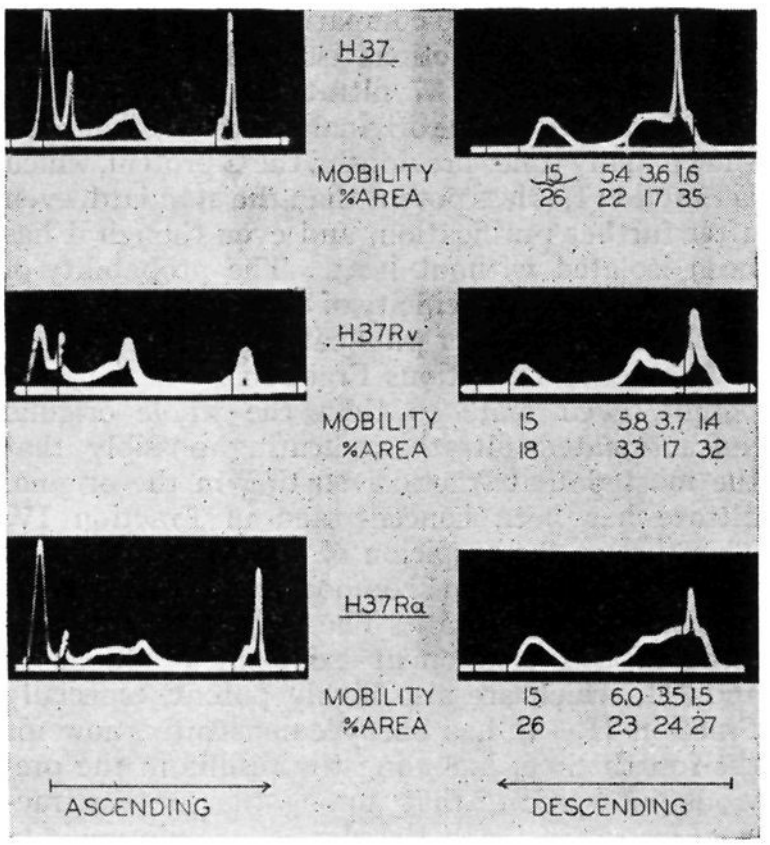

Fig. 4.—Comparison of H 37, H 37 Rv and H 37 Ra heated aqueous bacillary extracts.

fact that the II, III and IV fractions display progressively slower major protein fractions, in addition to the polysaccharide fractions. While the mobilities and concentrations measured on the ascending sides are obviously much less accurate than they are on the descending sides, the resolution of components is better, as seen in Fig. 5. Thus Fraction II contains a major component with mobility 4.8, Fraction III with a mobility of 4.0 and Fraction IV one with mobility 2.5. These may correspond to mobilities on the descending sides of 3.7, 3.3 and 2.1, respectively. This separation assumes greater significance when considered in conjunction with the potencies of the fractions described in the next section.

Further chemical study of these fractions is in progress and will appear in a separate communication. The low nitrogen contents of the II, III and IV fractions (Table I) indicate that a large percentage of these fractions is of non-protein nature and is undoubtedly carbohydrate.

