THE PROTEINS IN UNHEATED CULTURE FILTRATES OF HUMAN TUBERCLE BACILLI

II. Determination of Serological Properties

By JANET R. McCARTER,* Ph.D., and ELLEN B. BEVILACQUA, Ph.D.

(From the Departments of Agricultural Bacteriology and Chemistry, The University of Wisconsin, Madison)

(Received for publication, November 12, 1947)

INTRODUCTION

Preparations obtained from unheated culture filtrates of tubercle bacilli by fractionation with ammonium sulfate were sufficiently distinctive according to their sedimentation and diffusion diagrams (1) to warrant a detailed quantitative investigation of their serological identities. Qualitative tests had previously indicated some differences in the precipitinogenic activities of purified protein derivative (P.P.D.) tuberculins (2) prepared in different ways, and these variations could be correlated with different degrees of activity in eliciting skin reactions in human beings (3). It was our hope that a correlation of the serological and the physicochemical data would indicate, first, the number of proteins in unheated culture filtrates and, second, whether or not skin activity resided in any particular kind or condition of protein.

By means of quantitative precipitin and precipitin absorption tests two distinct antigens were found among the protein fractions separated from the culture filtrates of both fully virulent and slightly virulent human tubercle bacilli: the presence in the various fractions of these two antigens could be correlated with the appearance on the sedimentation diagrams of components with constants of about 3.4 S and of about 2 S (4). The material giving the higher rate was in a fraction precipitated by 0.25 saturated ammonium sulfate at pH 4.4 which was practically homogeneous antigenically. The fractions obtained by full saturation with ammonium sulfate contained only the second serological entity. Fractions obtained by 0.5 and 0.75 saturation were mixtures of the two, according to both precipitin tests and sedimentation diagrams. A third antigen, and the one most active in eliciting antibody formation, was found only in the fractions from the virulent culture filtrate. The chemical and physical nature of this antigen is unknown since we have demonstrated its presence only by serological means.

That the two protein antigens were entities was shown also by another type of serological test; namely, the passive transfer of skin sensitivity to normal guinea pigs by the serum of guinea pigs sensitized to our protein fractions (4).

* John Simon Guggenheim Memorial Fellow 1944–45, Department of Medicine, College of Physicians and Surgeons, Columbia University.

All fractions proved to have skin activity for tuberculin-sensitive human beings or tuberculous guinea pigs. Thus this property cannot be associated with one or the other of the two protein antigens. With the antigenically homogeneous fractions it became possible to demonstrate that the subject tested rather than the kind of protein is responsible for certain differences in the nature of the skin reactions in human beings. To cite an example, those persons who started to react almost immediately upon the injection of a tuberculin responded early to either of the antigens. Also, a tuberculin-sensitive individual, whether he had active tuberculosis, or calcified lesions only in his lungs, or no signs of tuberculosis upon chest x-ray, reacted alike to the serologically distinct fractions.

The two protein antigens both proved to be capable of sensitizing the skin of normal guinea pigs (4): the sensitizing doses were of the order of magnitude of those commonly used in skin testing tuberculous guinea pigs. However, the sensitization was against only the homologous fraction. The skin reactions differed somewhat from those in tuberculous guinea pigs in certain characteristics, the significance of which is not known. The results leave it an issue as to whether or not the fundamental mechanism is the same for infected and protein-sensitized animals. Possibly a tuberculous animal gives a skin reaction only to a protein against which it has been specifically sensitized: if this is so, tuberculous human beings who reacted to the two protein antigens must have been specifically sensitized by both of them.

EXPERIMENTAL

Preparation of Fractions.—Culture filtrates were prepared both from a virulent (DT[1]) and from a slightly virulent (TB–1[1]) strain of human tubercle bacillus. The particular cultures of the virulent strain used for seeding were the second subcultures from ones shown to have full virulence for guinea pigs. The slightly virulent strain in doses of 1 mg. inoculated subcutaneously in finely divided suspensions produced tubercles in guinea pigs but the animals were still living after a year. This culture can be identified as a tubercle bacillus from its history of original virulence, from the fact that it had been successfully used during some 20 years to make the tuberculin for state cattle testing, and from the cultural characteristics of rate of growth and growth temperature requirements under given conditions.

