

A STUDY OF THE BINDING BETWEEN SULFADIAZINE
AND SERUM PROTEINS

By

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and Serum Proteins**

I. Introduction.....	1
II. Experiments and Results.....	3
A. Bactericidal activity.....	3
B. Isolation and identification of serum proteins.....	6
1. Precipitation between pH 9.2 and 7.0.....	6
2. Crystal formation at pH 9.2.....	24
3. Precipitation at pH 4.0.....	29
C. Solubility of sulfadiazine.....	35
III. Discussion.....	38
IV. Summary.....	46
V. Acknowledgment.....	47
VI. Literature Cited.....	48

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The results of a large number of experiments conducted in this laboratory have shown that serum proteins removed by acid precipitation of sulfadiazine from a solution of sodium sulfadiazine and bovine Brucella anti-serum have bactericidal properties against Brucella organisms in the presence of complement and traces of sodium sulfadiazine. In vitro experiments have shown that the drug in dilutions of $1:10^3$ to $1:10^5$ in liquid culture medium retard growth for only a 24 hour period. In view of the differences in the growth inhibiting of the drug alone and in the presence of antibody and complement, it became of interest in this laboratory to investigate the binding between the drug and the various protein components of serum. An investigation of this nature may serve to throw light upon probable "reactions" between sulfa compounds and blood serum constituents when these drugs are used as therapeutic agents in human and animal bacterial diseases.

Studies of this problem strongly indicate that sulfadiazine binds mainly with the globulin fraction of serum in an alkaline range and that the binding is through the free amino groups of the proteins. Practically no binding occurs with the free carboxyl groups of the protein. Evidence has also been obtained that reaction of sulfadiazine with serum in an acid range results in binding of the drug to the albumin fraction.

Davis (6, 7, 8) had demonstrated that sulfathiazole binds with

the albumin fraction of $(\text{NH}_4)_2 \text{SO}_4$ separated human serum and that there is practically no binding to the globulin fraction at pH 7.4. The conditions under which his experiments were conducted differ from the conditions used in this laboratory. Davis used $(\text{NH}_4)_2 \text{SO}_4$ separated serum, whereas the results obtained in this laboratory were from whole serum.

EXPERIMENTS AND RESULTS

A. Bacteriocidal activity.

The addition of dilute phosphoric acid to a clear solution of sodium sulfadiazine dissolved in bovine serum results in the precipitation of a white, paste-like substance. Examination under a microscope shows that the precipitate is of a crystalline structure, but this structure differs from the crystalline structure of pure sulfadiazine freshly precipitated from an aqueous solution of sodium sulfadiazine with dilute phosphoric acid. Investigation of this paste-like precipitate revealed that serum proteins are removed from the solution with the precipitated sulfadiazine.

Protein thus removed from a Brucella anti-serum has very high bacteriocidal activity against Brucella organisms in the presence of fresh normal rabbit serum to serve as complement and traces of sodium sulfadiazine (9). This anti-serum protein fraction is prepared in the following fashion: 1.0 gram of sodium sulfadiazine is dissolved in 5.0 ml. of Brucella anti-serum. A clear solution results with a pH of about 9.2. Dilute phosphoric acid (.15N) is added dropwise while the solution is stirred with a mechanical stirrer. Precipitation occurs immediately with the first few drops of the acid. Addition of the acid is continued until the pH of the solution is lowered to pH 7.0. The solution is filtered and the precipitate washed with 5 to 6 portions of .86% saline. The precipitate is dispersed in a small amount of saline and .1N sodium hydroxide is added dropwise to the well stirred slurry. The precipitate gradually dissolves and at pH 10.0 a clear, pale yellow solution usually results. The solution is dialyzed against physiological saline at

4°C. to remove the sodium sulfadiazine.

A representative sample which was used for a bacteriocidal test was prepared as given in the procedure above. The dialysate was filtered through a D8 Hormann sterilizing pad in a Seitz filter. The concentration of the sodium sulfadiazine, as determined by the Bratton and Marshall (3) method, was found to be 8.0 mg/ml. The protein nitrogen content (semi-micro Kjeldahl) was .33 mg/ml. Two series of two-fold dilutions were made of the sterile solution in 5 ml. amounts of fortified tryptose medium in test tubes. The fortified tryptose medium contains tryptose, glucose, thiamine hydrochloride and sodium chloride. The concentration in each dilution is indicated in Table I. To each dilution of one series, 0.2 ml. of fresh, normal rabbit serum was added as complement in order to measure the activity of the protein nitrogen. Each dilution in both series was then inoculated with 10^5 Brucella abortus cells. The tubes were shaken and incubated at 37° C. for 72 hours.

It will be noted from the results that the solution containing only sodium sulfadiazine and the anti-serum protein fraction had only a temporary inhibiting effect on the growth of the organisms. The maximum inhibiting dilution was no higher than that of sodium sulfadiazine alone. The addition of complement to the dilutions of the anti-serum protein fraction and sulfadiazine created a bacteriocidal complex which caused the death of the organisms in all the dilutions except the last. As will be demonstrated later, the major portion of this anti-serum protein fraction contains a high percentage of γ -globulin, and, therefore, contains Brucella antibodies.

Table I

Bactericidal action of Na-sulfadiazine and protein removed from Brucella anti-serum.

Complement added to each tube (a)	Concentration of agents in tubes, $\times 10^{-3}$ ^{mg.} ml. medium									Bacteria control 5 ml. of medium
	NaSD	80	40	20	10	5	2.5	1.25	0.62	
	Protein Nitrogen	3.3	1.6	0.8	0.4	0.2	0.1	0.05	0.025	
	Incubation period hours	Degree of growth, turbidity								
0.2 ml.	24	-	-	-	-	-	-	-	1+	2+ ^(b)
	72	-	-	-	-	-	-	-	2+	6+
None	24	-	-	-	-	-	1+	2+	2+	2+
	72	4+	4+	4+	4+	4+	4+	6+	6+	6+

10^5 Br. abortus added to each dilution and controls.

(a) Fresh, normal rabbit serum.

(b) 0.2 ml. of fresh, normal rabbit serum added to control tube.

- = no visible growth. + = degree of growth.

B. Isolation and identification of serum proteins.

1. Precipitation between pH 9.2 and 7.0.

Because of the bactericidal effect of the mixture of the protein fraction, sodium sulfadiazine and complement, it became of interest to determine the nature of the proteins removed with the precipitation of the sulfadiazine, as discussed above.

Preliminary work on the problem was to investigate the quantity of protein removed with varying portions of sodium sulfadiazine dissolved in 5.0 ml. of the serum. A series of samples was set up using 0.2 gms., 0.5 gms., 0.8 gms., 1.25 gms., 1.75 gms., and 2.25 gms. each of which was dissolved in 5.0 ml. of serum. Dilute (.15N) phosphoric acid was added dropwise to the agitated solutions. The pH of each solution was gradually lowered from the initial pH of 9.2 to pH 7.0. The samples were filtered through 2-#1 Whatman filter papers placed in a Büchner Funnel. The precipitates were rinsed several times with .86% saline to remove the soluble substances. They were then removed from the filter papers, dispersed in saline and re-filtered and rinsed. The precipitates were placed in the original beakers and dissolved while agitated with .1 N sodium hydroxide to pH 10.0. The resulting solutions were clear or slightly opalescent and pale yellow. This work was done as quantitatively as possible. The alkaline solutions were placed in cellophane tubing and dialyzed at 4°C. against .86% saline for several days with daily saline changes to completely remove the sodium sulfadiazine. The sodium sulfadiazine was removed so that the protein nitrogen value determined by semi-micro Kjeldahl method was direct and not by difference since sodium sulfadiazine contains 20.6% nitrogen. After

dialysis the volume of each solution was measured, a semi-micro Kjeldahl determination made, and the presence of sodium sulfadiazine checked by the Bratton and Marshall procedure. The total nitrogen value was calculated on the basis of the total volume of the solution and then multiplied by 6.25 to convert to grams of protein. The protein removed for each weight of sodium sulfadiazine used is recorded in Tables II, III, and IV. Three different bovine sera were used; #991 (8.5% protein) contained Brucella agglutinins in a titer of 1:10,000, #1441 (7.5% protein) came from an immunized cow of a low agglutinin titer, and #1443 (6.9% protein) was from a non-infected cow. More protein was removed from #991 because of the higher γ -globulin content present in this serum. The reason for this conclusion will become more apparent later. Figs. 1, 2, and 3 illustrate graphically the results given in the tables mentioned above. The curves in each case show a steep slope up to about 0.8 gram sodium sulfadiazine. In fact, this portion of the curve is almost a straight line.

With each drop of dilute phosphoric acid that is added to a solution of sodium sulfadiazine and serum, sulfadiazine precipitates and also removes protein from the serum. The pH of the solution gradually decreases. Three samples of 1.0 gram sodium sulfadiazine and 5.0 ml. of #991 serum were set up to determine the amount of protein removed by sulfadiazine with the addition of dilute phosphoric acid when the final pH of each solution was 8.5, 8.0, and 7.5 respectively. The procedure was essentially as given above. The data in Table V illustrate that the quantity of protein nitrogen removed varies with the final pH of the solution. The ratio of

Table II

Protein removed from a Brucella anti-serum (No. 991
bovine) during precipitation of sulfadiazine.

Sample No.	1	2	3	4	5	6
Ml. serum 8.5 per cent protein	5.0	5.0	5.0	5.0	5.0	5.0
Gm. NaSD	0.20	0.50	0.80	1.25	1.75	2.25
Gm. protein removed	0.046	0.110	0.163	0.209	0.249	0.252

Table III

Protein removed from a bovine serum (No. 1441,
immunized) during precipitation of sulfadiazine.

Sample No.	1	2	3	4	5	6
Ml. serum 7.5 per cent protein	5.0	5.0	5.0	5.0	5.0	5.0
Gm. NaSD	0.20	0.50	0.80	1.25	1.75	2.25
Gm. protein removed	0.043	0.097	0.142	0.171	0.180	0.197

Table IV

Protein removed from a normal bovine serum
(No. 1143) during precipitation of sulfadiazine.

Sample No.	1	2	3
Ml. serum 6.9 per cent protein	5.0	5.0	5.0
Gm. NaSD	1.25	1.75	2.25
Gm. protein removed	0.174	0.191	0.201

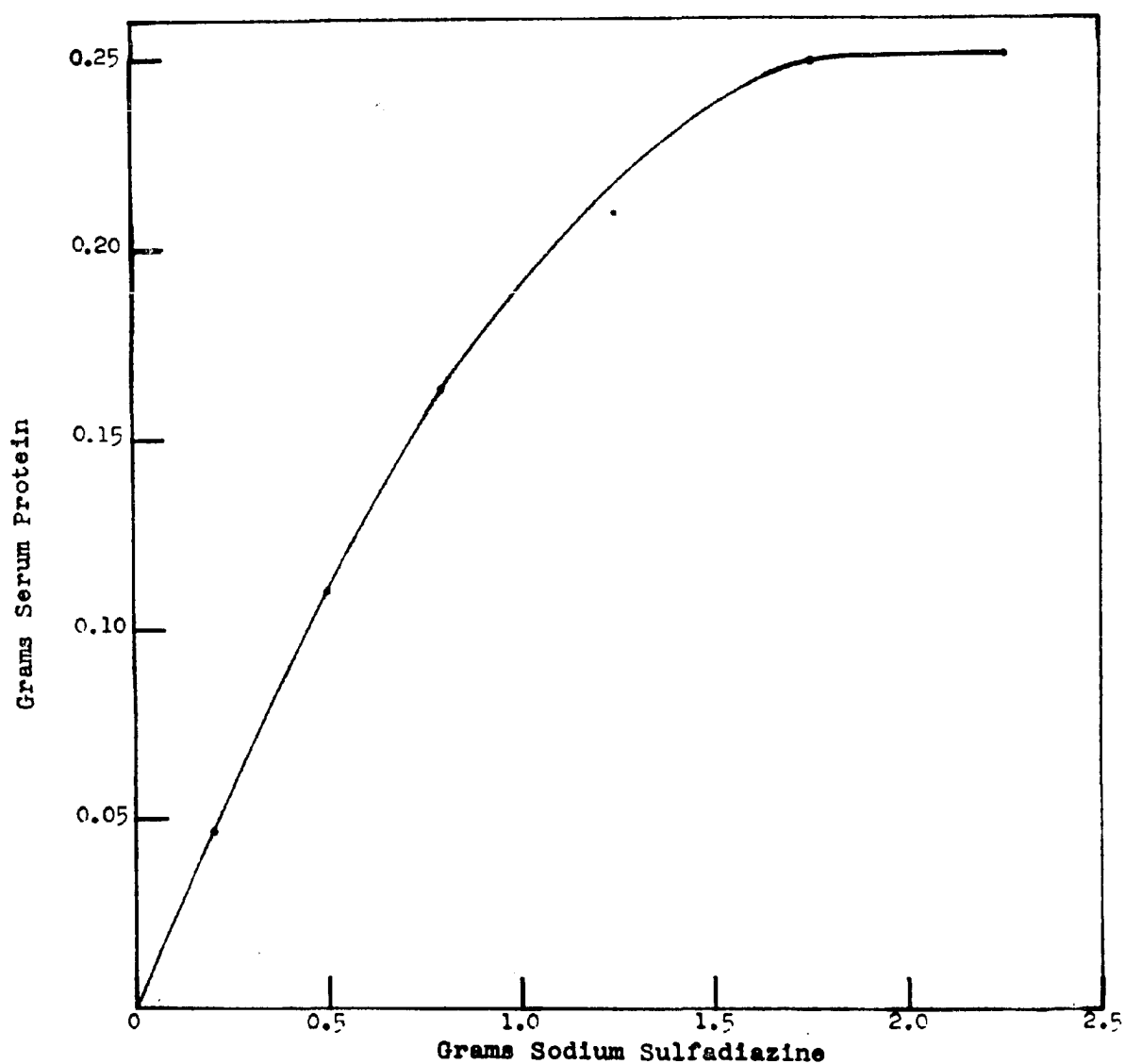


Fig. 1. Serum protein removed from solutions of 5.0 ml. No. 991 serum with varying quantities of sodium sulfadiazine.