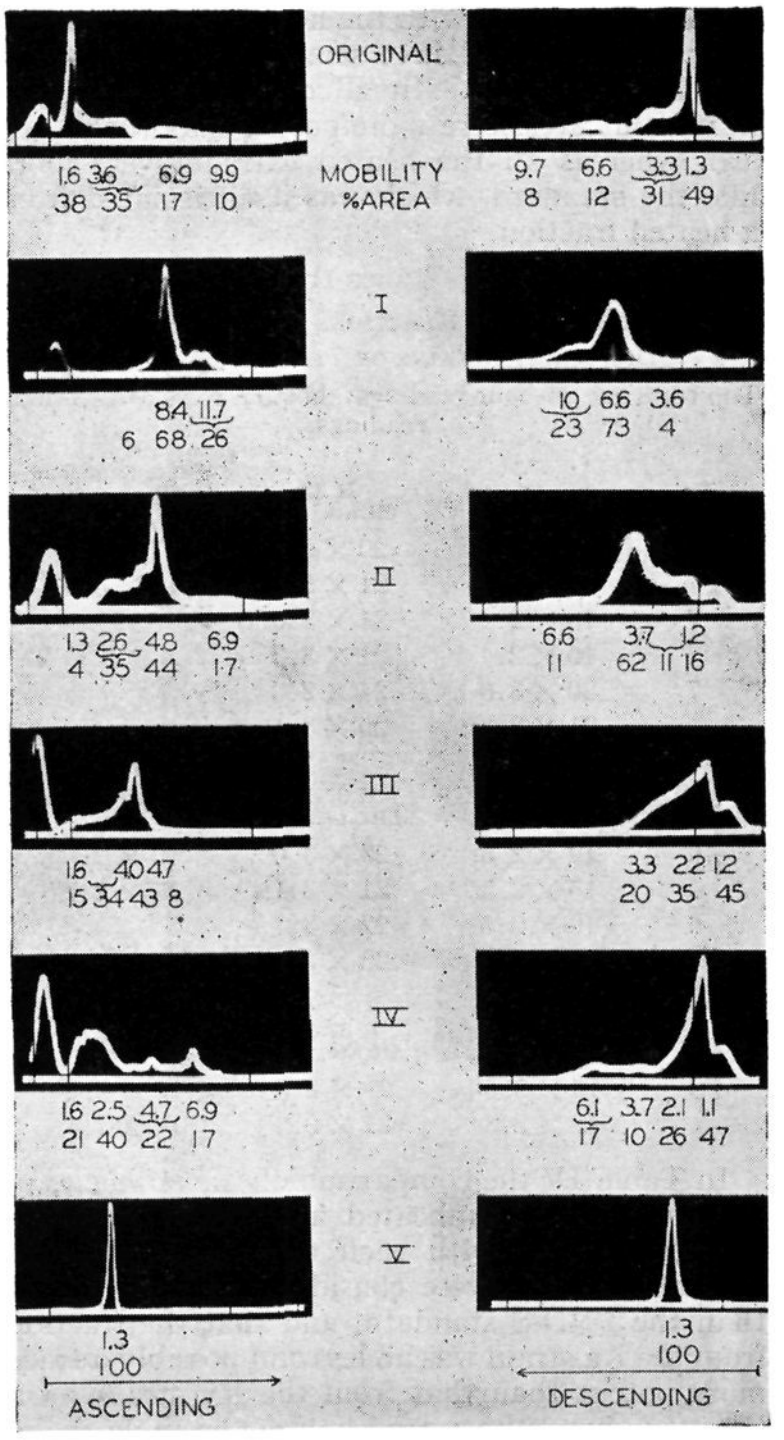

Fig. 5.—Fractionation of tuberculin 100.

Comparative Potencies of Fractions

Simultaneous intradermal tests on at least 6 guinea pigs known to be highly sensitive to tuberculin, because of previous inoculation with tubercle bacilli, were made in each series of experiments with a number of fractions. Of each fraction, 0.1 ml. containing either 0.002 mg. for more sensitive guinea pigs, or 0.005 mg. for less sensitive guinea pigs, of protein, calculated from its nitrogen content, was injected, and, therefore, comparison of the relative potencies in terms of protein could be made.

The reactions were read at twenty-four and forty-eight hours, the former being recorded in the tables as the top reactions and the latter as the lower reactions. The reactions were measured in millimeters as the longest diameter and then the diameter at right angles to this diameter. The depth of the reaction was also estimated in millimeters. For the sake of ease in comparison, only the average of the two superficial diameters is recorded, along with the depth of the reaction. In all series a test with the standard PPD-S was included. Only the average of the reactions found in the six to nine guinea pigs in each series is recorded.

While the size of the tuberculin reaction is not proportional to the dose administered, a difference of 10% in any of these recorded reactions means a highly significant difference in the potency of the fractions tested. In fact, it may indicate as much as a 40 to 50% difference in actual potent material present when small test groups are used. For a more comprehensive interpretation of the tuberculin test see the review on the Progress in the Chemistry of Tuberculin.[4]

Table III shows the comparison of the potency of the standard with the unheated tuberculin

(4) F. B. Seibert, *Advances in Tuberculosis Research*, **1**, in press (1950).

filtrates, as well as with the heated aqueous bacillary extracts of all three strains, the H 37, H 37 Rv and H 38 Ra. In all cases all of the unheated filtrates were more potent than the standard, whereas all the heated extracts were more like the standard, which was itself isolated from a heated fraction.

TABLE III

COMPARISON OF SKIN REACTIONS WITH FRACTIONS FROM DIFFERENT STRAINS OF TUBERCLE BACILLI

Top rows are 24-hour readings; bottom rows are 48-hour readings.

