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REITER PROTEIN ANTIGEN

I. RELATIONSHIP OF SOME PHYSICAL AND CHEMICAL CHARACTERISTICS TO SEROLOGICAL ACTIVITY*

BY

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The Italian workers D'Alessandro, Oddo, Comes, and Dardanoni (1949) and D'Alessandro and Dardanoni (1953) described the isolation of four serologically active components of Reiter's treponeme. These contributions represent milestones in the sero-diagnosis of syphilis since they were the initial systematic investigations into the serological components of treponemes. One of the components was considered to be a soluble protein and was identified by the letters "ATPS"—antigene treponemico proteico solubile. ATPS was shown to be serologically active when employed as antigen in a complement fixation test. In the present report ATPS is designated "Reiter protein antigen" or "Reiter protein" to specifically identify the treponemal source. Several clinico-serological evaluations have demonstrated that, when used in certain complement-fixation techniques, Reiter protein provides a highly specific antigen in a serological test for syphilis (Wallace and Harris, 1962). The use of the antigen in complement-fixation testing of clinical specimens has been designated the Reiter protein complement-fixation (RPCF) (Cannefax and Garson, 1957) and the Kolmer Reiter protein (KRP) test (Bossak, Falcone, Duncan, and Harris, 1958).

During the developmental phase of RPCF testing and antigen preparation, it was observed that the antigenic titre of several lots of Reiter protein was relatively constant from lot to lot. However, it was observed that there were physical, chemical, and certain serological differences between individual lots of Reiter protein prepared from organisms grown under identical cultural conditions and strict adherence to the D'Alessandro method of protein isolation. These differences were manifested by the presence or absence of zoning in the antigen titration, differences in the optical density, and variability of protein concentration with respect to an almost constant antigenic activity. The observed variation in protein concentration, with relatively constant serological activity, demonstrated that Reiter protein contains non-reactive as well as reactive protein.

It was the original intent of the investigations reported here to determine if a physical separation of reactive and non-reactive components could be effected which would provide a more constant relationship of chemical content and serologic activity. A completely satisfactory separation has not been accomplished.

The purpose of this report is to present the results of investigations which have demonstrated certain physical and chemical characteristics of the protein and other substances extracted from Reiter's treponeme by the cryolysis and ammonium sulphate precipitation method of D'Alessandro. These studies have demonstrated:

(1) The particulate nature of Reiter protein and the relative particle sizes,

(2) The effect of pH and ionic strength on the precipitation of the reactive component.

(3) The adsorption and elution of the reactive component from a cellulose ion exchanger.

(4) The density of the reactive component as determined by differential migration employing centrifugation and density gradient columns.

(5) The presence of variable amounts of carbohydrate.

Material and Methods

Reiter Protein Antigen.—Prepared according to the cryolysis and ammonium sulphate method of D'Alessandro and Dardanoni (1953). The antigens were preserved with 1:5000 merthiolate.

Ion Exchanger.—Diethylaminoethyl (DEAE) cellulose anion exchanger of Peterson and Sober (1956) was washed with 1 N NaOH followed by water to remove excess alkali and finally with the desired buffer until the filtrate was the same pH as the buffer.

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Complement-fixation Technique.—The 1/5 volume Kolmer complement-fixation technique (Kolmer, Spaulding, and Robinson, 1951) has been used throughout these studies. The technique is described in detail in the "Manual of Serologic Tests for Syphilis" (1959).

Determination of Particle Size.—The Millipore filter in the 100–5000 mμ range has been used for demonstrating the variability and the upper and lower limits of particle size of reactive Reiter protein.

Protein Migration under Centrifugal Force.—Relative centrifugal forces (R.C.F.) of 30,000 and 177,000 have been employed in studies of differential migration of Reiter protein. The Servall SS-1 has been employed with the 30,000 RCF studies and the Spinco Model L preparative ultracentrifuge with the swinging bucket attachment and density gradient tubes was employed in the 177,000 R.C.F. studies. Density gradient tubes were prepared by layering the following sucrose solutions: 26, 45, 64, and 79 per cent.