The following synthetic medium (Henley (5)) was used to grow the organisms:

$K_2HPO_4 \cdot 7H_2O$	50 gm.
Sodium citrate, $5\frac{1}{2}H_2O$	25 "
$MgSO_4 \cdot 7H_2O$	40 "
Glycerine	3,500 cc.
Ferric citrate	3.2 gm.
Asparagine	250 "

The ingredients were put into solution and added in order named and the volume made up to 50 liters. The pH of the medium before autoclaving was 7.0. The cultures were incubated

[1] The DT culture came from the Bureau of Animal Industry, and was kindly sent to us by Dr. Florence Seibert. The culture TB–1 also originally came from the Bureau of Animal Industry.

at 37°C. and held at this temperature for varying lengths of time after marked autolysis had taken place. To determine the effect of length of autolysis upon the protein content of the filtrates, two lots of virulent cultures were set up at different times and one held for a total incubation of 9 weeks and the other for 21 weeks. The cultures of the slightly virulent strain were held 9 weeks before harvesting. For investigating the effect of heat upon the protein content of the filtrates, cultures from the lot held 21 weeks were treated in three different ways. One part, the cultures from 15 of the original 50 liters of medium, was made as an old tuberculin (O.T.); *i.e.*, the cultures were held for 4 hours in a steamer with the liquid about 100°C., the cells filtered off through paper, and the filtrate evaporated on a steam bath with a jet of air blowing over the surface, to one-tenth the original volume of the culture fluid. A second part of the cultures from 6.5 liters of original medium was held in the steamer for 2 hours (the method used recently for making P.P.D. tuberculins (2)) and allowed to cool in air to room temperature. The third part of the culture filtrate originally representing 24 liters was not heated; after filtration to remove the cells it was held at 5°C.

Cellular material was removed by filtration through paper, then coarse and finally fine Mandler filters. The filtrates were immediately concentrated and washed by ultrafiltration with phosphate buffer (0.005 M, pH 7.4 to 7.5) at 5°C. (2). Throughout the process merthiolate was maintained at a concentration of 1/10,000.

To ascertain whether we might be losing any significant constituents by ultrafiltration, parts of the ultrafiltrates were in turn concentrated and washed in the alundum cups impregnated with heavier cellulose acetate films. To prevent any changes which might occur upon standing in solution, all preparations were lyophilized immediately after they had been washed by ultrafiltration.

Fractionation was carried out, as previously described (1), on the preparation from the 21-week virulent culture filtrate and on that from the 9-week slightly virulent culture filtrate. This consisted essentially in a preliminary partition with acetate buffer at pH 4.4, and a fractionation of both acid supernatant and precipitate with ammonium sulfate to give successively precipitates at 0.25, 0.5, 0.75, and full saturation.[2] Estimations of protein, polysaccharide, and nucleic acid contents were made on each fraction as previously described. These fractions were used for detailed serological investigation.

---

[2] The fractions will be given here the same designations as in the first paper of this series (see Fig. 1 (1)). The following fractions were obtained from the acid supernatant:
from the part precipitated in a preliminary treatment with 0.5 saturated ammonium sulfate at pH 7.2

B by treatment with acetate buffer at pH 4.4
HS¼ by 0.25 saturation with ammonium sulfate at pH 4.4
G " 0.5 " " " " " " 4.4
K " 1.0 " " " " " " 4.4

from the part soluble in 0.5 saturated ammonium sulfate

A by 0.75 saturation with ammonium sulfate at pH 7.2
J " 1.0 " " " " " " 4.4
M soluble in 1.0 saturation with ammonium sulfate at pH 4.4.

The other fractions were precipitated from the acid precipitate as follows:—

E by 0.25 saturation with ammonium sulfate at pH 7.2
N " 0.5 " " " " " " "
P " 0.75 " " " " " " "
Q " 1.0 " " " " " " 4.4.