No. pigs	PPD-S, mm.	H 37 filtrate, mm.	H 37 heated aqueous bacillary extract, mm.
7	16 × 1.8	20 × 2.3	17 × 2.1
	18 × 2.7	21 × 3.0	18 × 3.0
7	18 × 2.4	21 × 3.0	
	16 × 2.4	21 × 3.3	
7	20 × 3.0	24 × 2.6	
	21 × 3.3	25 × 3.1	
	PPD-S, mm.	H 37-Rv filtrate, mm.	H 37-Rv heated aqueous bacillary extract, mm.
9	19 × 2.5	23 × 3.0	19 × 2.7
	15 × 2.3	21 × 3.1	17 × 2.7
6	19 × 2.5	22 × 3.1	
	19 × 3.0	22 × 3.3	
	PPD-S, mm.	H 37-Ra filtrate, mm.	H 37-Ra heated aqueous bacillary extract, mm.
8	19 × 2.5	21 × 2.5	17 × 2.4
	17 × 2.3	21 × 2.3	16 × 2.4

In Table IV the comparison of the potencies of the concentrated unheated filtrates of the three different strains with each other showed again that all of them were considerably more potent than the PPD-S standard, and that the fraction from the Ra strain was no less and possibly a trace more potent than that from the Rv strain. On the other hand the second half of the table shows that the heated aqueous extract from the Rv strain was more potent than that from the Ra strain. Moreover, the potency of all the heated aqueous extracts was only slightly greater than that of the standard PPD-S. These differences in potencies of the different fractions are undoubtedly dependent upon the relative amounts of the different proteins present.

TABLE IV

COMPARISON OF SKIN REACTIONS WITH FRACTIONS FROM VIRULENT AND AVIRULENT BACILLI

No. pigs	PPD-S, mm.	Unheated filtrate fractions from H 37, mm.	H 37 Rv, mm.	H 37 Ra, mm.
8	17 × 2.1	23 × 3.0	22 × 3.1	23 × 3.1
	18 × 2.4	24 × 3.1	23 × 3.3	24 × 3.1
	PPD-S, mm.	Heated aqueous bacillary extracts from H 37, mm.	H 37 Rv, mm.	H 37 Ra, mm.
8	19 × 2.6	19 × 2.6	20 × 2.6	18 × 2.6
	17 × 2.7	19 × 2.7	20 × 2.7	18 × 2.7

(5) F. B. Seibert and E. DuFour, *Am. Rev. Tuberc.*, **58**, 393 (1948).

Table V shows the comparison of the potencies of the different fractions isolated, as described above, from the H 37 filtrate, with that of the standard and with the original filtrate. As has been shown many times previously, the C protein, which is Fraction I, is less potent than the standard, even after further purification, and even though it has been isolated without heat. The probability of a lower degree of specificity of this fraction has been emphasized in earlier publications.[2,5]

Of the other fractions Fraction IV is the most potent, even more so than the whole original unfractionated filtrate, indicating possibly that the most potent fraction existing in the original filtrate has been concentrated in Fraction IV. This throws the suspicion of greatest potency on the slowest protein component with mobility about 2.1 (see Fig. 5). The diagrams show that some of this component exists in Fractions II and III, which are also highly potent, especially Fraction II. It has been demonstrated now for the fourth time, including the results in the previous publication,[2] that among the protein fractions migrating with the slowest mobilities, is to be found the most potent one. No titration for determining the exact potency ratio has as yet seemed justified, since the electrophoretic diagrams show that still more work must be done to gain true electrophoretic homogeneity in all fractions, except Fraction V, which is polysaccharide II. This fraction has been shown previously[3] to be devoid of potency.

Further chemical studies are being made on these fractions as well as further purifications. The concentrated filtrates and extracts of the H 37 Rv and H 37 Ra strains will also be fractionated.