The tubes were allowed to develop the gradients in the refrigerator overnight and provided a density range of 1·1 to 1·4. Density gradient tubes were also prepared by layering the following solutions:

45 per cent. sucrose in 75 per cent. saturated ammonium sulphate,
26 per cent. sucrose in 75 per cent. saturated ammonium sulphate,
75 per cent. saturated ammonium sulphate,
50 per cent. saturated ammonium sulphate.

These sucrose-ammonium sulphate gradients provide, a range of 1·3 to 1·8. The solutions were coloured red, blue, and yellow by adding small amounts of commercial food colours. The use of these coloured substances did not interfere with migration measurements and provided a check of the efficiency of the layering technique and the degree of diffusion at the interfaces after development of the gradient overnight in the refrigerator.

Quantitative Determination of Protein.—Nitrogen was determined by the Nesslerization method of Koch and McMeekin (1924), using the Klett-Summerson photoelectric comparator for the measurement of colour development. Nitrogen values were converted to protein by multiplying by 6·25.

Quantitative Determination of Carbohydrate.—The orcinol method of Heidelberger and Kendall (1936) was used with the colour development determined with the Klett-Summerson photo-electric comparator. Calibration analyses were made with glucose solutions.

Results

Variation in Protein and Carbohydrate.—It has been observed by simple inspection that there are differences in degrees of opalescence among several lots of Reiter protein antigen. Also, some lots have a greenish-yellow tint. On the assumption that some of these differences might be caused by variations in extractable protein and carbohydrate from Reiter's treponeme these components were studied by the quantitation of total nitrogen and carbohydrate. Table I shows the quantitative results obtained with six lots of Reiter protein antigen. It will be noted that the total protein concentration of the six lots varied from approximately 200 to 800 mg. per cent. Lots have been prepared which contain less than 200 mg. per cent. protein and occasionally a lot may exceed 800 mg. per cent. protein. It will also be noted in Table I that the range of carbohydrate concentration with the six lots of Reiter protein antigen was 30 to 60 mg. per cent. The range of all lots thus far tested has been 10 to 60 mg. per cent. carbohydrate. It should be emphasized that the protein and carbohydrate variations demonstrated in Table I were observed with lots of Reiter protein antigen having a constant serologic titre of 1 : 128.

TABLE I
VARIATION OF PROTEIN AND CARBOHYDRATE IN SIX LOTS OF REITER PROTEIN ANTIGEN
Serological titre of each antigen 1:128

Reiter Protein Antigen Lot	Protein (mg. per cent.)	Carbohydrate (mg. per cent.)
R	214	36
Z	429	60
Q	482	30
AA	589	60
Y	589	60
X	804	38

Particulate Nature of Reiter Protein and Range of Sizes of Particles.—The variation in opalescence with constant serological activity from lot to lot suggested that filtration employing known pore sizes might be used to remove the opalescence and that the relationship of opalescence and serologic activity might be determined. The results of one filtration experiment is shown in Table II (opposite). The serological activity may be completely removed, with this lot of antigen, by filtration through a 100 mμ filter. It can also be seen that the particle sizes, for the most part, lie in the 100 to 800 mμ range. It is quite evident that considerable portions of the protein and carbohydrate are serologically inactive. With this lot of antigen, 47·5 per cent. of the total protein and 75 per cent. of the total carbohydrate were demonstrated to be serologically inactive. While this experiment definitively demonstrates the particulate nature of the reactive protein it does not demonstrate that only the reactive protein is particulate.