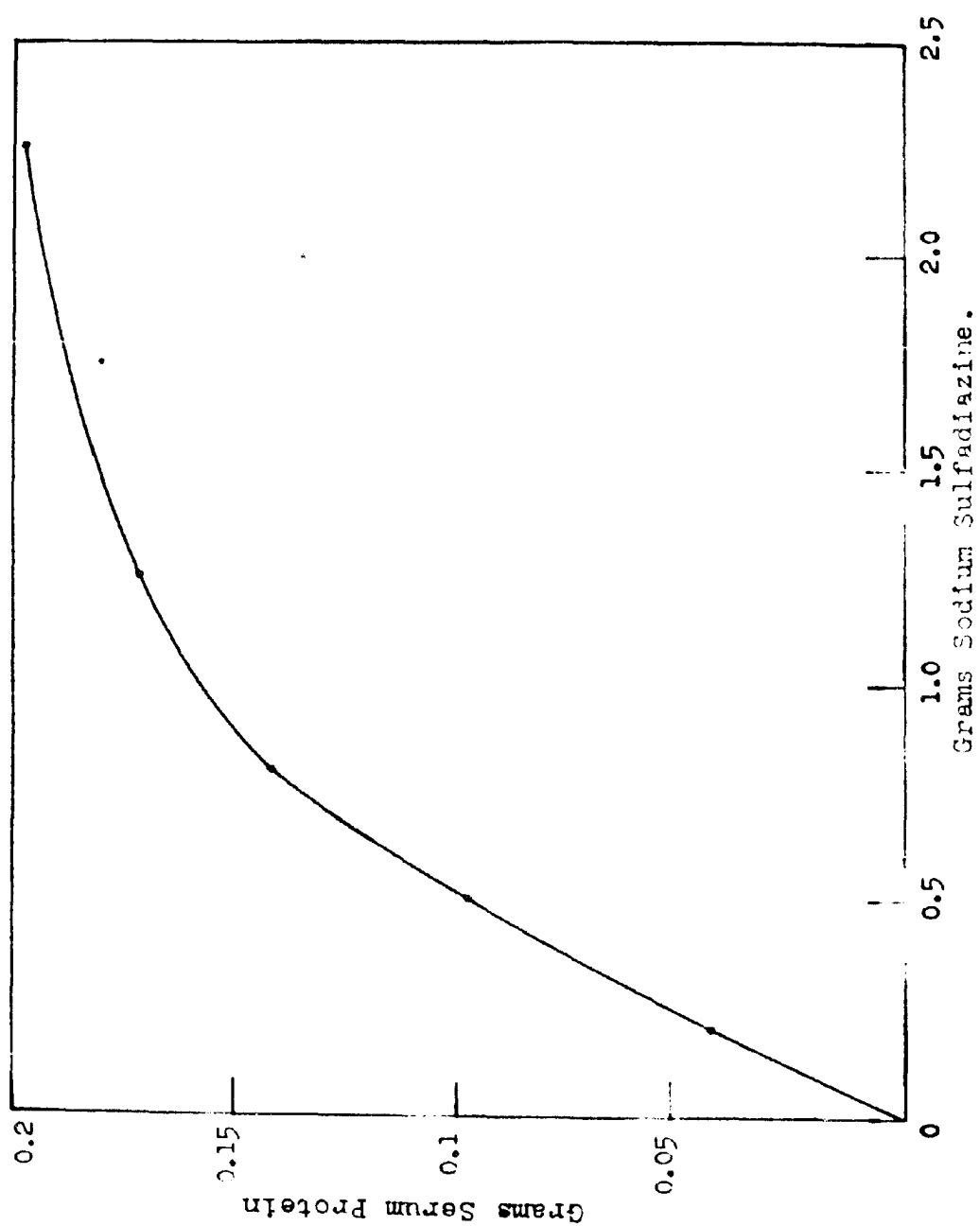


Fig. 2. Serum protein removed from solutions of 5.0 ml. No. 1441 serum with varying quantities of sodium sulfadiazine.

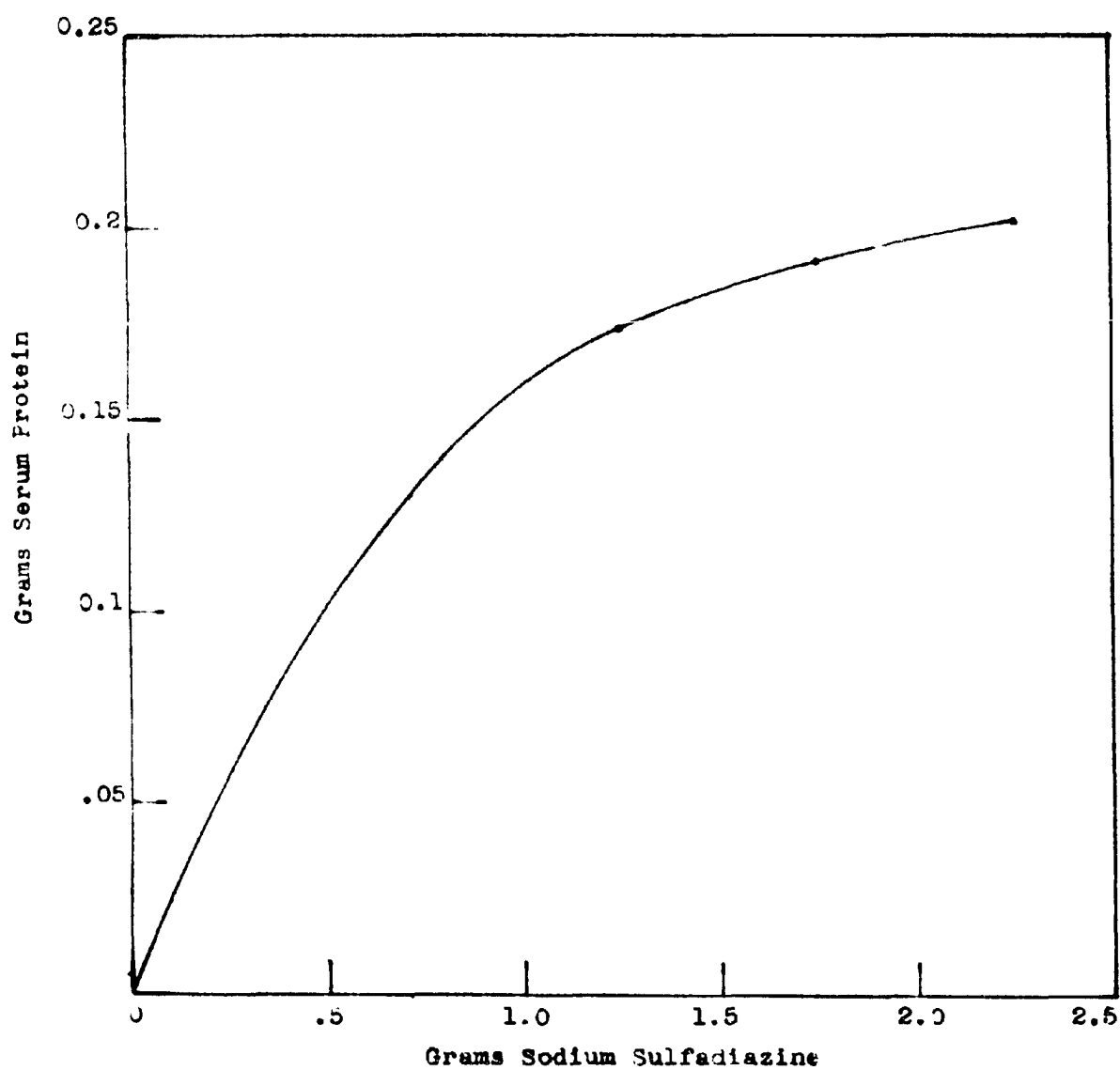


Fig. 3. Serum protein removed from solutions of 5.0 ml. No. 1443 serum with varying quantities of sodium sulfadiazine.

mg. protein N removed is quite constant. One mg. of sodium sulfadiazine precipitated removes on the average .033 mg. of protein nitrogen. The results are illustrated graphically in Fig. 4. The graph of serum protein nitrogen removed plotted against pH forms a smooth curve and shows clearly that the serum protein nitrogen removed is a function of the final pH.

Identification of the components of the total protein removed by the addition of acid to a solution sulfadiazine and serum was determined electrophoretically with the aid of the Tiselius (19) apparatus as modified by Longsworth and MacInnes (13). In order to obtain sufficient serum protein for each determination, 50.0 ml. samples of serum were used. To separate 50.0 ml. samples of #991 serum was added 5.0, 8.0, 12.5, and 22.5 grams of sodium sulfadiazine. Each sample was treated with dilute (.15N) phosphoric acid to pH 7.0, filtered, and the precipitate rinsed with .86% saline. The filtrates were saved. The precipitates were dissolved with .1N sodium hydroxide to pH 10.0. The resulting solutions were dialyzed at 4°C. against 10 liters of .86% saline for 9 days with daily saline changes to remove the sodium sulfadiazine. To increase the protein percentage of the solutions to slightly above 2.0% for electrophoresis studies, the solutions were concentrated by rapid evaporation in cellophane tubing under an over-head fan at 37°C. After evaporation, sodium sulfadiazine and nitrogen determinations were made. The samples which had been treated with 12.5 and 22.5 mgs. of sodium sulfadiazine were diluted to 2.0% with barbital buffer of pH 8.6 and .1 ionic strength and equilibrated by dialysis against this buffer. The other two samples which had been treated with 5.0

Table V

Protein removed from No. 991 serum during the precipitation of sulfadiazine between the pH figures as indicated in the table.

Sample No.	1	2	3
Ml. serum	5.0	5.0	5.0
Gm. NaSD	1.0	1.0	1.0
Initial pH	9.2	9.2	9.2
Final pH	8.5	8.0	7.5
NaSD content, mg.	578.7	730.4	777.0
Protein N, mg.	18.0	23.5	27.0
$\frac{\text{mg. protein N}}{1.0 \text{ mg. NaSD}}$	.031	.032	.035

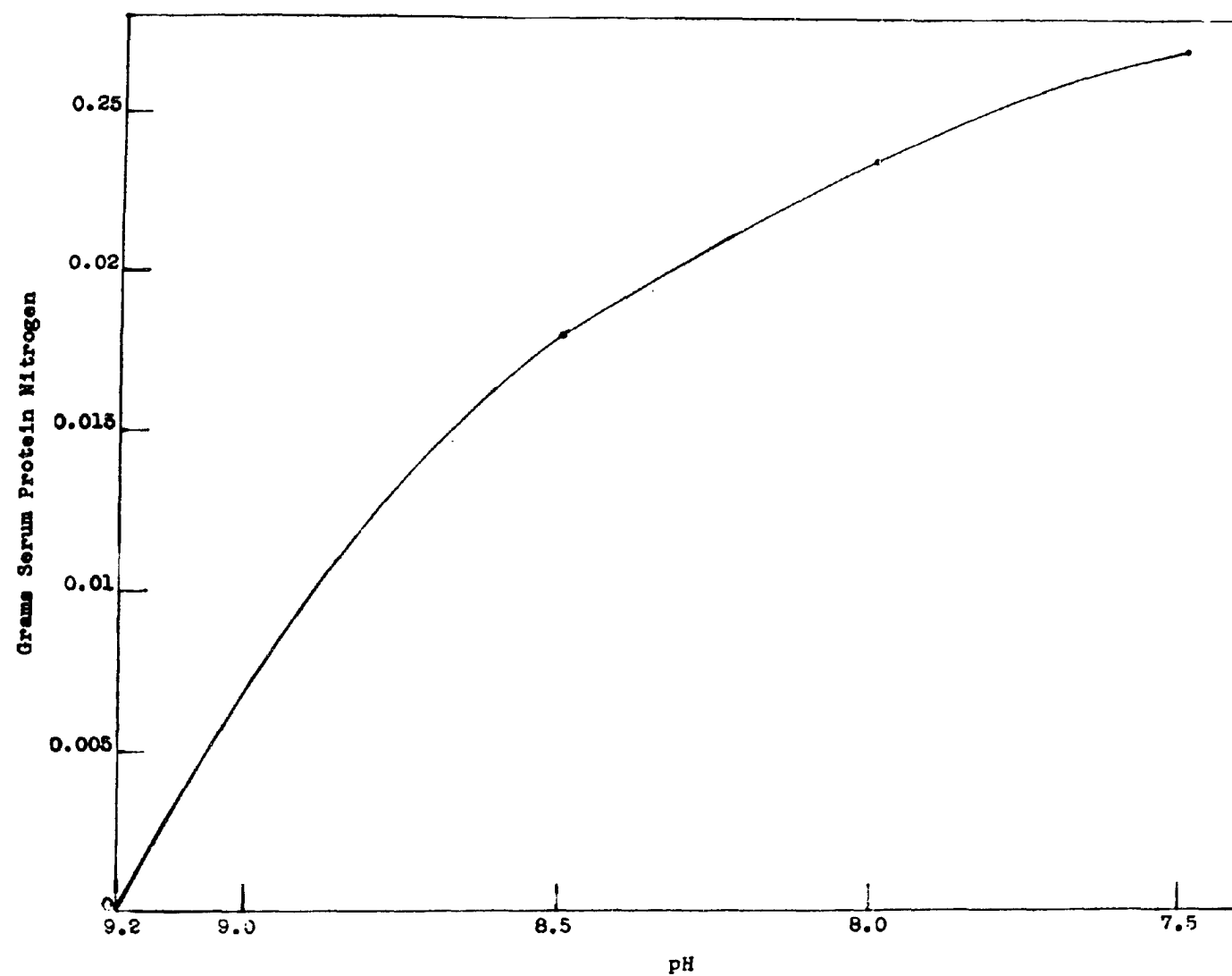


Fig. 4. Serum protein nitrogen removed from solutions of 5.0 ml. No. 991 serum and 1.0 gm. sodium sulfadiazine by the precipitation of sulfadiazine from the initial pH 9.2 to 8.5, 8.0 and 7.5 respectively.

and 8.0 grams sodium sulfadiazine respectively were discarded after the total protein nitrogen removed had been determined.

Sodium sulfadiazine was determined from the filtrate solutions and was found to vary from 0.20 to 0.24 grams in each of the four filtrates. This means that the precipitation of the sulfadiazine from its sodium salt is practically 100% at pH 7.0. The filtrates for electrophoretic studies were prepared and treated in the same way as the precipitate solutions. Electrophoretic scanning patterns (13) were made on all of the filtrates.

In Table VI are recorded the grams of protein removed in the precipitate and the grams of protein that remained in the filtrate. The recovery of total proteins as compared to proteins originally present in the 50.0 ml. of serum varied from 90.6 to 94.5%. Probable losses occurred in the precipitate, washings of the precipitate, losses in the dialysing tubing, etc. The shape of the curves in Fig. 5 shows that protein is removed proportionately to the quantity of sodium sulfadiazine used.

The electrophoretic patterns furnish a visual picture of protein components that are removed during the precipitation of the sulfadiazine from its sodium salt with acid, and the protein components that remain behind in the filtrate. By comparing patterns of the filtrate with that of the original untreated serum it is obvious that the precipitation of the sulfadiazine removes protein mainly from the globulin region, especially the γ -globulin. This is more evident in the patterns of the proteins removed with the precipitate. Figs. 6, 7, and 8 demonstrate graphically the results recorded in Tables VI and VIII-XIV, which were calculated from the descending

Table VI

Protein removed from a Brucella anti-serum
(No. 991 bovine) during precipitation of sulfadiazine.