Discussion

A comparative analysis was made in 1930 by Cooper[6] on the dissociated forms of the BCG organism and his report showed higher content of lipid in the R than in S form, but a smaller amount of material soluble in aqueous solvents, possibly glycogen and proteins. He repeatedly mentioned the highly colloidal nature of the S solutions. It is clear from the present work that this opalescence was due to the high content of Polysaccharide II in the S fractions. His results on the substances in the culture media of these two strains showed a similar contrast between the R and S forms.[7]

Steenken[8] in 1941 studied the protein and carbohydrate contents and biological properties of fractions isolated from the culture media of his two dissociated strains, H 37 Rv and H 37 Ra. The growth on a modified Proskauer and Beck's medium was very different in the two cases; "a dry, brittle, dense and heaped-up growth of the Ra variant, and moist, tenacious, thin and

(6) F. B. Cooper, *J. Biol. Chem.*, **88**, 485–491 (1930).
(7) F. B. Cooper, *ibid.*, **88**, 493 (1930).
(8) W. Steenken, Jr., *ibid.*, **141**, 91 (1941).

TABLE V

COMPARISON OF SKIN REACTIONS WITH FRACTIONS OF UNDISSOCIATED H 37 TUBERCULIN

Top rows are 24-hour readings; bottom rows are 48-hour readings.

No. pigs	PPD-S, mm.	H 37 Original filtrate, mm.	Fraction I, mm.	Fraction II, mm.	Fraction III, mm.	Fraction IV, mm.
6	20 × 2.7		18 × 2.0	24 × 3.3		
	19 × 2.8		18 × 2.3	25 × 3.5		
7	18 × 2.4	21 × 3.0			21 × 3.0	23 × 3.6
	16 × 2.4	21 × 3.3			21 × 2.8	21 × 3.6
6	19 × 2.5	22 × 3.1		21 × 3.1	20 × 2.8	22 × 3.3
	19 × 3.0	22 × 3.3		20 × 3.3	18 × 2.8	24 × 3.5
			(I Purified)			
7	20 × 3.0	24 × 2.6	18 × 2.8			
	21 × 3.3	25 × 3.1	17 × 2.6			

diaphanous growth of the Rv variant." The proteins were isolated by the method used for making PPD and the carbohydrates were also isolated. Greater yields of both were obtained from the Rv variant, although the Ra yielded more organisms. Electrophoretic diagrams made in our laboratory of his concentrated crude filtrates showed differences, not very striking, but suggestive of the ones reported in this paper on the concentrated filtrates. The differences found were less evident and significant than those reported in this paper, possibly because his solutions had been heated whereas ours were not.

It is interesting that in this study also, more polysaccharide was found in the unheated filtrate of the virulent than of the avirulent strain. While this fact may be associated with the difference in virulence of these two dissociants from a single strain, nevertheless one must consider the facts that a large amount of polysaccaride was also previously found[2] in the avirulent (BCG) strain as well as in another moderately virulent human strain (A 33), whereas on the contrary, only a trace was found in the virulent human (DT) strain. Further study will have to be made of the effect of these respective isolated polysaccharide fractions directly on normal and sensitized cells and this work is in progress in our laboratory.

Still another fact concerning the carbohydrate in these fractions may be of significance. When calculation was made of the total yields of protein, based on the nitrogen contents of the different fractions, and this was compared with the total contents of the electrophoretic components, exclusive of the slowest component, which was obviously polysaccharide II, it was clear that the components consisted of more than just protein. The differences, shown in the last column of Table II, probably represent carbohydrate which was bound to the protein fractions and which traveled with them in the electrophoretic field. It is interesting that in the case of both unheated filtrates and heated aqueous extracts, more carbohydrate appeared to be bound to the fractions of the avirulent than of the virulent strain.

With respect to the nucleic acid constituents, with mobilities of 13.3 to 15.6, relatively more was found in the unheated filtrate and in the heated aqueous extract of the avirulent than in the virulent strain, while the actual yields (Table II) in the two heated extracts were the same. In view of these results, speculation concerning the presence of a transforming factor in the virulent strain, as would be suggested by analogy with the pneumococcus strains, is prohibited.

The demonstration that considerable nucleic acid is extracted by heat from the bacillary bodies, whereas it is found in only an insignificant amount in the original filtrates, explains why it is found in commercial Old Tuberculins.[9] However, since it has been found earlier[10] to have no tuberculin potency, it can be considered to be merely an additional impurity in the Old Tuberculins.

It is, furthermore, highly significant that all three heated aqueous bacillary extracts were less potent than the unheated fractions, which would presumably contain mainly undenatured protein. It is to be expected that the proteins in Old Tuberculin would be similar to these heated proteins, and their potencies were of the same order as these heated fractions and also of the proteins found in the PPD-S, which was also produced from a heated tuberculin. It has been shown[10] that heated tuberculin protein fractions are less antigenic than unheated proteins and, therefore, are less likely to give rise to false positive skin reactions, and this is the reason why all skin testing has so far been done with such fractions. Careful study of the sensitizing ability of the unheated fractions, when given in the minute doses needed for a skin test, will have to be made before such fractions could be used for this purpose.