TABLE II

PROTEIN, CARBOHYDRATE, AND SEROLOGICAL ACTIVITY FOLLOWING SUCCESSIVE FILTRATIONS FROM 5,000 TO 100 mμ FILTERS

Pore Diameter (mμ)	Antigen Doses		Protein		Carbohydrate	
	Number per ml.	Per cent. of Original	mg. Per cent.	Per cent. of Original	mg. Per cent.	Per cent. of Original
5,000	1,280	100	275	100	40	100
3,000	640	50	260	94·9	40	100
1,200	640	50	220	80	35	87·5
800	640	50	220	80	35	87·5
650	320	25	220	80	35	87·5
450	160	12·5	190	69·1	40	100
300	40	3·1	150	54·5	35	87·5
100	0	0·0	130	47·5	30	75
Unfiltered	1,280		275		40	

Effect of pH on Solubility of Reiter Protein, Carbohydrate, and Serological Activity.—The possibility was considered that Reiter protein might be separated into reactive and non-reactive components by selective precipitation. The precipitability characteristics of Reiter protein were therefore studied in relation to hydrogen ion concentration and ionic strength. Aliquots of Reiter protein were dialyzed against Miller and Golder (1950) buffers ranging from pH 2 to 7 with a constant ionic strength (μ) of 0·01. Varying degrees of precipitation occurred which was dependent upon the pH. The precipitates were separated by centrifugation and the precipitates and supernatants examined separately. The precipitated fractions were suspended with D'Alessandro and Dardanoni (1953) buffer to the original volume of the aliquot and the separate fractions were dialysed against four changes of buffer with 8 to 16 hours between changes to fresh buffer. The results obtained with one lot of Reiter protein antigen are shown in Table III. It can be seen that at pH 5 and below, the serological activity is contained in the precipitated fractions and the total reactivity is diminished with each lower pH unit until all the reactivity is lost at pH 2. At pH 6 and 7, the serological activity is found principally in the supernatant fractions. It will be noted that the fractions with the greatest reactivity were also those with the greatest amount of protein and carbohydrate with the exception of pH 5. At pH 5 the principal serological activity was associated with the least amount of protein compared with the reactivity and protein of the other fractions. Also, at pH 5 there was a partitioning of the carbohydrate between the supernatant and precipitate fractions. In all other instances the total demonstrable carbohydrate was found to be contained in either the supernatant fraction (pH 6 and 7) or in the precipitate fraction (pH 2, 3, and 4). In each of these fractions the carbohydrate was found to be contained totally in the fraction exhibiting the principal serological activity.

Effect of Ionic Strength at pH 5 on Solubility of Reiter Protein and Serological Activity.—Since the solubility of a given protein is dependent upon both pH and ionic strength the solubility characteristics of Reiter protein at a constant pH and variable ionic strength were investigated to determine if ionic strength might be made use of for the separation of reactive and non-reactive Reiter protein. The pH value of 5 was chosen as the constant pH since, as shown in Table III, the precipitated fraction contained the greatest serological activity with the least total protein when compared with the other pH values at the constant ionic strength of 0·01. Aliquots of one lot of Reiter protein antigen were dialysed against the various ionic strength buffers, the precipitated fractions were collected by centrifugation, mixed with D'Alessandro buffer to the original volume of the aliquot and dialysed against four changes of D'Alessandro buffer at 8 to 16 hour intervals. The results of one experiment are shown in Table IV (overleaf). At pH 5, with $\mu = 0{\cdot}05$ and $\mu = 0{\cdot}1$, the precipitated fractions contained the number of antigen doses of the original and the protein represented approximately 37 and 46 per cent., respectively, of the original concentration of protein. It can also be seen that at ionic strength of less than 0·05 and more than 0·1 the serological activity and total protein are diminished. These

TABLE III

EFFECT OF pH ON PRECIPITABILITY OF REITER PROTEIN, CARBOHYDRATE, AND SEROLOGICAL ACTIVITY

	Original	pH 2*		pH 3		pH 4		pH 5		pH 6		pH 7	
		S**	P***	S	P	S	P	S	P	S	P	S	P
RPCF Test (doses/ml.)	2,560	0	0	0	640	0	1,280	320	1,280	1,280	320	1,280	320
Protein (mg. per cent.)	107	78	43	32	56	13	80	35	56	78	13	80	13
Carbohydrate (mg. per cent.)	16	0	10	0	10	0	11	10	8	12	0	12	0

* Ionic Strength = 0·01. ** S = Supernatant fraction. *** P = Precipitate fraction.