The subscript "v" identifies the fractions from the virulent culture filtrate and "av" those from the slightly virulent.

Antiserum Preparation.—Antisera were produced against certain of the fractions by the intracutaneous injection into rabbits of the materials at 3 day intervals to a total of approximately 100 mg. of protein per animal.

Specific Precipitation.—The fractions were characterized as to their antigens by determining the amounts of specific precipitate nitrogen with the procedures of Heidelberger and Kendall (6) and Heidelberger, Kendall, and Soo Hoo (7). All precipitations and operations were carried out at 0–5°C. The amounts of fraction protein nitrogen used as precipitants were the smallest quantities which would give maximal precipitates as based on preliminary tests; one-half these amounts of antigen were used for all succeeding absorptions. The first precipitates were taken out after 48 hours and subsequent absorptions were each held for 1 week. All antisera had been tested for polysaccharide antibodies with the carbohydrate we had isolated from the virulent culture filtrate (fraction M) and with one of those obtained from tubercle bacillus cells[3] by Heidelberger and Menzel (8). A few sera showed very slight precipitates with polysaccharides; they were preliminarily absorbed with the cell polysaccharide, 5 μg. per cc. of antiserum: this quantity was sufficient to absorb the polysaccharide antibodies but small enough not to cause precipitation of protein antibodies by the contaminating protein.

RESULTS

When the fractions from the unheated virulent and slightly virulent culture filtrates were tested with an antiserum against the unfractionated unheated virulent culture filtrate, at least three serological entities were found. The results are shown in Table I. The combined quantitative and absorption data show that the J_{av} and the HS¼[4] fractions are distinct and that the P_v fraction contains at least one other antigen.

Further evidence that the isolated fractions HS¼ and J_{av} are different antigenically is provided in the data in Table II. The use of the O.T. antiserum as a source of antibodies for the "saturated" antigen[5] was justified by the following considerations, and thus in the preparation of the antiserum inroads did not have to be made into the small yields of the J fractions. The O.T. contained none of the 0.25 antigen[5] as even large amounts failed to cause precipitation in the HS¼ antiserum. The small amount of precipitate obtained with the J_{av} fraction in the HS¼ antiserum can be explained by assuming a slight admixture of the "saturated" protein in the HS¼ preparations: this has been later corroborated by other types of biological tests. This would explain why relatively large amounts of HS¼ fractions brought down precipitates in the O.T. serum while small quantities did not, and also why long continued absorptions with HS¼ fractions will eventually exhaust the O.T. antiserum of precipitins for the J fractions.

[3] Their label "$520B_2^{17}$." From their figures this polysaccharide contained 3.25 per cent nitrogen.

[4] Since the yield of the HS¼ fractions was so small, and since the sedimentation pictures and preliminary precipitin tests indicated the proteins were similar, the v and av fractions were combined and are referred to as HS¼.

[5] The antigen present in the HS¼ fractions will be referred to as the 0.25 antigen, that in the J fractions as the "saturated," and the other one present in the P_v fraction (Table I) will be called the third antigen.

The much greater antibody-eliciting potency of the third antigen present in the P_v fraction (Table I) and the further differentiation of this antigen from the 0.25 and the "saturated" antigens are indicated in Table III. The large amount of antibody for this third antigen in the serum described in the table made possible its detection without the use of absorbed sera or quantitative tests, but the quantitative data show strikingly its relative antigenic power.

TABLE I

Amounts of Precipitates Formed When Three Different Protein Fractions Were Added to an Antiserum Prepared by Injecting Unheated Unfractionated Ultrafiltered Culture Filtrate of Virulent Tubercle Bacillus

Antiserum*	Fraction used as precipitant	No. of absorption	Precipitant protein N added per cc. antiserum	N precipitated per cc. antiserum
			μg.	μg.
Unabsorbed	P_v	1st	100	210
		2nd	50	60
		3rd	50	30
		4th	50	30
		5th	50	0
"	J_{av}	1st	80	40
		2nd	40	+‡
Absorbed with J_{av}	HS¼	1st	180	40
		2nd	90	+
Absorbed with J_{av} and then with HS¼	P_v	1st	10–100	Significant amount of precipitate not determined quantitatively

* For absorption with the P_v fraction 6 cc. of antiserum were used and with the J_{av}, 5 cc.
‡ Less than 10 μg.