Sample No.	1	2	3	4
Ml. serum	50.0	50.0	50.0	50.0
Gm. protein in serum	4.24	4.24	4.24	4.24
Gm. NaSD	5.0	8.0	12.5	22.5
Gm. protein removed with ppt.	0.99	1.33	1.75	2.40
Gm. protein in filtrate	2.99	2.52	2.13	1.43
Per cent recovery	94.5	91.0	91.6	90.6

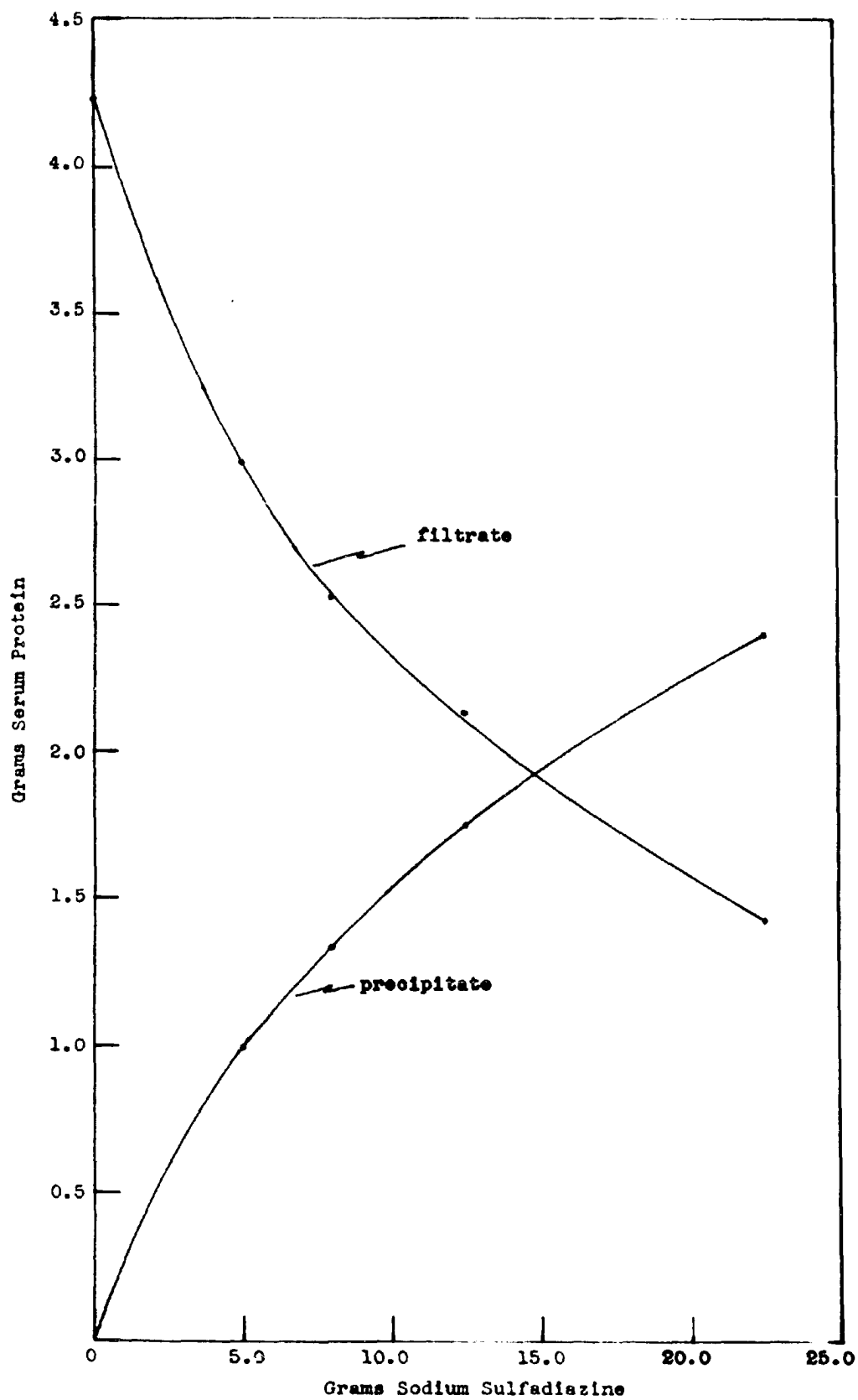


Fig. 5. Serum protein removed and serum protein in the filtrate from solutions of 50.0 ml. No. 991 serum and varying quantities of sodium sulfadiazine.

electrophoretic patterns. The curves in Fig. 6, drawn from the data obtained from the filtrates, represent the manner in which the protein components of the serum remain in the filtrate with increasing amounts of sodium sulfadiazine. The γ -globulin curve has the steepest slope, thus demonstrating that the sulfadiazine precipitate removes γ -globulin in preference to the other protein components in the serum. The α and β -globulin curves show that these components are also removed, but not nearly as rapid as the γ -globulin. The albumin curve has a peculiar shape. The curve starts with a rather steep slope, then tends to straighten out somewhat, and finally, beyond 8.0 grams of sodium sulfadiazine has a slope about as steep as the γ -globulin curve. Figs. 14 and 15 also show that the albumin content in the precipitate increases as the sodium sulfadiazine is increased from 12.5 grams to 22.5 grams. This can also be seen from the albumin curve in Fig. 8, in which the grams of albumin in the precipitate was plotted against grams of sodium sulfadiazine. The α , β and γ -globulin components present in precipitate were added together and plotted as shown in Fig. 8. In comparison, the globulins, mainly the γ -globulin, are removed from the serum to a much greater extent than the albumin fraction. The electrophoretic pattern, Fig. 9, illustrates the original No. 991 serum before treatment with sodium sulfadiazine. The electrophoretic patterns of the filtrates, Figs. 10, 11, 12, and 13 show a progressive decrease in the globulin region.

A similar set of results were obtained from the use of #1143 serum. The curves in Fig. 16 and 17 have the same general trend as those of #991 serum. It is of interest to note that the intersection

Table VII

Protein removed from a normal bovine serum
(No. 1443) during precipitation of sulfadiazine.

Sample No.	1	2	3	4
Ml. serum	50.0	50.0	50.0	50.0
Gm. protein in serum	3.43	3.43	3.43	3.43
Gm. NaSD	5.0	8.0	12.5	22.5
Gm. protein removed with ppte.	0.97	1.21	1.50	1.95
Gm. protein in filtrate	2.37	2.06	1.77	1.24
Per cent recovery	97.2	95.4	95.4	93.0

Table VIII

Data obtained from calculation of descending
electrophoretic pattern, Fig. 9. No. 991 untreated serum.

Serum protein	A	α	β	γ
Per cent from pattern	37.2	14.0	9.9	38.9
Gm. protein/50 ml. serum	1.58	0.59	0.42	1.65

Table IX

Data obtained from calculation of descending electrophoretic pattern of filtrate, Fig. 10.

Serum protein	A	α	β	γ
Per cent from pattern	45.5	8.5	9.8	36.2
Gm. protein	1.36	0.26	0.29	1.08
Per cent based on 50.0 ml. serum	32.1	6.0	6.9	25.5

Filtrate from 50 ml. No. 991 serum treated with 5.0 gm. NaSD.
Protein in filtrate = 2.99 gm. or 70.6 per cent protein remained
in filtrate.

Table X

Data obtained from calculation of descending
electrophoretic pattern of filtrate, Fig. 11.

Serum protein	A	α	β	γ
Per cent from pattern	52.2	7.4	9.5	30.9
Gm. protein	1.32	0.19	0.24	0.78
Per cent based on 50.0 ml. serum	31.1	4.4	5.6	18.4

Filtrate from 50 ml. No. 991 serum treated with 8.0 gm.
NaSD. Protein in filtrate = 2.52 gm. or 59.5 per cent
protein remained in filtrate.

Table XI

Data obtained from calculation of descending
electrophoretic pattern of filtrate, Fig. 12.

Serum protein	A	α	β	γ
Per cent from pattern	57.5	7.1	8.5	26.9
Gm. protein	1.22	0.15	0.18	0.57
Per cent based on 50.0 ml. serum	28.9	3.5	4.3	13.5

Filtrate from 50 ml. No. 991 serum treated with 12.5 gm. NaSD.
Protein in filtrate = 2.13 gm. or 50.3 per cent protein remained
in filtrate.

Table XII

Data obtained from calculation of descending electrophoretic pattern of filtrate, Fig. 13.

Serum protein	A	α	β	γ
Per cent from pattern	63.1	6.9	9.8	20.2
Gm. protein	0.90	0.10	0.14	0.29
Per cent based on 50.0 ml. serum	21.3	2.3	3.3	6.8

Filtrate from 50 ml. No. 991 serum treated with 22.5 gm. NaSD. Protein in filtrate = 1.43 gm. or 33.8 per cent protein remained in filtrate.

Table XIII

Data obtained from calculation of descending electrophoretic pattern of precipitate, Fig. 14.

Serum protein	A	α, β , and γ
Per cent from pattern	10.0	90.0
Gm. protein	0.17	1.58
Per cent based on 50 ml. serum	4.1	37.2

Precipitate from 50 ml. No. 991 serum treated with 12.5 gm. NaSD. Protein in precipitate = 1.75 gm. or 41.3 per cent of the protein in the serum was removed.

Table XIV

Data obtained from calculation of descending electrophoretic pattern of precipitate, Fig. 15.

Serum protein	A	α , β , and γ
Per cent from pattern	16.5	83.5
Gm. protein	0.40	2.00
Per cent based on 50 ml. serum	9.4	47.3

Precipitate from 50 ml. No. 991 serum treated with 22.5 gm. NaSD. Protein in precipitate = 2.4 gm. or 56.7 per cent of the protein in the serum was removed.

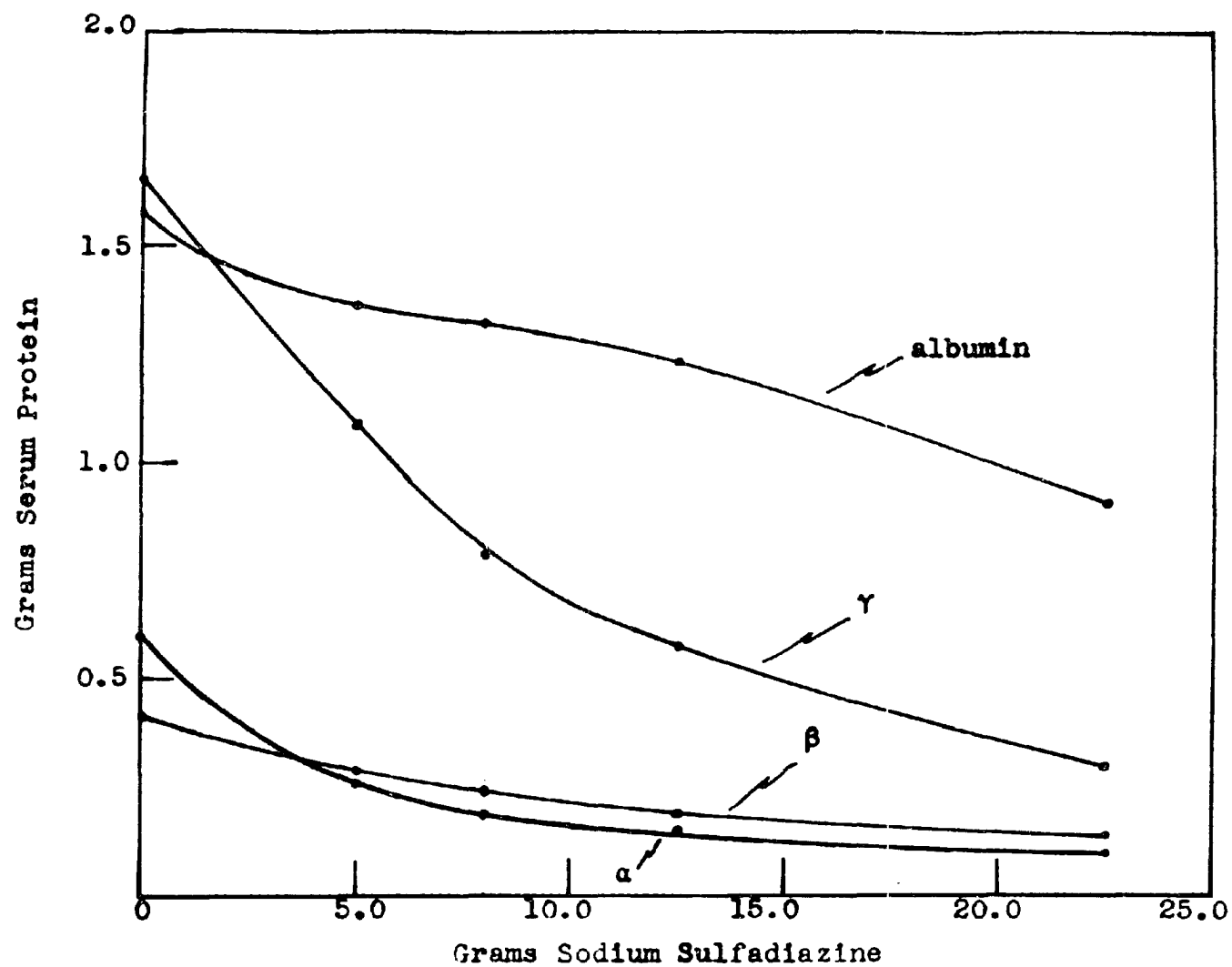


Fig. 6. Serum protein components in the filtrates from solutions of 50.0 ml. No. 991 serum and varying quantities of sodium sulfadiazine.

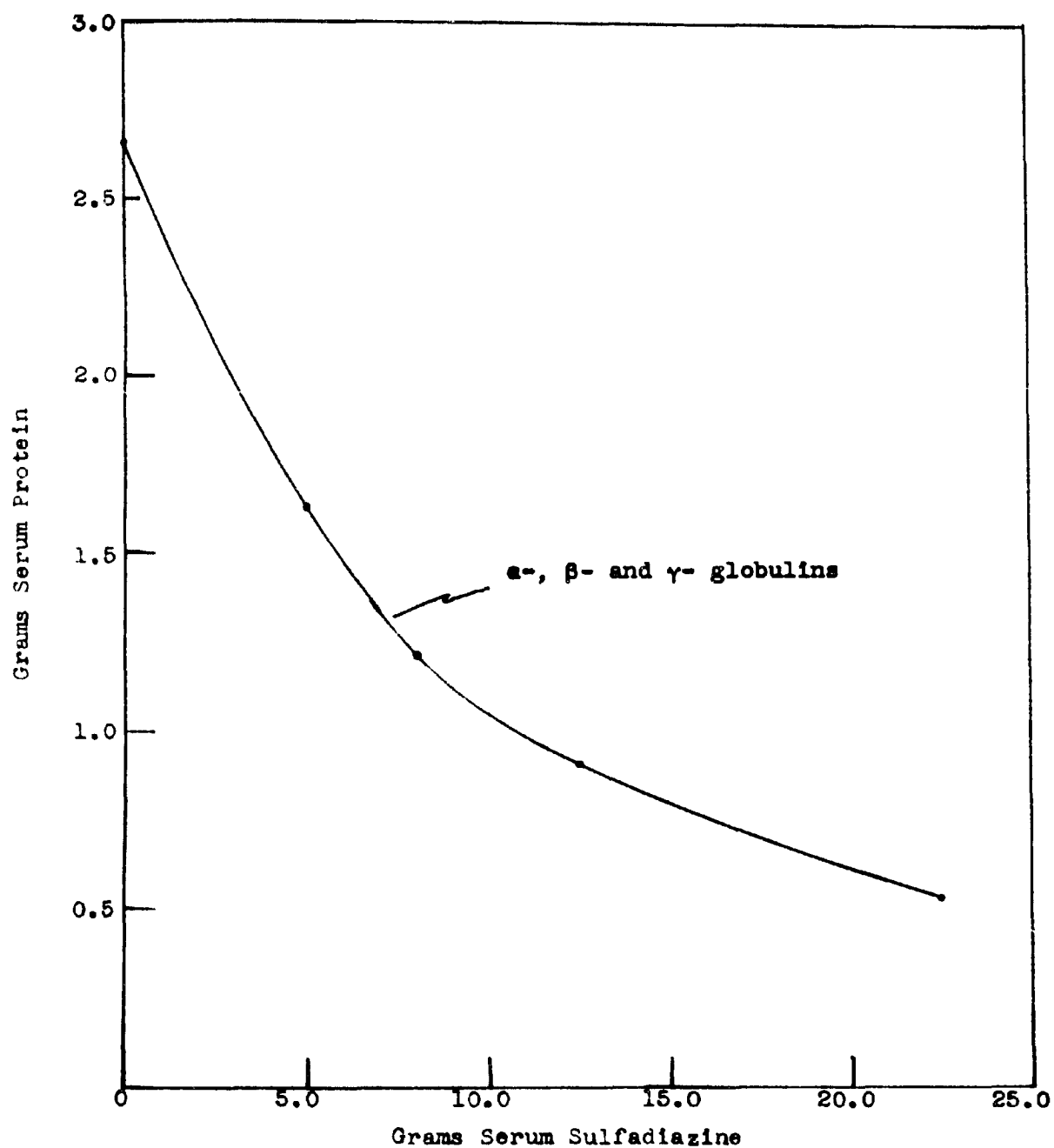


Fig. 7. α -, β - and γ -globulins in the filtrates from No. 991 serum plotted against varying quantities of sodium sulfadiazine.