TABLE IV

EFFECT OF IONIC STRENGTH AT pH 5 ON PRECIPITATION OF REITER PROTEIN AND SEROLOGICAL ACTIVITY

pH	Ionic Strength	Precipitate Fraction	
		Antigen (doses/ml.)	Total Protein (mg, per cent.)
5	0·001	Non-reactive	10
	0·01	640	24
	0·05	2,560	144
	0·1	2,560	176
	0·2	1,280	80
	0·4	160	10
Original Antigen ..		2,560	385

generalizations do not apply to all lots of Reiter protein.

Adsorption and Elution of Reiter Protein from an Anion Exchanger.—It has been demonstrated that diethylaminoethyl (DEAE) cellulose (Peterson and Sober, 1956), an anion exchanger, has a high capacity for the adsorption of certain proteins and that they may subsequently be eluted using mild measures. The possibility existed that Reiter protein might be adsorbed and eluted in a manner that might effect the separation of serologically reactive and non-reactive protein. DEAE cellulose was washed with 1 N NaOH, followed by water to remove excess alkali, equilibrated with phosphate buffer, pH 7·1, $\mu = 0·1$, and collected by sedimentation. Reiter protein, at the same pH and ionic strength, was well mixed with the prepared DEAE cellulose. After 30 min. at room temperature, the cellulose was again sedimented and the supernatant removed. The supernatant was devoid of serological activity. The elution of Reiter protein, batchwise, from three equal parts of DEAE adsorbed Reiter protein employing pH 5 and three ionic strengths (1·0, 0·5, 0·1) is shown in Table V. It can be seen that the elution of serologically reactive protein increases from nil at $\mu = 0·1$ to complete serological recovery at $\mu = 1·0$.

TABLE V

ELUTION OF REITER PROTEIN FROM DEAE ION EXCHANGER

pH	Ionic Strength	Total Doses	Protein (mg. per cent.)
5	1·0	1,280	106
	0·5	160	43
	0·1	None	37
Original Antigen ..		1,280	137

It can be seen, also, that, with complete recovery of serological activity, most of the original protein is also recovered. In this experiment the recovered protein amounted to approximately 77 per cent. of the original protein concentration.

Sedimentation Characteristics of Reiter Protein as found with Ultra-centrifugation and Density Gradient Tubes.—Fig. 1 (opposite) shows two density gradient tubes which were prepared by layering sucrose solutions which provided a gradient at 1·1 at the top to 1·4 at the bottom. The tube on the left shows the appearance of the layer of Reiter protein at the top of the tube and the tube on the right the appearance following 16 hours' centrifugation at 177,000 × G. It can be seen that a wide haze is present in the area of 1·1 density and that a narrow dense area occurred at the 1.4 density layer. By means of a tube-slicer the fractions that were separated in the hazy and dense areas were obtained and examined for serological activity and protein concentration after dialysis against D'Alessandro and Dardanoni (1953) buffer. It was found that almost all the serological activity and original protein concentration were recovered in the dense area (Sp. Gr. 1·4). Under the conditions of this experiment it appears that the density of serologically active and non-reactive Reiter protein is approximately 1·4.

Fig. 2 (opposite) shows two density gradient tubes prepared by layering solutions of ammonium sulphate and ammonium sulphate plus sucrose to provide a density gradient of 1·3 to 1·8. The tube on the left shows the appearance before, and that on the right the appearance after, centrifugation at 177,000 × G. The intended use of the ammonium sulphate solutions was to determine if the centrifugation of the protein through 50 and 75 per cent. saturated ammonium sulphate solutions would result in a re-precipitation of the protein which might produce density differences between serologically active and non-reactive protein. Fig. 2 shows a wide, hazy zone near the top of the tube (density 1·3) and a dense, graded zone near the lower third of the tube (density 1·5). The serological activity and most of the original concentration of protein were recovered in the dense zone. The observed difference in the density of the protein in sucrose solutions (1·4) and in sucrose-ammonium sulphate solutions (1·5) may be the result of particle dehydration in the concentrated ammonium sulphate.