The data thus reveal the presence of a major antigen in the HS¼ fractions, another in the J_{av} fraction, a combination of these two in the A_v fraction, and a third antigen in the A_v and P_v fractions from the virulent culture filtrate.

To assay all the fractions from the virulent and slightly virulent culture filtrates, three antisera were used; namely, (1) to test for 0.25 antigen, antiserum to HS¼ with small amount of antibody to "saturated" antigen absorbed out with J_{av}; (2) to test for "saturated" antigen, antiserum to A_{av}, absorbed with HS¼; (3) to test for the third antigen, antiserum to unheated unfractionated virulent culture filtrate, absorbed with HS¼ and with J_{av}.

The results presented in Table IV indicate that in general fractions obtained by 0.25 saturation with ammonium sulfate contained the 0.25 antigen, likewise those precipitated by saturated ammonium sulfate had the "saturated"

TABLE II

Amounts of Precipitates Formed in Homologous and Cross-Reactions of 0.25 and "Saturated" Antigens in Their Antisera

Substance used to produce antiserum	Substance used to absorb antiserum	Substance used as precipitant	No. of absorption	Precipitant protein N added per cc. antiserum*	N precipitated per cc. antiserum
				μg.	μg.
HS¼		HS¼	1st	10	90
			2nd	5	10
			3rd	5	0
			4th	8	+
			5th	8	+
"		J_{av}	1st	1‡	10
			2nd	0.5	0
			3rd	0.5	0
			4th	0.5	0
"		$O.T._{v}$ unfractionated		160§	0
				16§	0
$O.T._{v}$ unfractionated		J_{av}	1st	30	30
			2nd	15	+
			3rd	15	+
" "		HS¼	1st	90	20
			2nd	45	+
			3rd	45	+
			4th	45	+
			5th	45	0
" "	J_{av}	HS¼		80	0
" "	HS¼ 3 times	J_{av}		5	++‖
" "	HS¼ 5 times	J_{av}		5–50	0

* For each set of absorptions 3 cc. of each antiserum were used.

‡ Larger ratios of antigen to antibody gave no precipitate and were undoubtedly in the antigen excess zone.

§ Nitrogen of portion precipitable by alum.

‖ Not determined quantitatively.

antigen, and those obtained by 0.5 or 0.75 saturation had both antigens. The third antigen was present in all the preparations from the virulent culture filtrate except in HS¼ and in J. Thus J_{v} and J_{av} have the same antigenic constitution, as well as $HS¼_{v}$ and $HS¼_{av}$.

TABLE III

Comparative Amounts of Precipitates Formed by the Three Antigens in an Antiserum against Fraction A_v

Fraction used as precipitant	Precipitant protein N added per cc. antiserum	N precipitated per cc. antiserum
	μg.	μg.
HS¼	80	45
J_{av}	80	++*
P_v	160	730
A_v	160	740

* Not determined quantitatively but approximately same visible amount of precipitate as yielded by HS¼.

TABLE IV

The Distribution of the Three Antigens in the Fractions from the Virulent and the Slightly Virulent Culture Filtrates

Fraction used as precipitant*	Antiserum against		
	0.25 antigen	"Saturated" antigen	Third antigen
$HS¼_v$	+	0	0
G_v	+	+	+
A_v	+	+	+
J_v	0	+	0
K_v	0	+	+
E_v	+	0	+
N_v	+	+	+
P_v	+	+	+
Q_v	0	0	+
G_{av}	+	+	0
A_{av}	+	+	0
J_{av}	0	+	0
N_{av}	+	+	0
P_{av}	+	+	0

* For each individual test quantities corresponding to 0.15 cc. of undiluted antiserum were used. The amount of fraction protein used as antigen varied with the particular antiserum: in general it was twice as much of the fraction protein to be tested as of the homologous fraction which would give maximal precipitate with the antiserum. This quantity was always considerably less than the amount of homologous fraction which would be in the antigen excess zone. For the 0.25 antibody the ratio was 300 μg. of fraction protein per cc. of serum; for the saturated antiserum 500 μg. of protein; and for the "third" antibody 100 μg. of protein. Negative results were checked with a range of dilutions of each fraction.