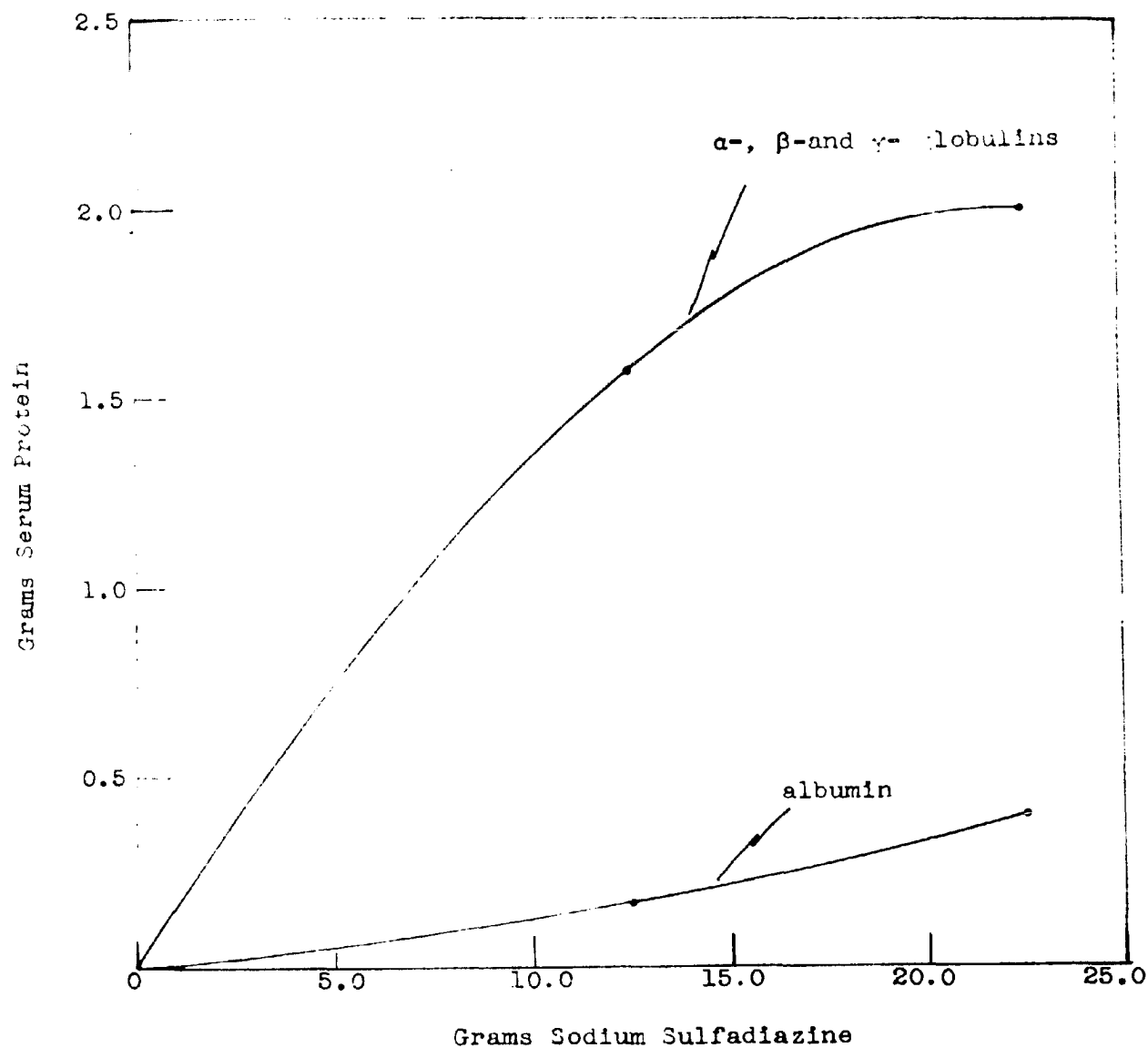


Fig. 8. α -, β - and γ -globulins and albumin removed from solutions of 50.0 ml. No. 991 serum with varying quantities of sodium sulfadiazine.

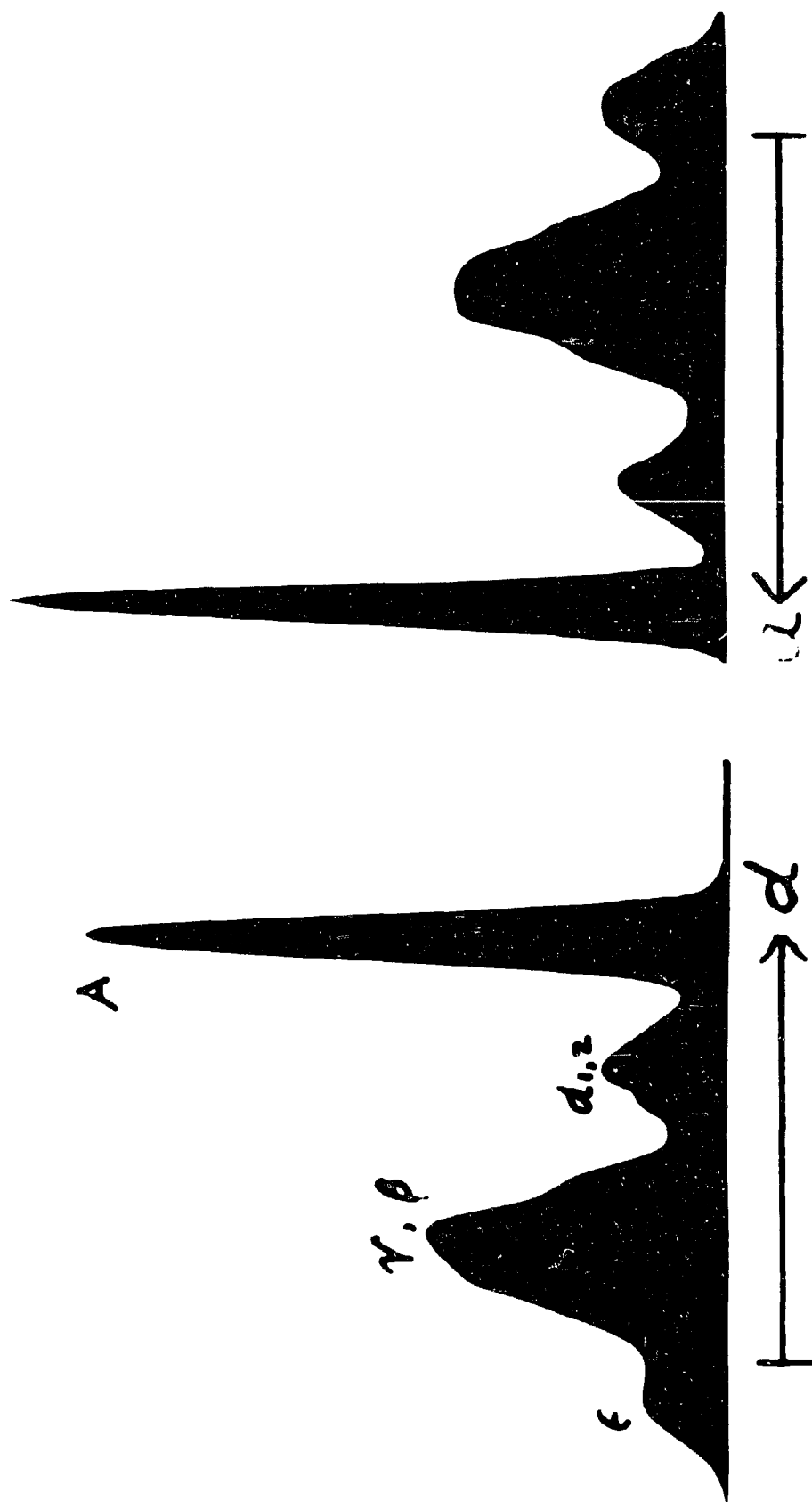


Fig. 9. Bovine serum No. 991. Electrophoresis for 9,000 seconds at 6.05 volts per cm. Protein conc. 2 per cent.

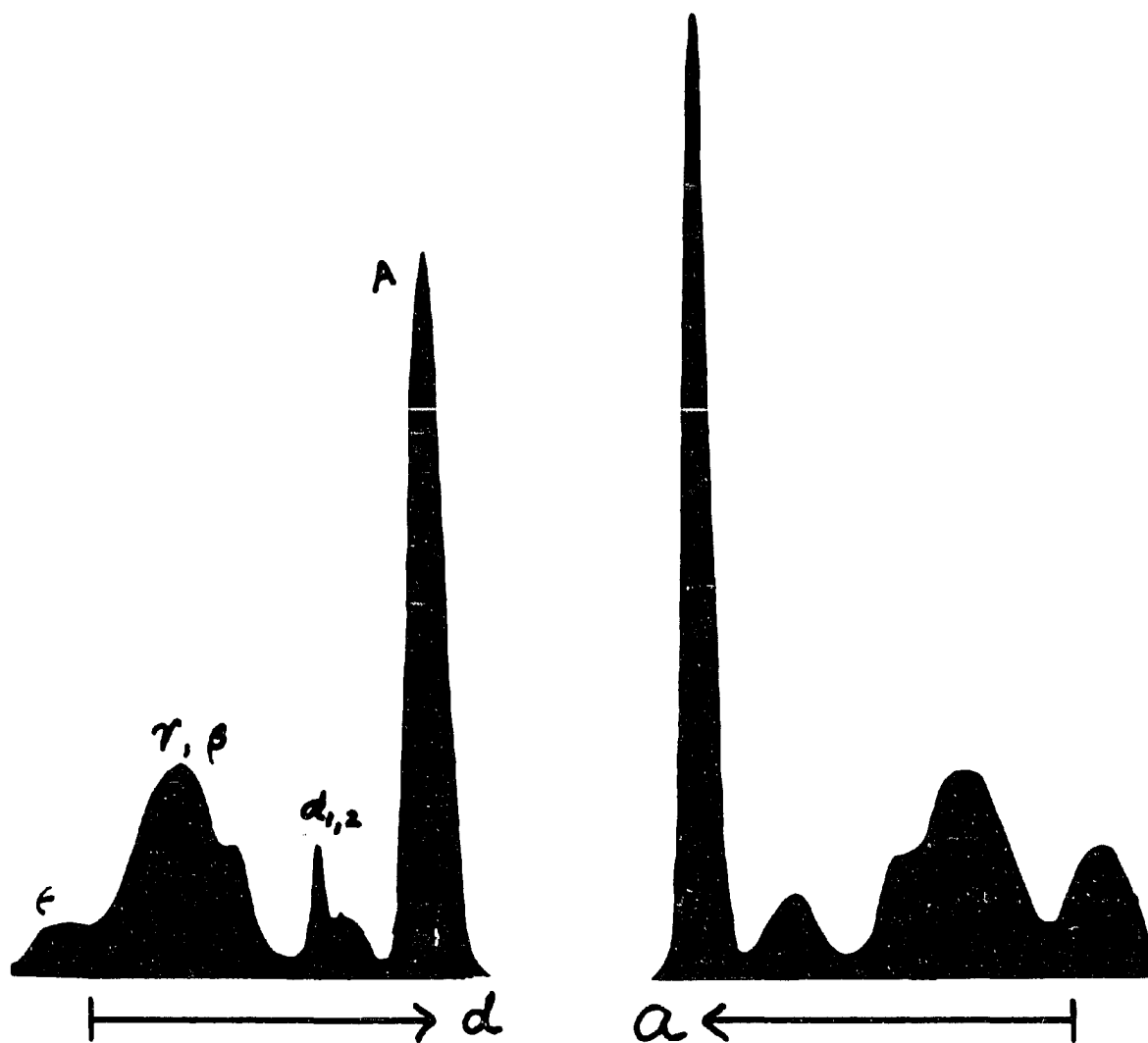


Fig. 10. Composition of filtrate from a solution of 50 ml. No. 991 serum and 5.0 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.77 volts per cm. Protein conc. 2 per cent.

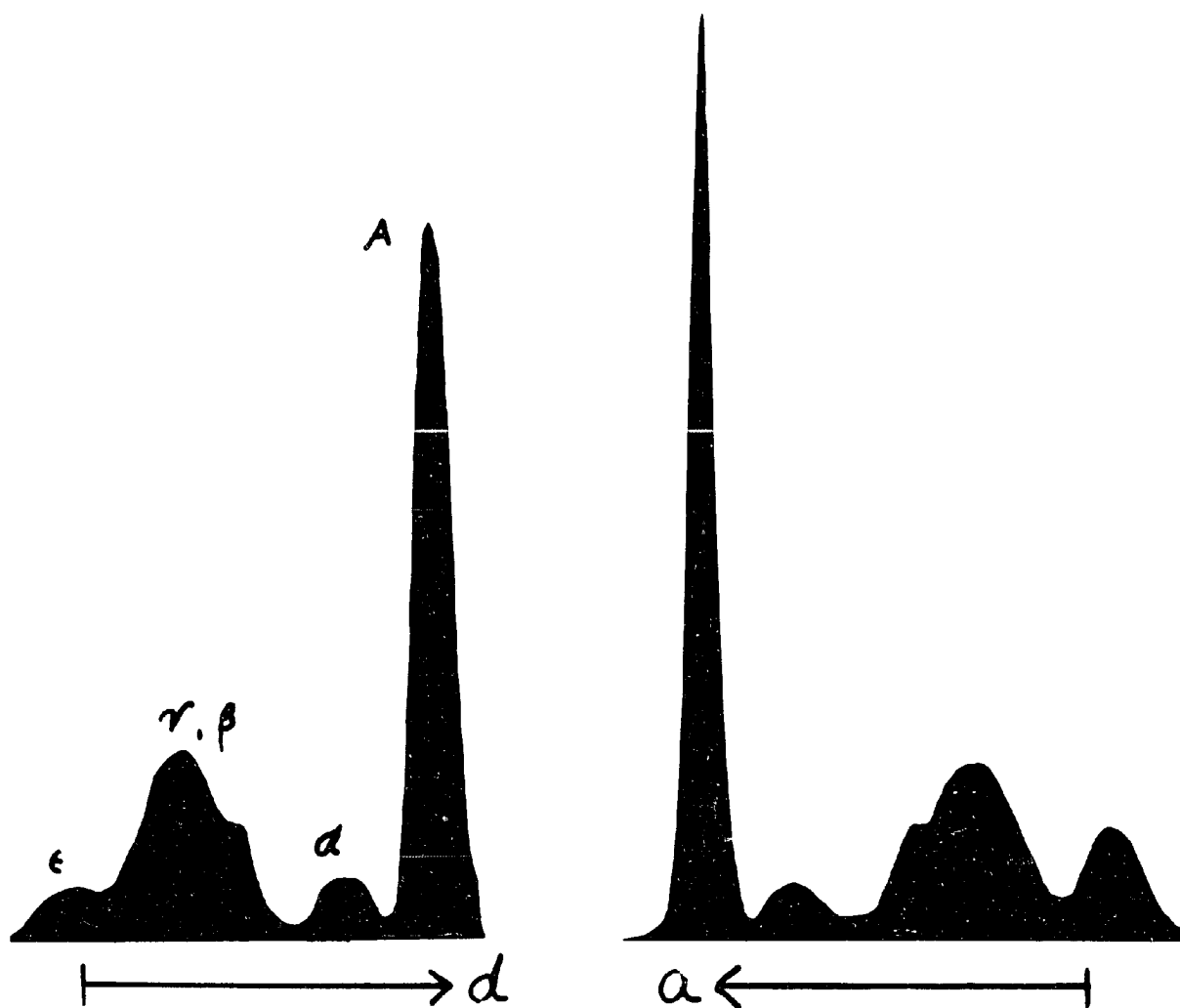


Fig. 11. Composition of filtrate from a solution of 50 ml. No. 991 serum and 8.0 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.94 volts per cm. Protein conc. 2 per cent.