The greatest potency apparently exists in the proteins with slowest electrophoretic mobilities and it is probable, therefore, that the fraction with greatest specificity will also be found among this group of proteins. However, the presence of these proteins does not appear to be linked with virulence since relatively less of them was found in the unheated filtrate, as well as in the heated aqueous extract of the virulent than in that of the

(9) F. B. Seibert, K. O. Pedersen and A. Tiselius, *J. Exp. Med.*, **68**, 413 (1938).

(10) F. B. Seibert, *Chem. Revs.*, **34**, 107 (1944).

avirulent strain, as seen in Table I, and in actual total yield, shown in Table II, no difference existed between the two strains.

On the other hand, the fact that there was more, both relatively and actually, of the faster protein component (mobility 5.8–6.0) in the heated aqueous extract from the virulent than from the avirulent strain, might suggest some connection between this component and virulence. Since two proteins, B and C, are found in this peak[2,5] and since the C protein has seemed to be less specific in its potency, it is possible that the B protein may prove to be of significance as far as virulence is concerned. This will be investigated.

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Summary

1. Electrophoretic diagrams of concentrated filtrates of unheated tuberculin serve for the evaluation of the relative amounts of the different protein, polysaccharide, nucleoprotein and nucleic acid components present, all of which have been isolated and identified.

2. The mobilities and relative proportions of these components correspond very closely in different batches made from the same strain at different times.

3. The relative percentage of the components varies greatly between different strains, even though the mobilities correspond closely.

4. In a study of differences between the undissociated H 37 and the dissociated H 37 Rv and H 37 Ra strains, the Rv strain yielded in the unheated filtrate more polysaccharide II, than did the Ra strain.

5. Extracts of the bacillary bodies of all three strains made by autoclaving them with water showed the presence of a large amount of nucleic acid not found in the original filtrates. The extract of the H 37 Rv bacilli showed the presence of more polysaccharide II and more of the faster protein with mobility 5.8–6.0, but the same amount of the slower proteins with mobilities around 3.7, and of the free nucleic acid, as did the Ra strain. The possible relationship between virulence and these findings, as well as those listed under (4), is discussed.

6. An extensive fractionation of the H 37 filtrate was carried out and further resolution of the slowest proteins was achieved.

7. Comparative skin potency tests showed that the slowest protein was the most potent one. Furthermore, unheated filtrates of the H 37, as well as of the dissociated strains, H 37 Rv and H 37 Ra, were all more potent than the heated aqueous extracts of the bacillary bodies. These latter were similar in potency to the standard PPD-S. The unheated filtrate from the H 37 Ra strain was as, or more, potent than that from the H 37 Rv strain, but it was less potent when the heated aqueous extracts of these two strains were compared.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY SCHOOL OF MEDICINE]

The Interaction of Insulin with Thiocyanate and other Anions. The Minimum Molecular Weight of Insulin[1]

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Introduction

Recent studies have indicated that the minimum molecular weight of insulin is approximately 12,000[3,4] and that in aqueous solutions, this insulin "monomer" is in equilibrium with higher, tri- or tetrameric aggregates. The equilibrium is greatly dependent on protein concentration, *p*H, and ionic strength of the medium.[4]

Preliminary to an electrophoretic investigation of insulin in the isoelectric range it was found[5] that between *p*H 5 and 8 thiocyanate ions increase appreciably the solubility of this protein. This was also reflected in a shift in the *p*H mobility curve, corresponding, at *p*H 5.5, to the binding of approximately 2 equivalents of thiocyanate ions per 12,000 g. of insulin. In contrast, in acid solutions in which insulin is highly soluble in the absence of thiocyanate, the addition of these anions resulted in almost complete precipitation of the protein. Subsequent quantitative studies on the effect of thiocyanate on the solubility of insulin,[6] carried out in relation to protein concentration, temperature and *p*H have led to the conclusion that the precipitating action of thiocyanate is directed primarily toward the tri- or tetrameric form of insulin and that any factor which shifts the molecular equilibrium toward the aggregated state likewise promotes precipitation.

In the work presented herein, an attempt was made to evaluate quantitatively the effects of

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