The individuality and the antigenicity of the 0.25 and "saturated" antigens have been demonstrated also by skin tests on both actively and passively sensitized guinea pigs. For active sensitization J_V was injected into seven guinea pigs. Fifty μg. doses of protein were given intracutaneously on the belly in 0.1 cc. volumes at 5- to 6-week intervals. Small but definite skin reactions appeared in response to the second injections and the inflammation elicited increased markedly up through the fourth injections. When it was judged that the animals would give maximal skin reactions, they were tested with the sensitizing fraction and, 72 hours later, with one heterologous antigen. To control the effect of one injection on the succeeding injection, the tests were repeated. An animal thus received four injections, two of homologous and two of a heterologous fraction, with 72 hours between each injection. The solutions were put in intracutaneously on the flank and as usual in a volume of 0.1 cc. Reactions in the skin of the flank have better definition than those in the abdominal skin. Only one fraction was given at a time since previous work on animals sensitized with heat-killed tubercle bacilli had indicated that the skin reaction to one fraction might be inhibited by the simultaneous injection of another fraction. The reactions to two equal doses of the same fraction given 144 hours apart were of approximately the same intensity and therefore the method of comparing fractions by injecting them 72 hours apart was deemed sufficiently accurate.

TABLE V

Specificity of Skin Reactions in Guinea Pigs Actively Sensitized with Fraction J_V When Tested with the 0.25 and "Saturated" Antigens

Animal No.	Test fraction	Amount of test fraction protein injected	No. of previous injections of J_V	Skin reactions after 18 hrs.*
		μg.		mm.
1	J_V	10	Six	21 × 21 (× 2)
	B_V	20		7 × 7 (× 1)
	J_V	2		9 × 9 (× 1.5)
2	B_V	20	Three	10 × 10 (× 1)
	J_V	20		28 × 26 (× 2.5)
3	M_V	1,000 (polysaccharide)‡	Four	22 × 22 (× 2.5)
	J_V	50		23 × 23 (× 3)

* The dimension in parentheses is the height of the inflamed area estimated for comparative purposes by measuring the thickness of the doubled skin at the center of the reaction and dividing by two.

‡ Estimated from N content to contain about 50 μg. of protein.

Typical results appear in Table V.[6] All the skin reactions started almost immediately with erythema, edema, and slight purplish discoloration at the center. They reached their height at 18 to 19 hours when they showed slight erythema, rather firm edema, and lavender to reddish purple centers not over 3 mm. in diameter. By 48 hours the reactions had almost disappeared. (How-

[6] Note that B_V was used in place of HS¼ since our supplies of the latter fractions were insufficient. Serologically the B fractions were similar to HS¼ and physical chemical data indicated the B fractions to be slightly denatured HS¼.

ever, it should be noted here that a stronger reaction was obtained in one guinea pig sensitized with B_v in the same way as the J_v animals. In this particular animal 100 μg. were given for the fourth sensitizing dose, and the reaction was still marked, although decreasing, at 48 hours, and had an ulcerated center 10 mm. in diameter.) The reactions varied in general from those seen in animals sensitized with tubercle bacilli in that the amount of central necrosis was relatively small compared to the volume of the edema, although reactions have been seen in guinea pigs sensitized with heat-killed tubercle bacilli which reached maximal size at 24 hours. At that time also the latter reactions could not be distinguished by their other characteristics from simultaneous reactions in the animals sensitized with the J_v fraction.