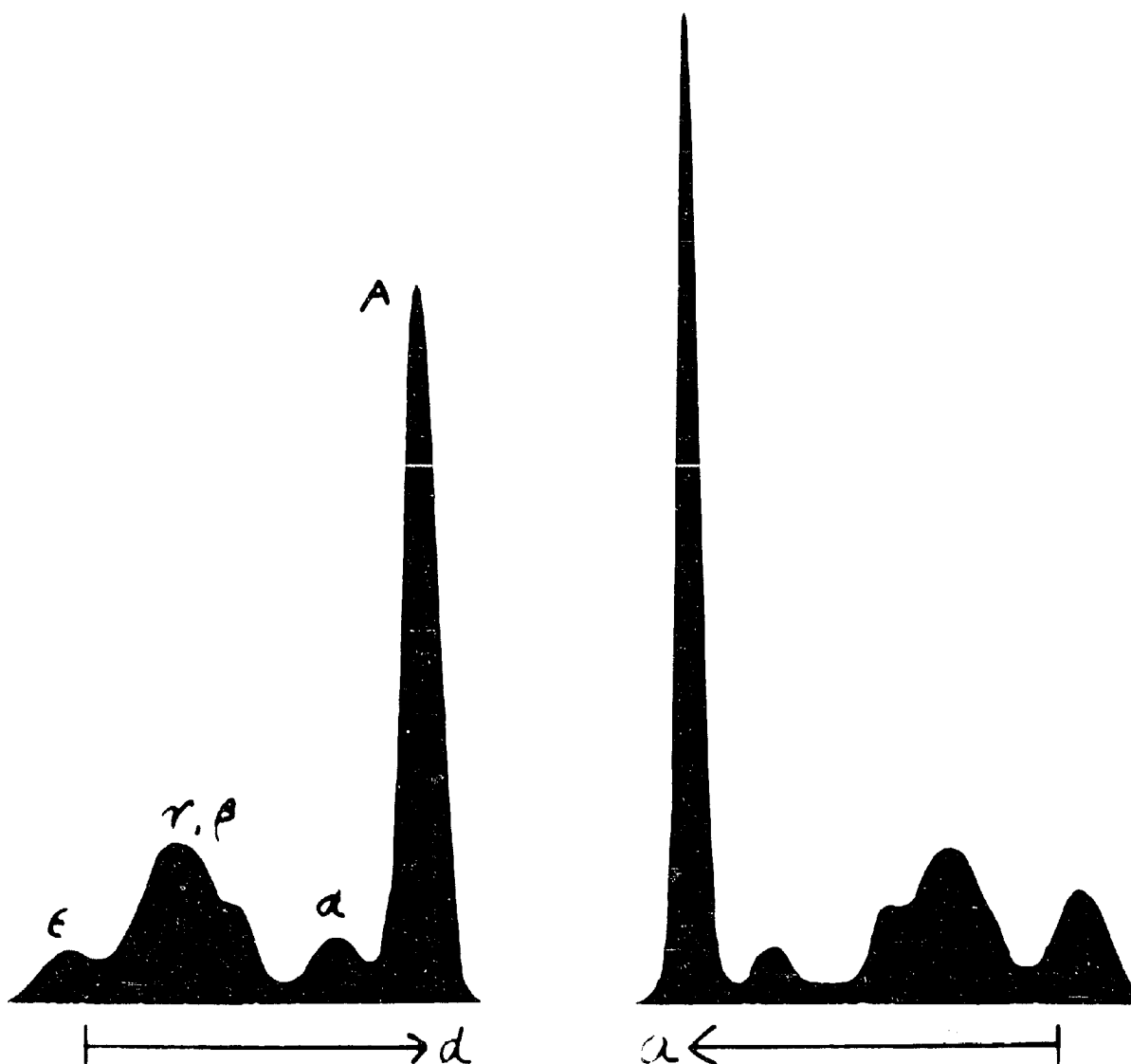


Fig. 12. Composition of filtrate from a solution of 50 ml. No. 991 serum and 12.5 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.83 volts per cm. Protein conc. 2 per cent.

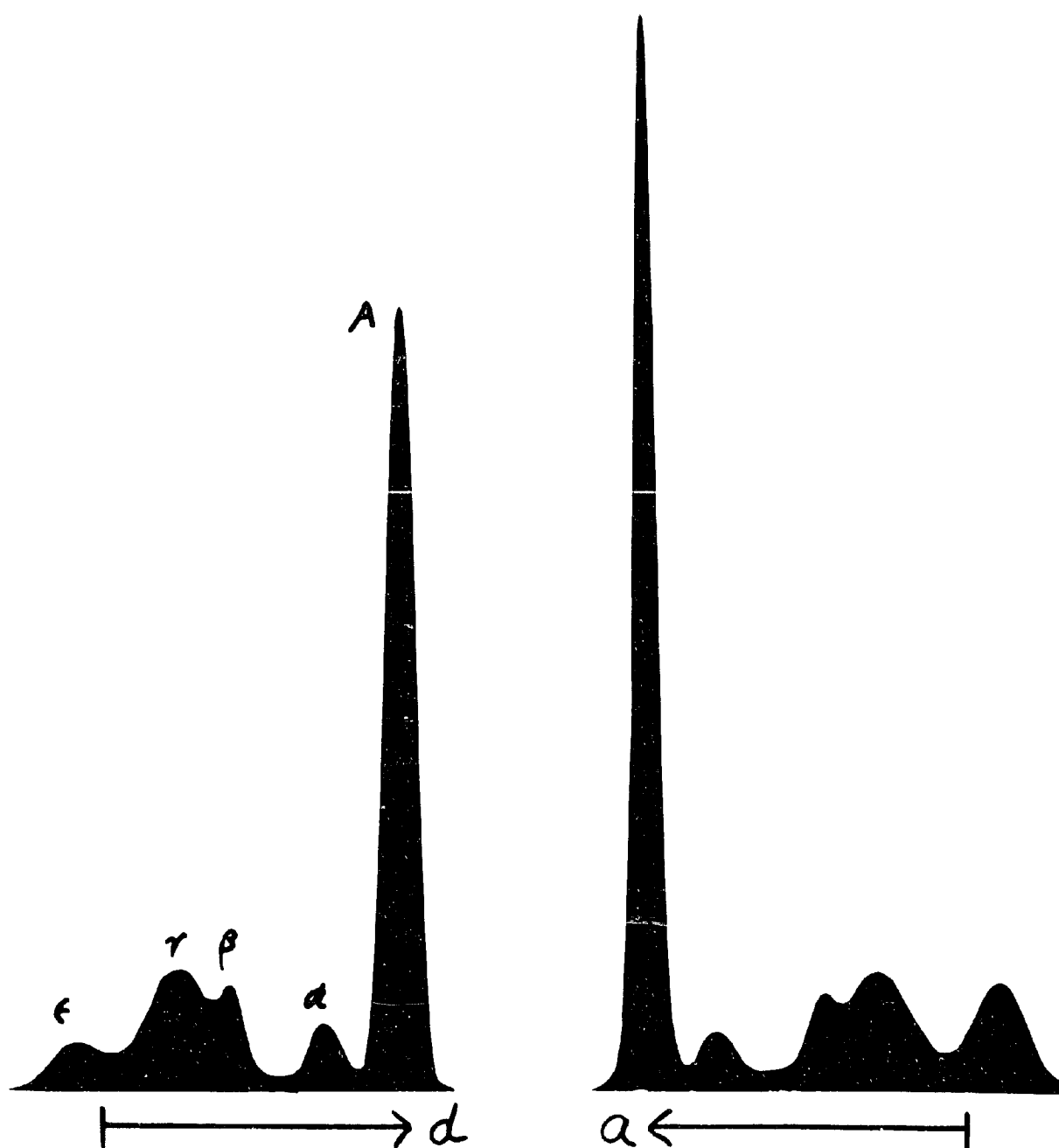


Fig. 13. Composition of filtrate from a solution of 50 ml. No. 991 serum and 22.5 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.88 volts per cm. Protein conc. 2 per cent.

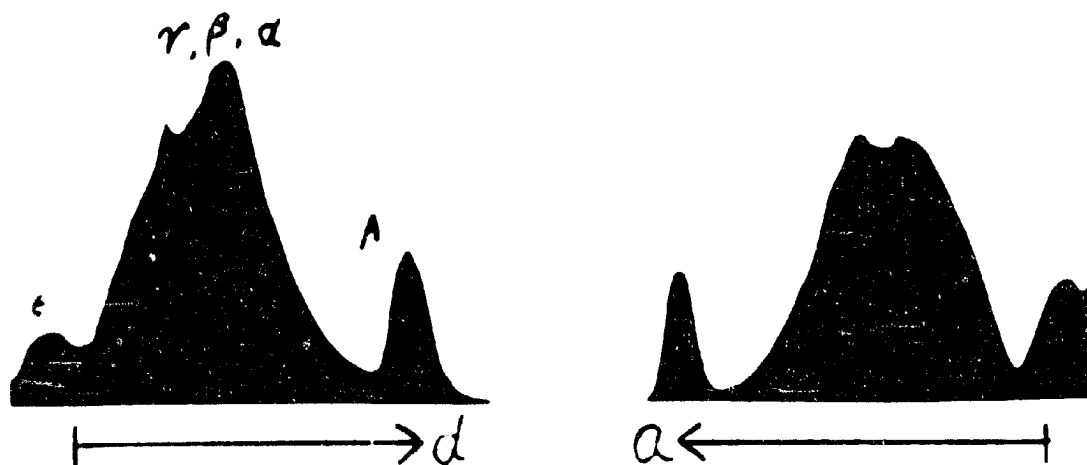


Fig. 14. Composition of protein fraction from a solution of 50 ml. No. 991 serum and 12.5 gm. sodium sulfadiazine removed by precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.80 volts per cm. Protein conc. 2 per cent.

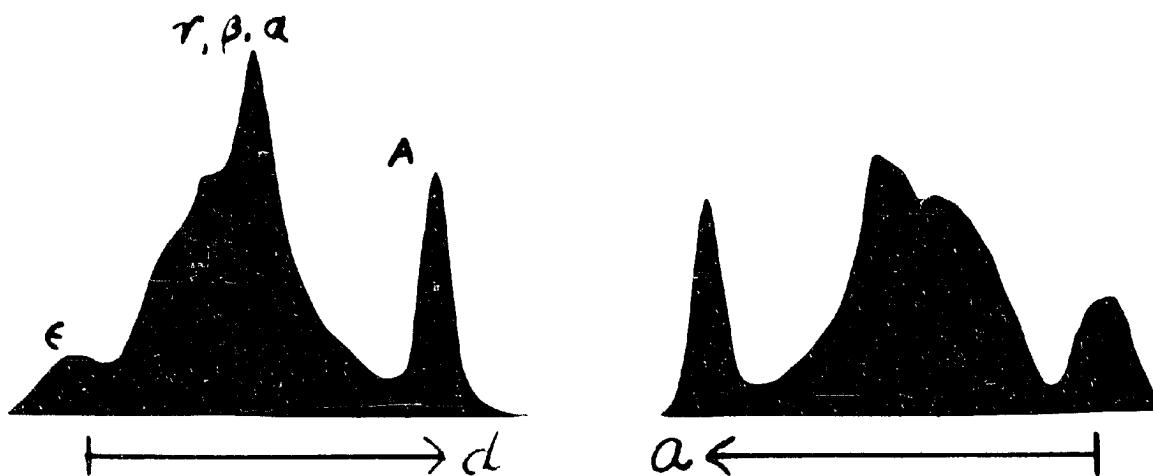


Fig. 15. Composition of protein fraction from a solution of 50 ml. No. 991 serum and 22.5 gm. sodium sulfadiazine removed by precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 6.01 volts per cm. Protein conc. 2 per cent.

of the curves in Fig. 5 and Fig. 16 occurs at about 15 grams sodium sulfadiazine. This point represents the number of grams of sodium sulfadiazine needed to divide the grams of protein recovered equally between the precipitate and the filtrate. Figs. 18, 19, 20, and 21 illustrate the removal of protein from the globulin region with increasing quantities of sodium sulfadiazine.

2. Crystal formation at pH 9.2.

If a solution of sodium sulfadiazine and serum is allowed to stand at 4°C., or at room temperature or at 37°C., crystalline material forms. A reaction takes place between the sulfadiazine anion and the serum proteins. The crystalline material forms more rapidly at 37°C. than at lower temperatures since the rate of reaction is faster at the higher temperature. After preliminary investigations, it was found that the crystals consisted of sulfadiazine and serum proteins. The nature of the proteins was determined.

In order to isolate sufficient serum protein by precipitation for an electrophoretic study, a large test sample was prepared. To 300 ml. of #991 serum was added sixty grams of sodium sulfadiazine. After 4 to 5 hours storage at 4°C., crystals began to form. The crystals were filtered after 12 days and stored in saline solution. The filtrate was again placed at 4°C. for further reaction to occur. More crystals occurred after 24 hours. The crystals were filtered after a few weeks reaction period. The crystalline material was combined and rinsed free of soluble materials with .86% saline. No further crystallization occurred in the serum filtrate, even after the addition of 5 grams of sodium sulfadiazine.

Table XV

Data obtained from calculation of descending electrophoretic pattern, Fig.18. No. 1443 untreated serum.

Serum protein	A	α	β and γ
Per cent from pattern	44.0	14.8	41.2
Gm. protein/50 ml. serum	1.51	0.51	1.42

Table XVI

Data obtained from calculation of descending
electrophoretic pattern, Fig. 19.

Serum protein	A	α	β	γ
Per cent from pattern	57.4	7.6	11.8	23.5
Gm. protein	1.36	0.18	0.28	0.56
Per cent based on 50 ml. serum	39.5	5.2	8.2	16.2

Filtrate from 50 ml. No. 1443 serum treated with 5.0 gm.
NaSD. Protein in filtrate = 2.37 gm., or 68.9 per cent
protein remained in filtrate.

Table XVII

Data obtained from calculation of descending electrophoretic pattern of filtrate, Fig. 20.

Serum protein	A	α	β	γ
Per cent from pattern	67.4	5.6	11.2	15.8
Gm. protein	1.19	0.10	0.20	0.28
Per cent based on 50 ml. serum	34.8	2.3	5.8	8.2

Filtrate from 50 ml. No. 1443 serum treated with 12.5 gms. NaSD. Protein in filtrate = 1.77 gm., or 51.6 per cent protein remained in filtrate.