Sedimentation of Serological Activity and Partial Separation of Protein and Carbohydrate at 30,000 R.C.F.—The centrifugation of one lot of Reiter protein antigen for 6 hours at 30,000 R.C.F. resulted

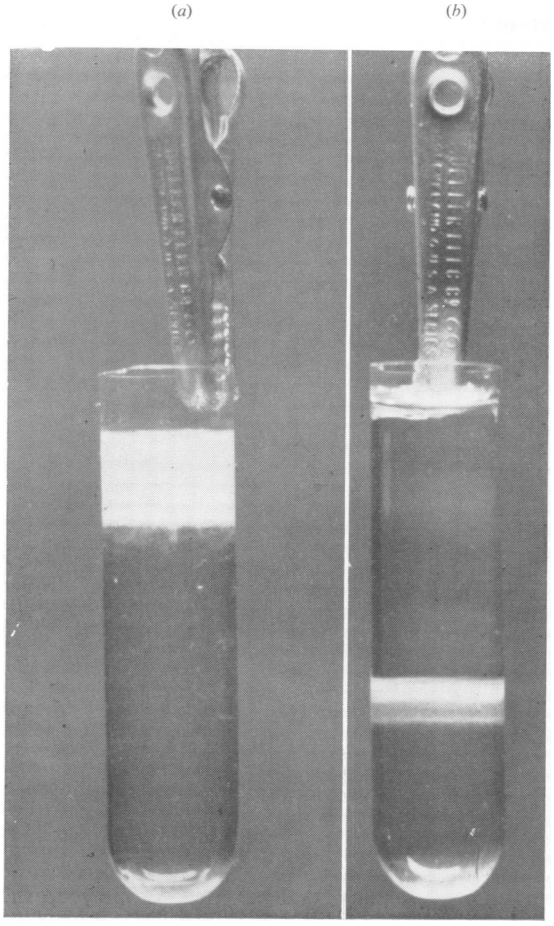

FIG. 1.—Density gradient tubes prepared by layering sucrose solutions. (*a*) RP layer at top of tube. (*b*) After 16 hrs centrifugation at 177,000 × G.

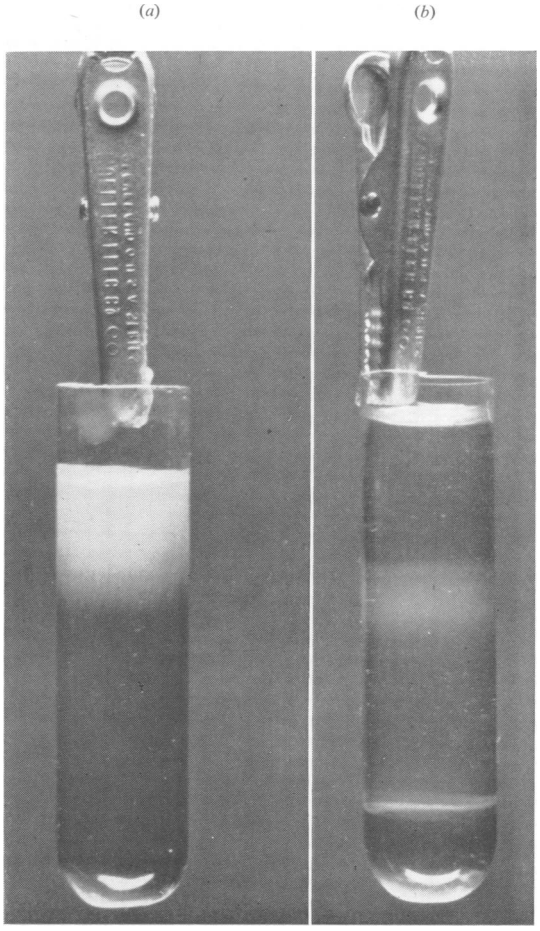

FIG. 2.—Density gradient tubes prepared by layering ammonium sulphate and ammonium sulphate plus sucrose. (*a*) Before centrifugation. (*b*) After centrifugation at 177,000 × G.