The primary injection of a fraction into the normal animal served as a control on the toxicity of the substance and on the condition of the guinea pig with respect to tuberculous infection. The usual response was an almost colorless edema, not over 10 mm. in diameter, which appeared almost immediately and lasted for only 6 to 8 hours. One animal was regarded as suspicious because it gave a primary reaction of slight, almost colorless edema 15 mm. in diameter at 18 hours. The subsequent reactions of this animal gave no indications that it was tuberculous and the conclusion was drawn that the toxic action of J_v varies slightly with the animal.

For making serological tests small amounts of serum were taken from each J_v-sensitized animal and from animals sensitized similarly with the fractions J_{av}, G_v, G_{av}, and B_v. Trial bleedings were made at various intervals but the most potent sera were obtained when the blood was taken about a week after an injection which had resulted in a maximal skin reaction. Precipitins could not be demonstrated in these sera but the presence of antibodies was made manifest by the moderately large skin reactions obtained when the sera were injected into the skin over the flanks of normal guinea pigs and the fractions injected 48 hours later. This type of reaction has been shown by Chase (9) to be given by guinea pigs injected with the sera from rabbits and guinea p gs sensitized with various proteins such as horse serum, and has been fully described more recently by the same author (10). The details of some of these passive transfer reactions with the sera of sensitized guinea pigs are given in Table VI.

Quantities of 0.1 or 0.15 cc. of the undiluted antiserum were injected into the skin of normal guinea pigs in the weight range of 250 to 300 gm. Animals of this size have been found to give optimal skin reactions of this type. When the fractions were given subcutaneously they were put over the belly and when intracutaneously they were put in locally as closely as possible to the site of injection of the antiserum. Where injections of two different fractions were given successively, the interval between was that denoted in the table as the time when the reaction to the first injection was negative. In the case of the last animal in Table VI the injections of the two antisera were made in the same animal and the reactions were simultaneous.

The data in Tables V and VI again indicate that the 0.25 and the "saturated" antigens are distinct, and that both have about the same potency as antibody-eliciting substances. The animals actively sensitized with the J fractions gave only slight skin reactions to the B_v fraction and the serum from the J-

TABLE VI

Skin Tests Showing Lack of Cross-Reactions to Fractions in Passively Sensitized Guinea Pigs

No. of guinea pig donor	Fraction used to sensitize donor	Test guinea pig No.	Test fraction	Test fraction protein	Route of injection*	Skin reaction	
						Time when maximal	Size in mm. and color‡
				μg.		*hrs.*	
4	J_{av}	40	J_{av}	30	sc	0.42	42 × 37 vfp− (++)
4	J_{av}	41	B_v	30	"	2	0
			J_{av}	30	"	0.5	27 × 21 vfp (+)
5	J_v	50	J_v	30	"	0.25	31 × 31 vfp+ (+++)
5	J_v	51	B_v	30	"	1.17	0
			J_v	30	"	0.28	32 × 32 vfp− (+)
6	B_v	60	B_v	30	"	0.58	57 × 38 fp (+++)
6	B_v	61	J_{av}	30	"	0.3	22 × 22 fp (±)
6	B_v	62	J_v	30	"	19	0
			B_v	10	"	3.5	45 × 35 alcls (++)
7	G_v	70	J_{av}	10	ic	3	18 × 17 vfp (+++)
7	G_v	71	B_v	10	"	0.2	25 × 14 vfp (++)
7	G_v	72	G_v	100	sc	0.42	33 × 29 vfp− (+)
8	G_{av}	72	"	"	"	"	35 × 30 vfp− (+)

* sc = subcutaneously; ic = intracutaneously.

‡ The number of +'s in parentheses is an estimation of the relative height, since measurement of height would have disturbed reaction. v = very; f = faint; p = pink; al = almost; cls = colorless.

sensitized guinea pigs transferred no sensitivity to the B_v fraction. Conversely the serum from the animal sensitized with the B_v fraction transferred to one animal a very slight sensitivity to the J fraction. Thus the data gave corroboration that the HS¼ has a small admixture of the "saturated" antigen. The G fractions were further shown to have both the 0.25 and the "saturated" antigens. The serum from the G_v- and that from the G_{av}-sensitized animal

transferred about the same degree of sensitivity to the G_v fraction. That is, the third antigen had not been effective in producing antibodies in the G_v guinea pig probably because such small amounts were given. From the tests in the actively sensitized animal the protein in the M_v fraction, the one made up almost entirely of polysaccharide, appeared to be the same as that in the J_v fraction.