Table XVIII

Data obtained from calculation of descending
electrophoretic pattern of filtrate, Fig. 21.

Serum protein	A	α	β	γ
Per cent from pattern	72.4	5.0	11.3	11.3
Gm. protein	0.90	0.06	0.14	0.14
Per cent based on 50 ml. serum	26.1	1.8	4.1	4.1

Filtrate from 50 ml. No. 1443 serum treated with 22.5 gm.
NaSD. Protein in filtrate = 1.24 gm., or 36.1 per cent
protein remained in filtrate.

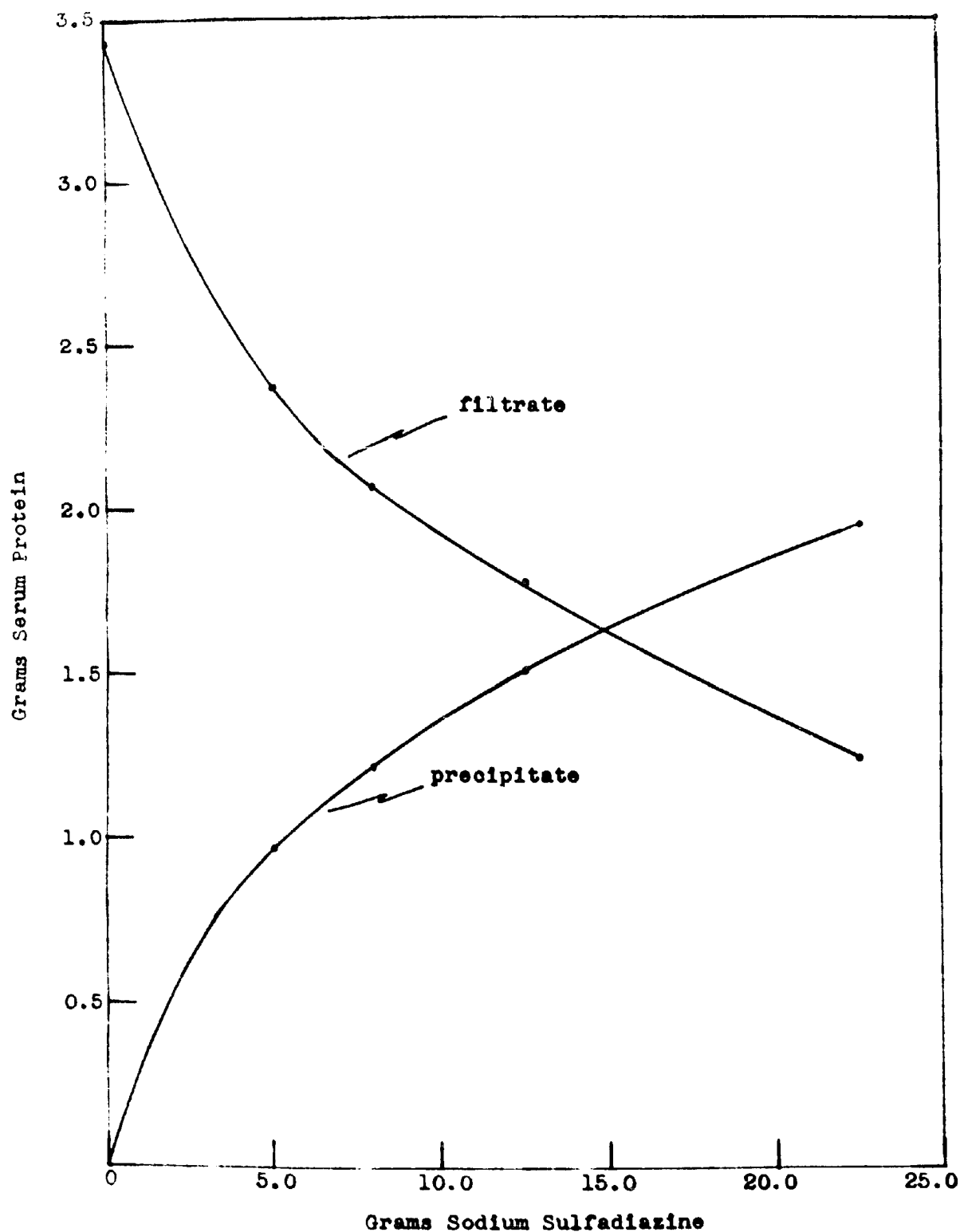


Fig. 16. Serum protein removed and serum protein in the filtrate from solutions of 50.0 ml. No. 1443 serum and varying quantities of sodium sulfadiazine.

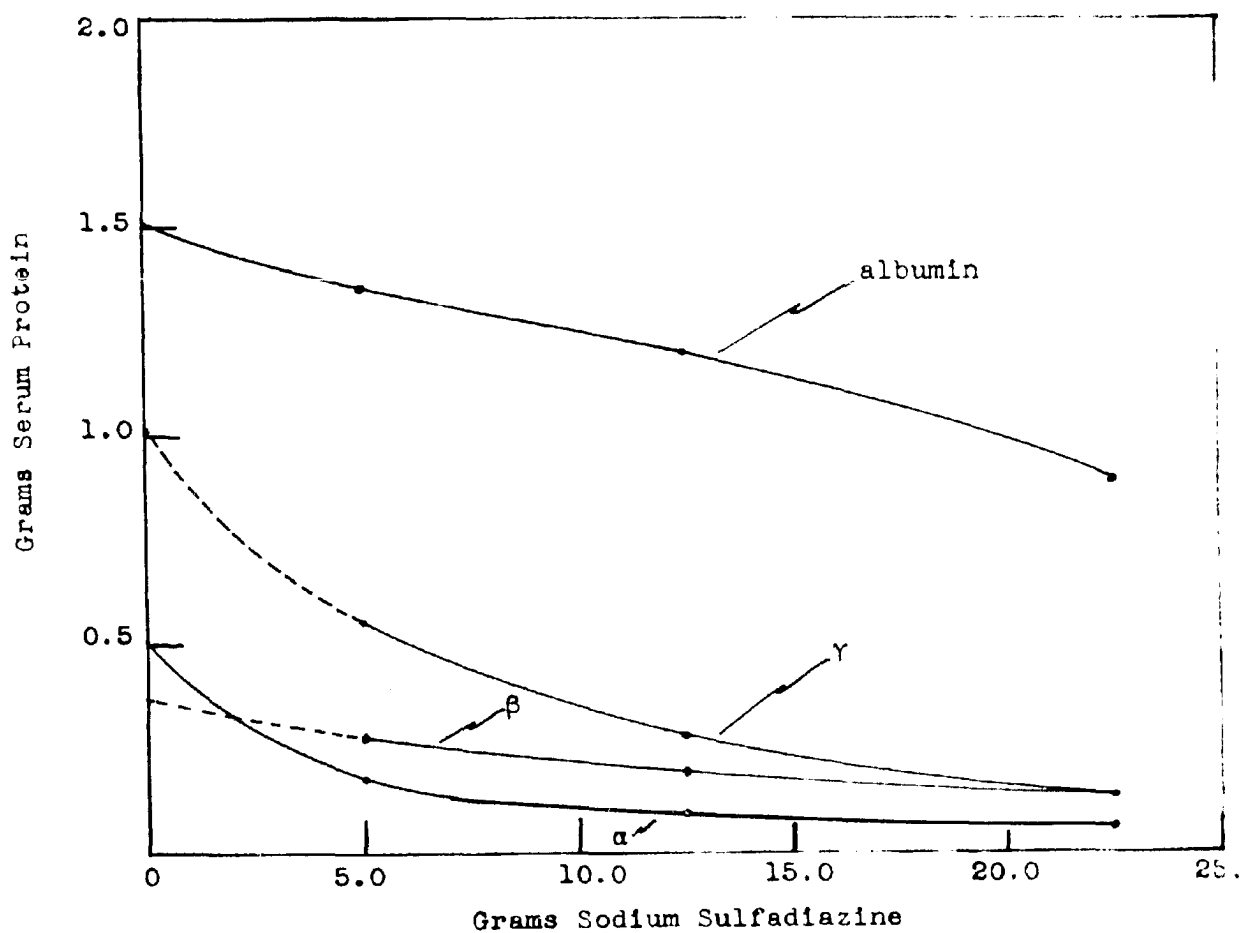


Fig. 17. Serum protein components in the filtrates from solutions of 50.0 ml. No. 1443 serum and varying quantities of sodium sulfadiazine.

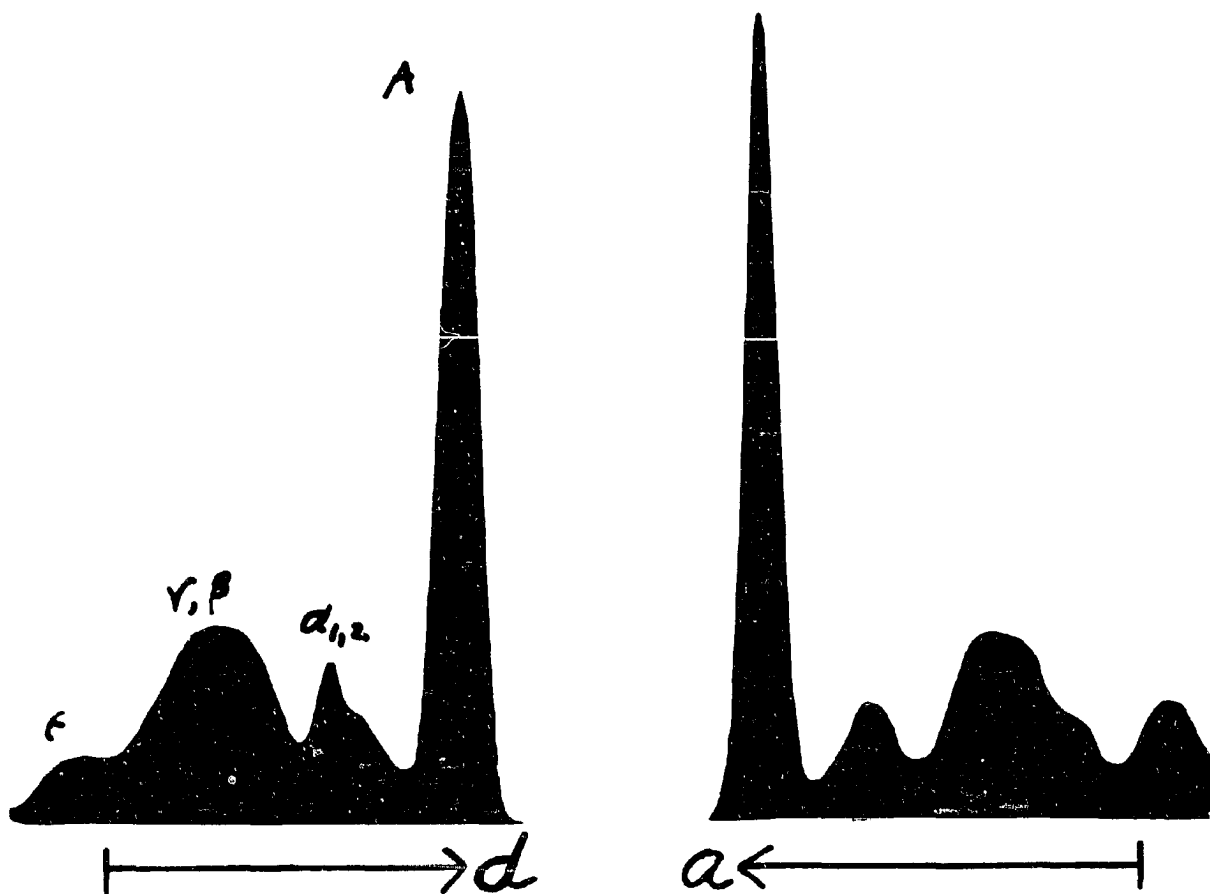


Fig. 18. Bovine serum No. 1443.
Electrophoresis for 9,000 seconds at 5.57
volts per cm. Protein conc. 2 per cent.

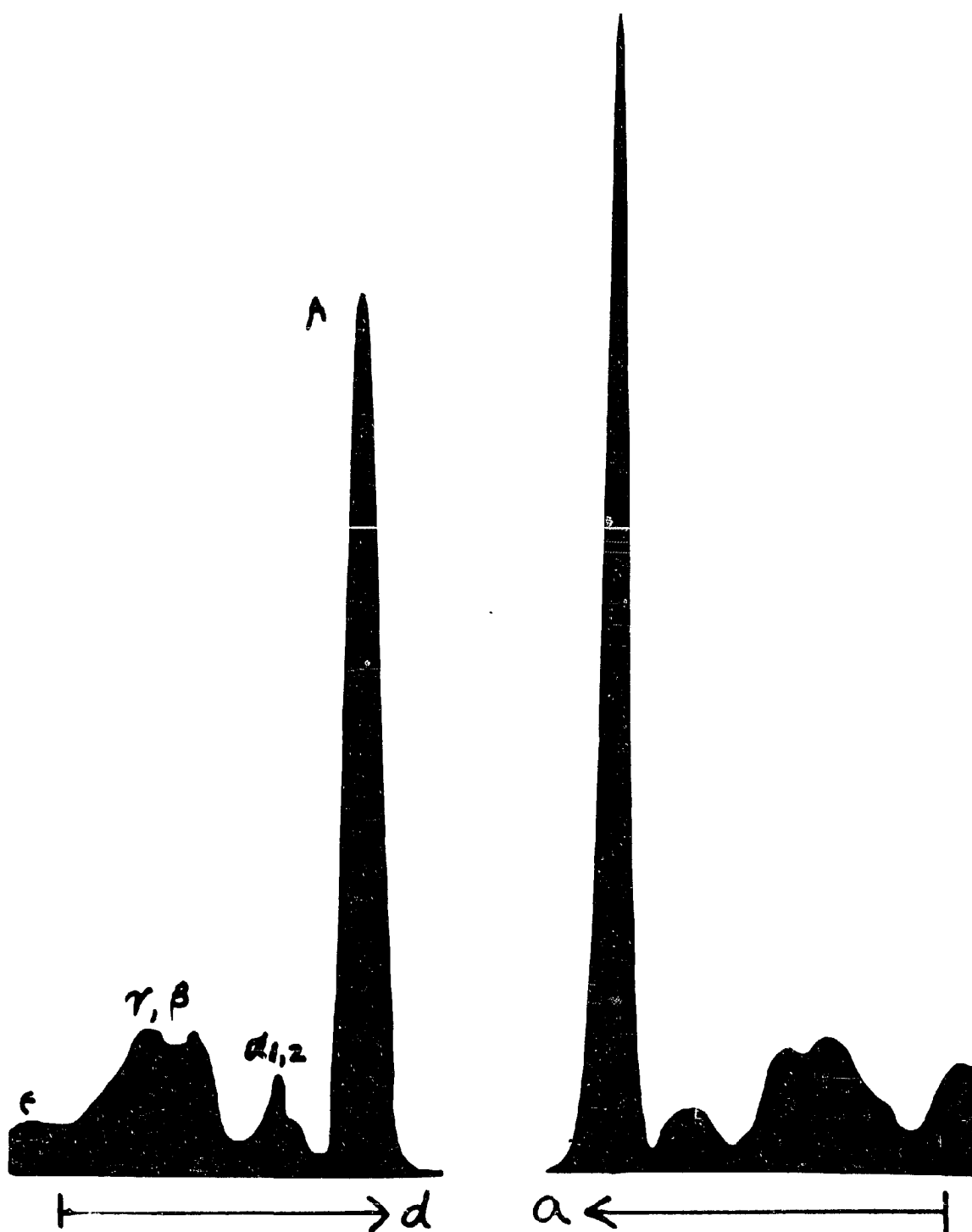


Fig. 19. Composition of filtrate from a solution of 50 ml. No. 1443 serum and 5.0 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.37 volts per cm. Protein conc. 2 per cent.