in the separation shown in Table VI. With this lot of Reiter protein antigen considerable separation of serologically active and inactive components was achieved when the values of the original antigen and those of the sediment and supernatant are compared. If the original values are compared with those of the supernatant, it will be seen that only a trace of serological activity is retained and that approximately 81 per cent. of the protein and 88 per cent. of the carbohydrate are associated with the trace of serological activity. Or, conversely, that the sediment contained 59 per cent. of the original serological activity in association with approximately 19 per cent. of the original protein and 12 per cent. of the original carbohydrate. The reactive Reiter protein was not purified to a greater degree with this lot of antigen by a second sedimentation and resuspension.

TABLE VI

SEPARATION OF PROTEIN, CARBOHYDRATE, AND SEROLOGICAL REACTIVITY OF REITER PROTEIN ANTIGEN AT 30,000 R.C.F.

	Original Antigen	6 hours' Centrifugation at 30,000 R.C.F.	
		Supernatant	Sediment
RPCF Test (doses /ml.)	2,560	20	1,280
Protein (mg. per cent.)	328	234	62
Carbohydrate (mg. per cent.)	34	28	4

Discussion

Experimental evidence has been presented which shows that different lots of Reiter protein antigen vary considerably in total protein and carbohydrate in relation to serological activity. Six lots of antigen with constant titres (1 : 128) have been shown to vary in total protein from approximately 200 to 800 mg. per cent. and in carbohydrate from 30 to 60 mg. per cent. Though not reported here, it has been observed that other lots of antigen also show similar variations in protein and carbohydrate values with constant serologic reactivities.

D'Alessandro and Dardanoni (1953) originally described Reiter protein antigen as a soluble protein (ATPS, antigene treponemico proteico solubile). Negative results with agar diffusion methods for showing antigen-antibody relationships and the failure of the reactive component to migrate when subjected to paper electrophoresis indicated the antigen was particulate rather than soluble. That the reactive component of the antigen is particulate rather than soluble, has been demonstrated by filtration of antigen through filters of decreasing pore sizes. The single lot of antigen reported in this paper was shown to have its principal reactivity associated with particles in the range of 100 to 800 mμ. Other lots of antigen have shown similar variations of particle sizes.

The filtration studies also demonstrated that considerable and variable amounts of protein and carbohydrate are serologically inactive. The single lot of antigen reported here was shown to have 47·5 per cent. of the protein and 75 per cent. of the carbohydrate serologically inactive. These studies have provided no information concerning the proportions of reactive and non-reactive components associated with that portion of the antigen shown to be serologically active. Though 25 per cent. of the total carbohydrate is associated with the reactive component of this lot of antigen, there is no evidence that the reactive component is, or is not, a carbohydrate-protein complex.

Since it is desirable to have an antigen in the greatest possible state of purity, purification was attempted by use of controlled pH and ionic strength buffers in an effort selectively to precipitate or separate the reactive and non-reactive components. Employing an ionic strength of 0·01 and pH values of 2 to 7, in steps of one pH unit, it was found that the reactivity was recovered from the precipitate at pH 4 and 5. Lower pH values destroyed reactivity and higher values did not result in the precipitation of the reactive component or of significant amounts of other protein or carbohydrate. There appeared to be a partitioning of the protein and carbohydrate between the supernatant and precipitate fractions at pH 5 with most of the reactivity associated with the precipitate fraction.

Further studies involving pH and ionic strength were made in which a constant pH of 5 was maintained while the ionic strength was varied from 0·001 to 0·4. It was found that the principal reactivity was contained in the precipitate obtained with ionic strengths of 0·05 and 0·1. However, the principal total protein recovery was also found at those ionic strengths. Therefore, the employment of constant pH and varied ionic strength or varied pH with constant ionic strength did not result in a selective precipitation or isolation of the reactive component in a much improved state of purity.