DISCUSSION

A close correlation exists between the results of the serological investigations and the sedimentation studies reported in the previous paper (1) in demonstrating the presence of the 0.25 and the "saturated" antigens in the various fractions separated from the filtrates of both the virulent and the slightly virulent cultures. However, the precipitin test is the more sensitive method, since it revealed the presence of two proteins in the G_{av} fraction which upon sedimentation was not resolved into two peaks. (The peaks for both the 3.3 S and the 2 S proteins do appear in the sedimentation diagrams of G_v, A_v, and A_{av}.)

These two antigens are proteins according to their behavior in sedimentation and in specific precipitation: that is, the mass of a fraction being sedimented as determined from the area under the curve and the quantity of the fraction necessary to bring down a given amount of specific precipitate N can both be correlated with chemically determined protein content. Although certain of the fractions, notably J_v and J_{av}, contain considerable percentages of polysaccharide, nevertheless the evidence is that the antisera prepared against the fractions are not directed to any measurable extent against their polysaccharide moiety. Thus, although the polysaccharide in the fractions will react in the horse antiserum (No. 5807L) (8) made against dead tubercle bacilli, the rabbit and guinea pig antisera prepared by injecting the fractions do not react with the polysaccharide in fraction M or with a polysaccharide from dead tubercle bacilli.

The two antigens are probably uniformly present in unheated culture filtrates of human tubercle bacilli: we have demonstrated their presence in 9- and 21-week old culture filtrates of a virulent strain and in the 9-week filtrate of a slightly virulent one. The HS¼ fractions from the three different filtrates were shown to be the same in sedimentation behavior in the ultracentrifuge, and to be the same as the one isolated by Seibert *et al.* (11).

The third antigen was found by means of the serological tests in both the 9-week and the 21-week culture filtrates of the virulent strain. Its nature and significance are not known. No evidence of its existence is found on the sedimentation diagrams of any of the fractions. Its presence is not correlated with the nucleic acid content of the fractions: although it is present in culture filtrates high in nucleic acid, it is absent from the J_v fraction which contains a large percentage of nucleic acid. It is probably present in very small quantities

in the fractions as relatively large amounts of the latter were required to bring about precipitation of the ant body to this antigen; furthermore, it did not sensitize the guinea pig injected with small quantities of the G_v fraction. This third antigen can cause errors in testing for the two known protein antigens unless its antibody has been demonstrated not to be present in the serum being examined.

These three antigens appear to be the major ones of unheated tubercle bacillus culture filtrates. Serological attempts to demonstrate others in either the crude preparations or the fractions were unsuccessful. The ultrafiltrates from the ultrafiltered culture filtrates when in turn concentrated by ultrafiltration had very weak skin activity for tuberculous guinea pigs and were therefore not studied further.

The HS¼ and the J fractions give a typical tuberculin type of skin reaction. This would seem to be the reason why O.T. can be used for intracutaneous testing even though the HS¼ protein is labile to heat. In cultures heated for 2 hours the 0.25 antigen could be demonstrated in two ways: the antiserum against the ultrafiltered but unfractionated culture filtrate contained antibodies for HS¼ and the same heated culture filtrate was precipitated by the antiserum against HS¼. The "saturated" antigen was also present and in much larger percentage in the 2-hour-heated preparations. In the culture filtrates heated for much longer periods to make a true O.T., however, the 0.25 antigen was absent although the "saturated" antigen remained and could still stimulate antibody formation. Undoubtedly much of the 2 S protein is changed markedly during the heating process because the material remaining in solution when the O.T. was fractionated with acetate buffer at pH 4.8 could not be sedimented at a measurable rate in the Svedberg ultracentrifuge. The acid precipitate was inhomogeneous upon ultracentrifugation. Thus the remaining protein must be denatured, but nevertheless some of the 2 S protein remained active and recognizable antigenically. (The third antigen was also heat-stable as its presence was determined serologically in the O.T.)