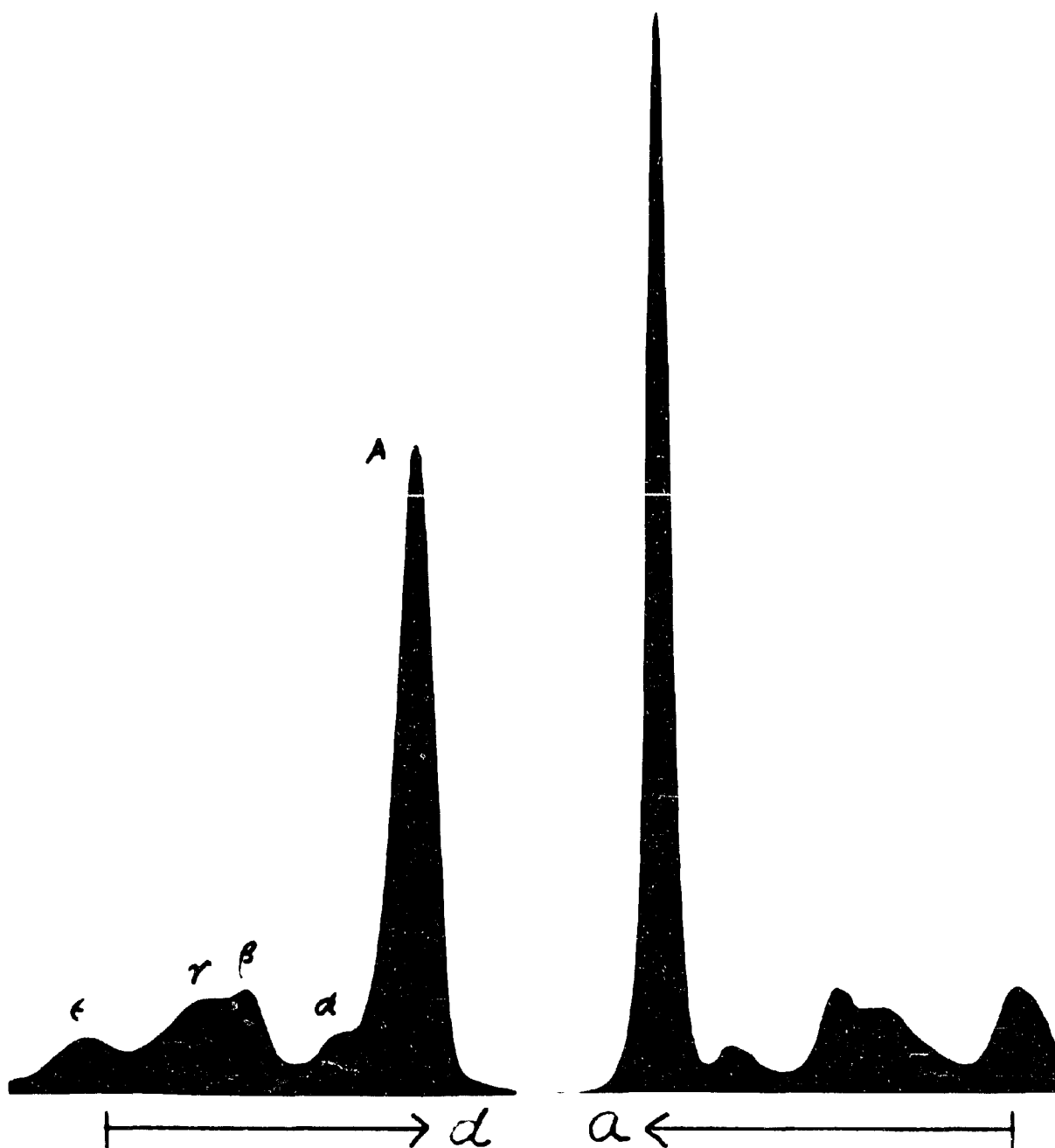


Fig. 20. Composition of filtrate from a solution of 50 ml. No. 1443 serum and 12.5 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.84 volts per cm. Protein conc. 2 per cent.

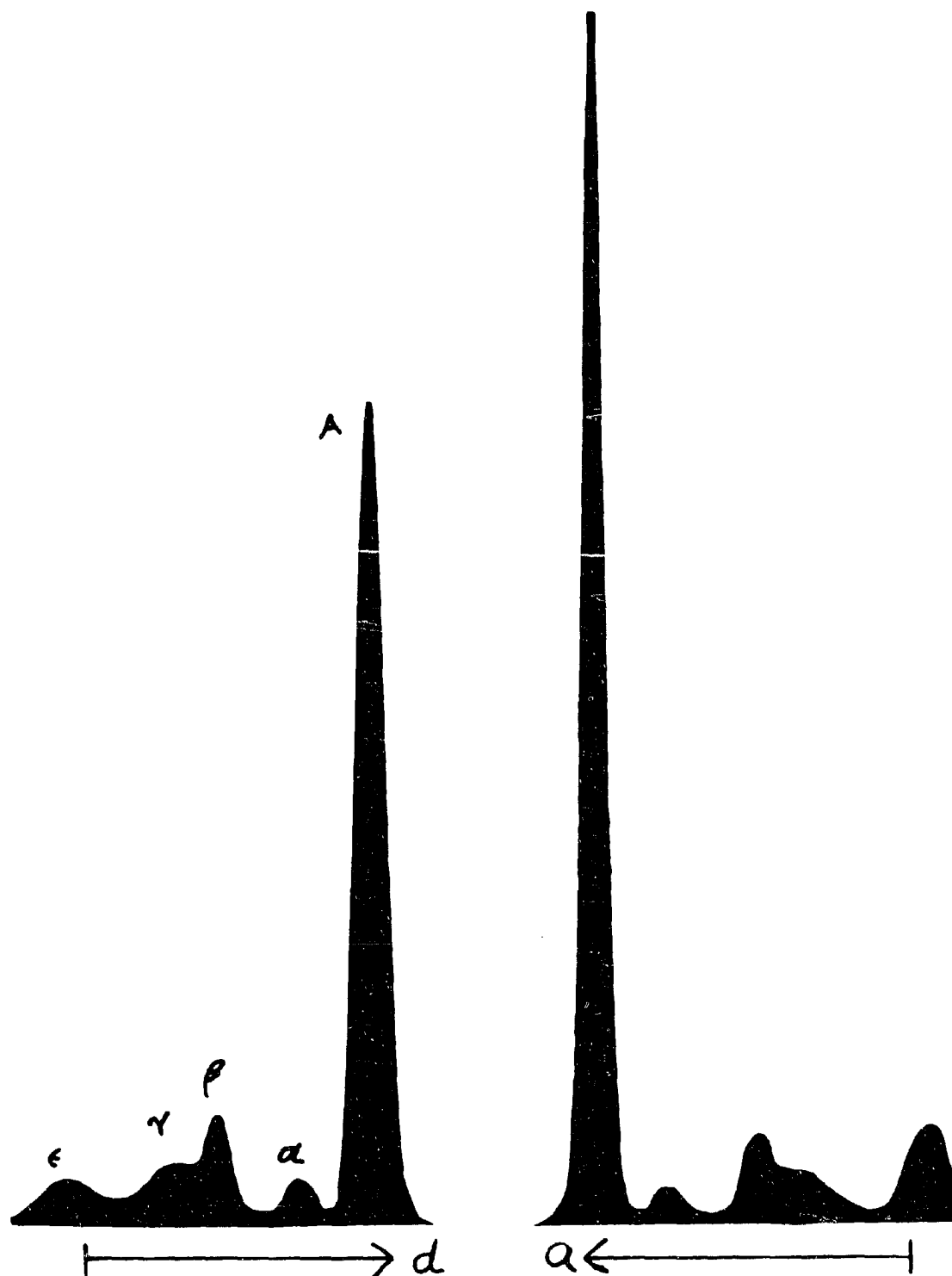


Fig. 21. Composition of filtrate from a solution of 50 ml. No. 1443 serum and 22.5 gm. sodium sulfadiazine after precipitation of sulfadiazine. Electrophoresis for 9,000 seconds at 5.82 volts per cm. Protein conc. 2 per cent.

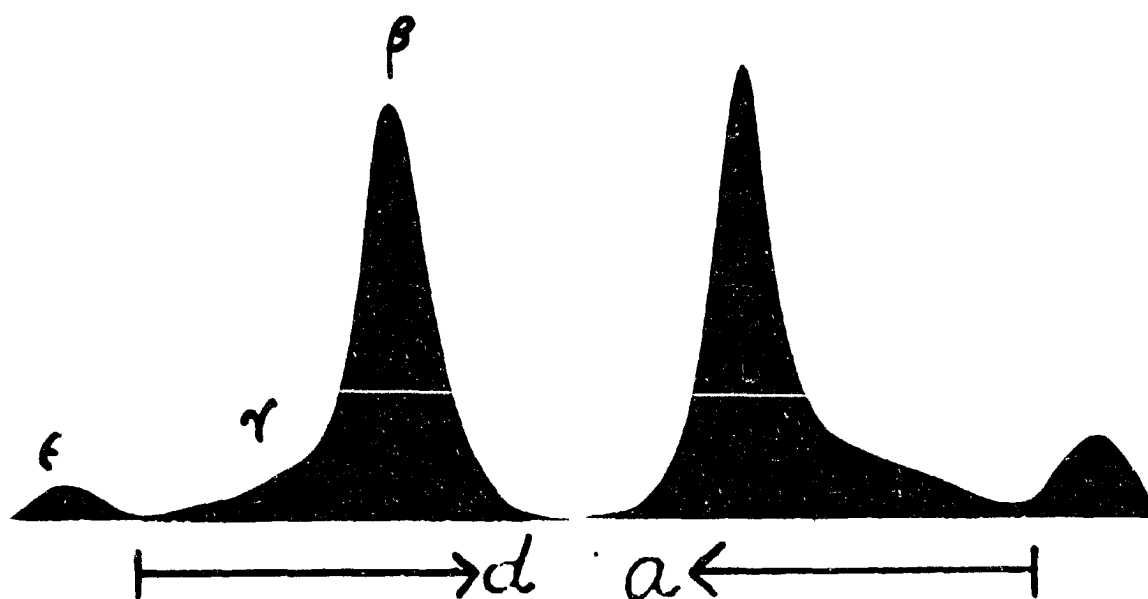


Fig. 22. Composition of protein fraction from a solution of 300 ml. No. 991 and 60 gm. sodium sulfadiazine removed by crystallization of sulfadiazine-protein complex at 4°C . Electrophoresis for 9,000 seconds at 5.96 volts per cm. Protein conc. 0.90 per cent.

The crystals, which went into solution with difficulty, were dissolved with .1N NaOH up to pH 10.0. The resulting pale yellow solution was placed in cellophane tubing and dialyzed against ten liters .86% saline for 6 days with daily saline changes to remove the sodium sulfadiazine. The protein content of the solution was 0.37 grams. The solution was concentrated to 0.90% protein and equilibrated by dialysis against barbital buffer in preparation for electrophoretic analysis. An electrophoretic scanning pattern, Fig. 22, revealed that the proteins removed from the serum consisted of 97.3% β -globulin and 2.7% γ -globulin. This serum protein component designated as β -globulin had a mobility between α - and β -globulin. It is possible that β -globulin has a greater mobility when other proteins are not present. Under the conditions of this experiment, the sulfadiazine anion had an affinity for β -globulin. The quantity of β -globulin removed, was very small. The original 300 ml. of serum contained 25.5 grams of protein. The amount of protein removed from the serum was 0.37 gram, or only 1.45% of the total protein. Of the protein removed, 1.41% was β -globulin and 0.04% was γ -globulin.

3. Precipitation at pH 4.0.

During the study of this problem, it was thought that the reaction between sulfadiazine and serum proteins was influenced by the charge of the protein molecules. The isoelectric points (15) of bovine serum proteins are:

albumin	4.45
α -globulin	4.98
β -globulin	5.28
γ -globulin	6.02

Since the precipitation studies had been carried on between pH 9.2 to 7.0, which is above the isoelectric points of the serum proteins, it was considered of interest to determine what the results would be if the charges on the serum proteins were reversed. A sample was precipitated at pH 4.0, which is below the isoelectric points of the proteins.

To 50 ml. of #991 Brucella anti-serum was added .15N phosphoric acid to lower the pH from 8.6 to 4.0. Ten grams of sodium sulfadiazine was dissolved in 250 ml. of distilled water. To the well agitated serum at pH 4, .15N phosphoric acid and the sodium sulfadiazine solution from separate burettes were added simultaneously. During the entire addition of the two reagents, the serum solution was maintained at pH 4.0. At this pH, the sulfadiazine precipitated with the removal of serum proteins. The solution was filtered through two #1 Whatman filter papers. The precipitate was rinsed with 25 ml. portions of 0.86% saline and these rinsings were allowed to drain in the original filtrate. The filtrate was saved. After more complete rinsing of the precipitate, it was dispersed in 0.86% saline and dissolved with .1N sodium hydroxide to pH 10.0. The alkaline solution was dialyzed at 4°C. against 0.86% saline to remove the sodium sulfadiazine and finally concentrated to increase the protein percentage of the solution. Nitrogen and sodium sulfadiazine were determined. A 2.0% protein solution was prepared for electro-

phoretic study. The sample was equilibrated against barbital buffer at pH 8.6 and .1 ionic strength.

The filtrate was made alkaline to pH 10.0. The sodium sulfadiazine in the total filtrate was found to be 90 mg. The solution was dialyzed, concentrated and prepared for an electrophoretic determination. The original untreated serum sample was also analyzed electrophoretically.

A comparison of the electrophoretic pattern of the proteins removed from the serum, Fig. 24, with that of the proteins remaining in the filtrate, Fig. 25, reveals that the major portion of the β , γ -globulins was not removed with the precipitate. It will be recalled that precipitation of sulfadiazine in the presence of serum between pH 9.2 to 7.0 removes the β , γ -globulins. It is noted with reference to Tables XIX, XX, XXI, and the patterns of the filtrate and precipitated proteins, Figs. 23, 24, and 25, that the total α -globulin fraction increased, while the β , γ -globulins and albumin fractions decreased. The reason for this discrepancy is unknown, but one may speculate that during the treatment at pH 4.0, the mobilities of a portion of the β , γ -globulins and albumin approached the mobilities of the α -globulins. The albumin fraction was found to be about equally divided between the filtrate and the precipitate, whereas the β , γ -fraction in the filtrate was 45.5% and in the precipitate, 6.6%. Discussion of the charges on the protein and possible reaction with the sulfadiazine will be given after some pertinent data have been presented.

Table XIX

Data obtained from calculation of descending electrophoretic pattern, Fig. 23. No. 991 untreated serum.

Serum protein	A	α	β and γ
Per cent from pattern	26.0	17.8	56.2
Gm. protein/50 ml. serum	1.12	0.77	2.43

Table XX

Data obtained from calculation of descending electrophoretic pattern of filtrate, Fig. 24.