During preliminary studies in which attemps were made to adsorb and elute the reactive component from various ion exchangers, it was found that diethylaminoethyl cellulose would adsorb Reiter protein and that it could be eluted by mild measures. Reiter protein was adsorbed at pH 7, $\mu = 0 \cdot 1$, and eluted batchwise at pH 5, $\mu = 1 \cdot 0$, which resulted in complete recovery of serological activity but was also associated with a high recovery of non-reactive components. Batchwise experiments were followed by experiments with column adsorption and various eluents which also did not give separation of active and inactive components.

Differential migration with isopycnic gradient centrifugation employing sucrose demonstrated that the serologically reactive component had a density of approximately 1·4. When sucrose plus ammonium sulphate gradients were employed, the density changed to 1·5. The density change may be explained by dehydration caused by relatively high concentrations of ammonium sulphate. The use of density gradients revealed the density of the reactive component but did not provide any substantial separation of reactive and non-reactive protein.

Centrifugation at the maximum speed of the Servall SS-1 centrifuge for 6 hours has demonstrated that partial purification may be accomplished with this simple technique with certain lots of antigen. The lot of antigen reported here was purified to the extent that 59 per cent. of the reactivity was contained in the sediment when resuspended to its original volume in fresh buffer. The resuspended antigen was associated with 19 per cent. of the original protein and 12 per cent. of the original carbohydrate. It has been found that successive sedimentation and resuspension does not result in a substantially greater degree of separation of reactive and non-reactive components. It has been found that different lots of antigen vary in the degree of

purification effected by simple sedimentation and resuspension.

It should, perhaps, be pointed out that the various values of serological activity, protein, and carbohydrate presented in the Tables and Figures were not obtained with a single lot of antigen. Some of the studies required considerable quantities of antigen which exhausted given lots and subsequent studies were then made with new lots of antigen. The observed differences between the values obtained with original antigens and the cumulative values of the recovered fractions are considered to be within the technical limits of the separation, recovery, and assay methods.

Summary and Conclusions

(1) The serologically active component of Reiter protein antigen considered by D'Alessandro and coworkers to be a soluble protein has been found to be particulate with particle sizes ranging from 100 to 800 mμ in a given lot of antigen.

(2) Different lots of Reiter protein antigen with the same serological titre have been shown to contain considerable and variable amounts of nonreactive protein and carbohydrate.

(3) It has been demonstrated that Reiter protein may be precipitated by the use of buffers of approximately pH 5 and ionic strength 0·01.

(4) Reiter protein can be adsorbed to diethylaminoethyl cellulose at pH 7 and eluted by use of buffers at pH 5 and ionic strength 1.

(5) Isopycnic gradient centrifugation with sucrose gradients demonstrated the reactive and nonreactive protein to have a density of approximately 1·4.

(6) The reactive components and part of the nonreactive components of Reiter protein antigen can be sedimented by centrifugation at approximately 30,000 R.C.F.

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L'antigène de la protéine de Reiter

RÉSUMÉ

(1) On a trouvé que le facteur actif sérologique de l'antigène de la protéine de Reiter, qu'Alessandro et ses collègues considèrent comme une protéine soluble, est composé de particules de 100 à 800 μ dans une quantité donnée d'antigène.

(2) Les quantités différentes avec le même titre sérologique contiennent des parties considérables et variables de protéine non-réactive et d'hydrate de carbone.

(3) La protéine de Reiter peut être précipitée par l'emploi de tampons de pH 5 et de force ionique de 0,01.

(4) La protéine de Reiter peut adsorber la cellulose diethylaminoethyl à pH 7 et peut être éluée par l'emploi de tampons de pH 5 et de force ionique de 1,0.

(5) La centrifugation de gradation isopyknique avec les gradients sucroses montra que la protéine réactive et non-réactive eut une densité d'à peu près 1,4.

(6) Les constituents réactifs and une partie des constituents non-réactifs de l'antigène de la protéine de Reiter peuvent être sédimentés par la centrigufation à une force relative (R.C.F.) de 30.000.