It is possible that the protein remaining in old tuberculins is denatured "saturated" or 2 S protein most of which is partially degraded. A fraction separated by Seibert from an O.T. was barely resolvable in the ultracentrifuge and proved to be relatively homogeneous with a constant of 1 S (3).[7]

The skin activity of the 1 S protein separated by Seibert from O.T. is quantitatively different from that of a fraction separated from a 2-hour-heated culture filtrate (the filtrate in this case being different from the one used to make the O.T., however)(3). When our O.T., 2-hour-heated tuberculin, and unheated culture filtrate, all concentrated and washed by ultrafiltration and all originating from the same culture filtrate, are compared on a nitrogen basis in skin

[7] P.P.D. 67–2.

tests on tuberculous guinea pigs, the unheated material is slightly stronger than the 2 hour-heated preparation and both are much more potent than the O.T. or even than the most active protein fraction made from the O.T. The greater potency of the unheated or 2-hour-heated proteins is not a desirable feature in a practical testing agent since all actively tuberculous human beings will react to the 1 S O.T. preparation (3). The unheated proteins have the disadvantage moreover of sensitizing the skin when given in very small doses whereas the 1 S protein is not antigenic (3).

As to the nature of the active skin agent in tuberculins, we must conclude that two completely different but equally potent proteins are present in the culture filtrate, or that the 3.4 S protein and the 2 S protein have a common skin-reactive grouping which does not influence the antigenic specificity of either one, or that some active substance is precipitated equally with both proteins.

The HS¼ fraction is shown to be a good precipitant in comparison with other known bacterial proteins: with 10 μg. of protein N 90 μg. of N were precipitated from 1 cc. of antiserum. The third unknown antigen is the best known precipitinogen among tuberculin or tubercle bacillus constituents in that about 700 μg. of specific precipitate N was brought down by about 150 μg. of fraction protein N so that more than 550 μg. of precipitate N must have come from the antibody.

SUMMARY

1. Only two serologically different proteins were found in the unheated culture filtrates of both virulent and slightly virulent tubercle bacilli. One of them was the protein which had a sedimentation constant of 3.4 S, and the other was in filtrate fractions with a constant of 2 S.

2. That these proteins were distinct was demonstrated by three methods: quantitative precipitin and precipitin absorption tests with rabbit antisera, skin tests in guinea pigs actively sensitized with the culture filtrate fractions, and skin tests in passively sensitized guinea pigs.

3. A third antigen of unknown nature was found by means of the precipitin tests, but only in certain fractions from the virulent culture filtrate.

4. The protein with the constant of 3.4 S could not be demonstrated serologically in an O.T. made from the same culture filtrate as the unheated preparation from the virulent organism.

The following acknowledgments are made with pleasure.

Dr. Michael Heidelberger, in whose laboratory the quantitative precipitin tests were carried out, gave help and encouragement in this phase of the work.

Dr. M. W. Chase of The Rockefeller Institute for Medical Research gave freely of his time in advising us how to carry out the tests on the passively sensitized guinea pigs.

BIBLIOGRAPHY

1. Bevilacqua, E. B., and McCarter, J. R., *J. Exp. Med.*, 1948, **87,** 229.
2. Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, 1941, **44,** 9.
3. McCarter, J. R., and Watson, D. W., *J. Immunol.*, 1941, **43,** 85.
4. McCarter, J. R., and Bevilacqua, E. B., *J. Bact.*, 1947, **54,** 68.
5. Henley, R. R., *Am. Rev. Tuberc.*, 1929, **19,** 660.
6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55,** 555.
7. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exp. Med.*, 1933, **58,** 137.
8. Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118,** 79.
9. Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52,** 238.
10. Chase, M. W., *J. Exp. Med.*, 1947, **86,** 489.
11. Seibert, F. B., Pedersen, K. O., and Tiselius, A., *J. Exp. Med.*, 1938, **68,** 413.