Serum protein	A	α	β and γ
Per cent from pattern	18.0	7.5	74.5
Gm. protein	0.47	0.20	1.97
Per cent based on 50.0 ml. serum	11.0	4.6	45.5

Filtrate from 50 ml. No. 991 serum treated with 10.0 gm. NaSD at pH 4.0. Protein in filtrate = 2.64 gm., or 61.1 per cent protein remained in filtrate.

Table XXI

Data obtained from calculation of descending electrophoretic pattern of precipitate, Fig. 25.

Serum protein	A	α	β and γ
Per cent from pattern	30.9	49.8	19.3
Gm. protein	0.50	0.74	0.29
Per cent based on 50.0 ml. serum	10.6	17.1	6.6

Precipitate from 50 ml. No. 991 serum treated with 10.0 gm. NaSD at pH 4.0. Protein in precipitate = 1.49 gm., or 34.3 per cent of the protein in the serum was removed.

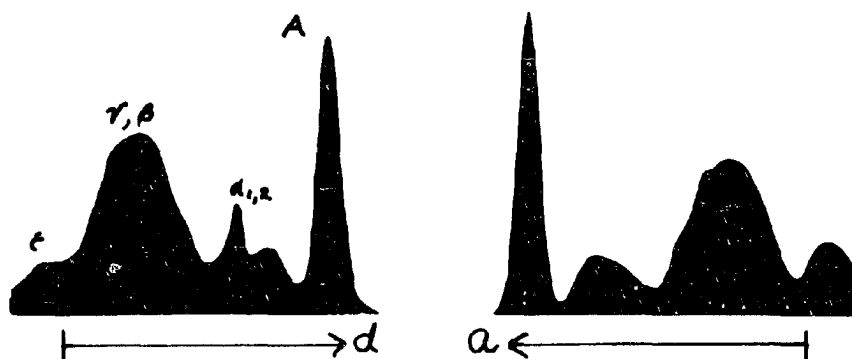


Fig. 23. Bovine serum No. 991. Electrophoresis for 9,000 seconds at 6.33 volts per cm. Protein conc. 2 per cent.

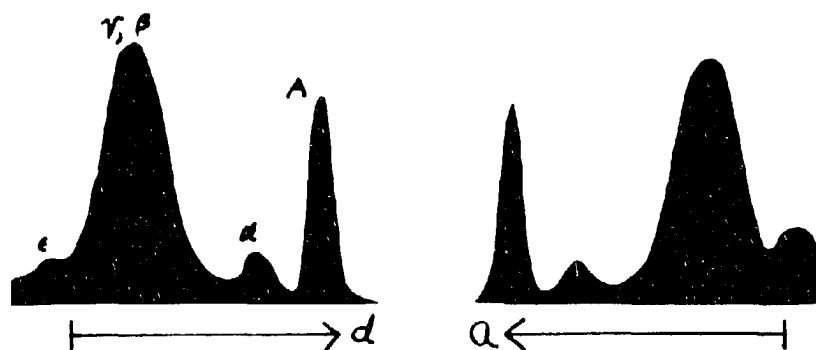


Fig. 24. Composition of filtrate from a solution of 50 ml. No. 991 serum and 10 gm. sodium sulfadiazine after precipitation of sulfadiazine at pH 4.0. Electrophoresis for 9,000 seconds at 5.93 volts per cm. Protein conc. 2 per cent.

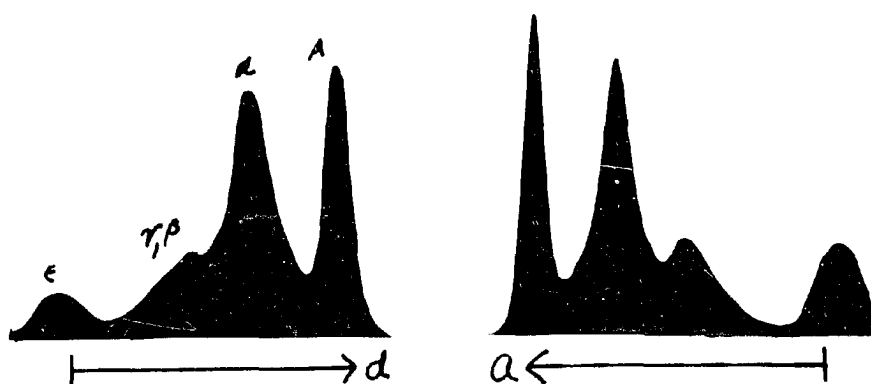


Fig. 25. Composition of protein fraction from a solution of 50 ml. No. 991 serum and 10 gm. sodium sulfadiazine removed by precipitation of sulfadiazine at pH 4.0. Electrophoresis for 9,000 seconds at 6.20 volts per cm. Protein conc. 2 per cent.

C. Solubility of Sulfadiazine.

During the course of this investigation, a method was developed to measure the relative "solubility" or reaction of sulfadiazine. This reaction was tested in a number of solutions and these results have strongly indicated a possible reaction between sulfadiazine and proteins. Sulfadiazine was found to be "soluble" in aqueous solutions of compounds of various structures. On the basis of these structures and the degree of "solubility", conclusions have been drawn as to reaction between sulfadiazine and proteins.

One molar solution of the compounds was made up in a phosphate buffer. The buffer (7) was made up as follows: .15 mol. sodium chloride, .005 mol. dipotassium acid phosphate, and .005 mol.

potassium dihydrogen phosphate are dissolved in distilled water and made up to one liter. One fortieth of a gram molecular weight of the compound was dissolved in 10 ml. of the phosphate buffer. The solution was adjusted to pH 7.7 with .1N sodium hydroxide. The solution was diluted to 25.0 ml. with phosphate buffer previously adjusted to pH 7.7. The resultant pH of the solution was 7.7. To this solution, placed in a 50 ml. Erlenmeyer flask, was added a slight excess of sulfadiazine powder. The solution was shaken on a Burrel Shaker for four hours at a moderate shaking speed. A shaking period of four hours was found to be optimum. After filtering the excess sulfadiazine on a number 50 Whatman filter paper, a sulfadiazine determination was made on the filtrate. The sulfadiazine found in the filtrate was a measure of the "solubility" or reaction. The "solubility" was calculated in milligrams of sulfadiazine reacted in 100 ml. of solution.

Solubilities of sulfadiazine in the sera and the distilled water given in Table XXII were determined by adjusting the pH as specified in the Table, adding the sulfadiazine and shaking for four hours and filtering. The sulfadiazine was determined from the filtrate.

The solubility or reaction of sulfadiazine was greatest in solutions at pH 7.7 of n-butyl amine, lysine, and arginine. The solubility of sulfadiazine in the other amino acid solutions at this pH was considerably lower. At pH 6.0, the isoelectric point of glycine and γ -globulin, the solubility of sulfadiazine in a glycine solution and in #991 and #1143 sera fell off sharply. Solubility in the two sera was also determined at pH 5.0, the isoelectric point of α -globulin, which is below the isoelectric points of β and γ -globulins and above the isoelectric point of albumin.

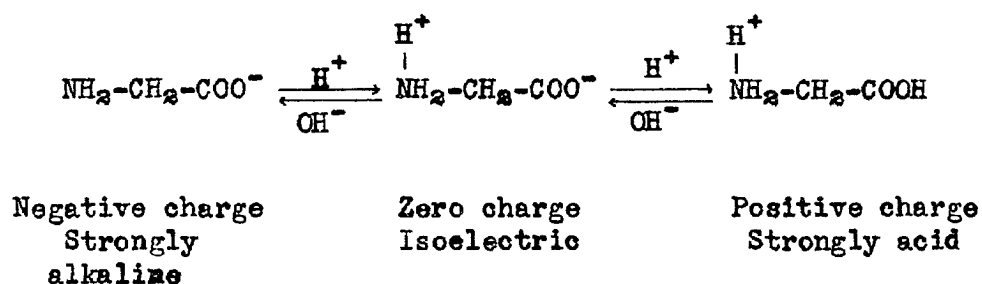
Table XXII

Solubility of sulfadiazine in 1.0 molar solutions of various substances. Solubility calculated in $\frac{\text{mg. SD}}{100 \text{ ml. solution.}}$

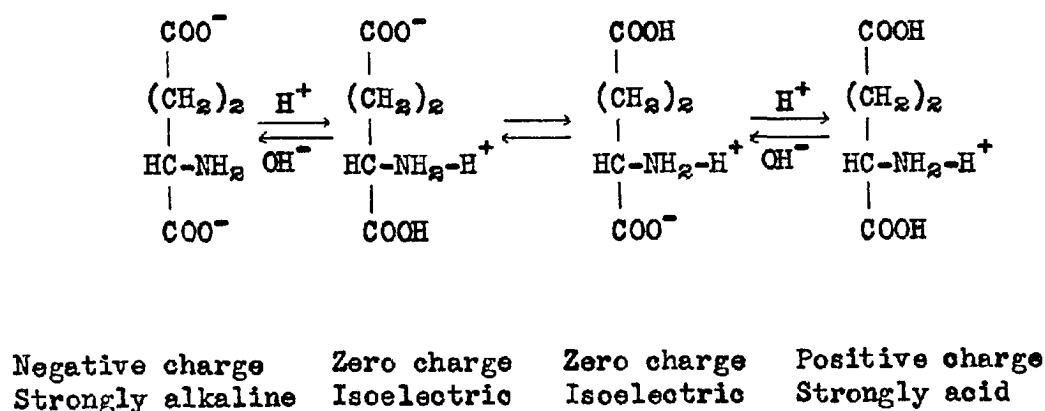
Substance	Solubility pH 7.7	Solubility pH 6.0	Solubility pH 5.0	Isoelectric Points
No. 991 serum	173	18	10	
No. 1441 serum	142	16	9	
Distilled water		7.5		
n-butyl amine	171			
Lysine	210			9.74
Arginine	230			10.76
Buffer	41	10	8	
Urea	44			
Tyrosine	48			5.66
Histidine	62			7.59
Acetic acid	63			
Threonine	67			
Glutamic acid	79			3.22
Glycine	80	8.5		5.97

DISCUSSION

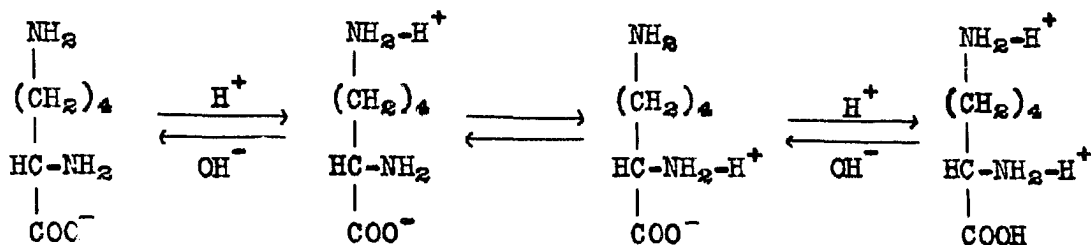
Amino acids are amphoteric compounds. Because of the carboxyl and amino group within the amino acid molecule, amino acids can exist in an aqueous solution as dipolar ions (17). A proton shift between the carboxyl and the amino group causes the following transformation (16) in glycine, for example:



Glutamic acid, which contains three dissociating groups, may exist in several different forms, depending upon the pH of the solution:

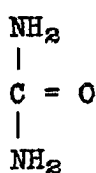


Lysine, which also contains three dissociating groups, may exist in several different forms, depending upon the pH of the solution:

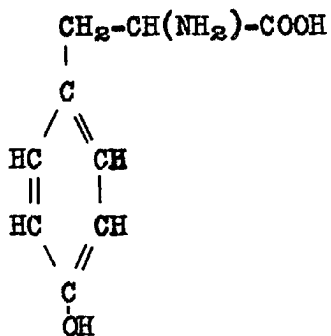


In Table XXII are given the pH of the isoelectric points (4) of these amino acids. It will be noted that the isoelectric points of lysine and arginine are pH 9.74 and 10.76 respectively. Since the "solubilities" were conducted at pH 7.7, which is below the isoelectric points of these two amino acids, then lysine and arginine each had a net positive charge. Histidine with an isoelectric point of 7.59 was either neutral or had a very slight net negative charge. The other amino acids listed in Table XXII had net negative charges. Solubility in a glycine solution was also conducted at the isoelectric point, pH 6.0. The solubility of sulfadiazine at this pH was slightly lower in the glycine solution than in the buffer. The glycine would have a net charge of zero. The slightly lower solubility than in the buffer may be due to a salting out effect.

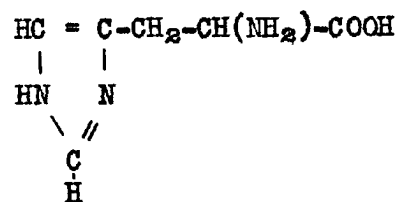
The chemical structure of the compounds listed in Table XXII are given below:



Urea



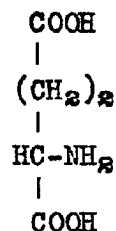
Tyrosine



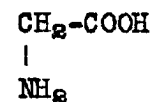
Histidine



Threonine



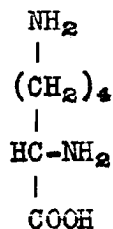
Glutamic Acid



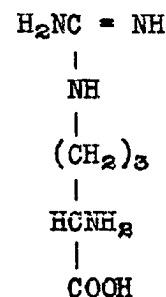
Glycine



n-Butyl amine



Lysine



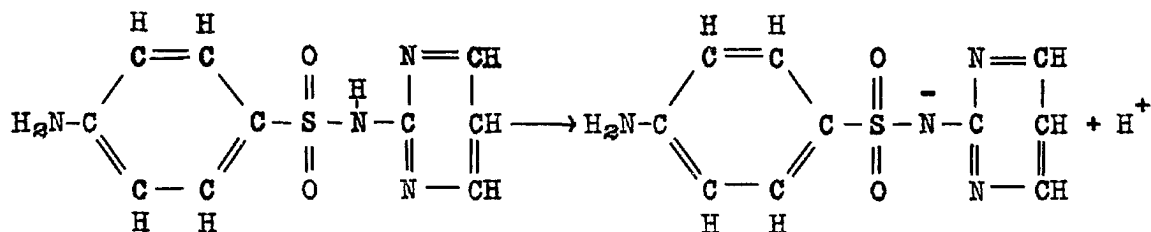
Arginine

Comparison of the structure of these compounds with the degree of solubility of sulfadiazine in 1.0 M solutions of these compounds indicates that certain radicals may be responsible for the increase in the solubility of sulfadiazine. Lysine, arginine and n-butyl amine have a profound effect on the solubility. Each of these compounds contains a group which confers a positively charged region within the molecules in a slightly alkaline range. At pH 7.7 lysine and arginine have positively charged regions at the ϵ -amino group, and the guanidinium group respectively.

From the data presented it can be concluded that the reaction between amino acids and sulfadiazine is due to the net positive charge residing in the vicinity of the "free" amino groups. The active point of lysine is the ϵ -amino group, and the active

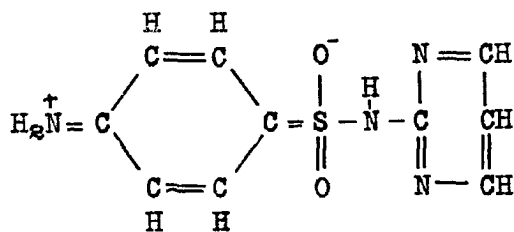
point of arginine may be the amino group in the guanidinium radical.

The ionization of sulfadiazine in an aqueous solution may be represented by the following equation:



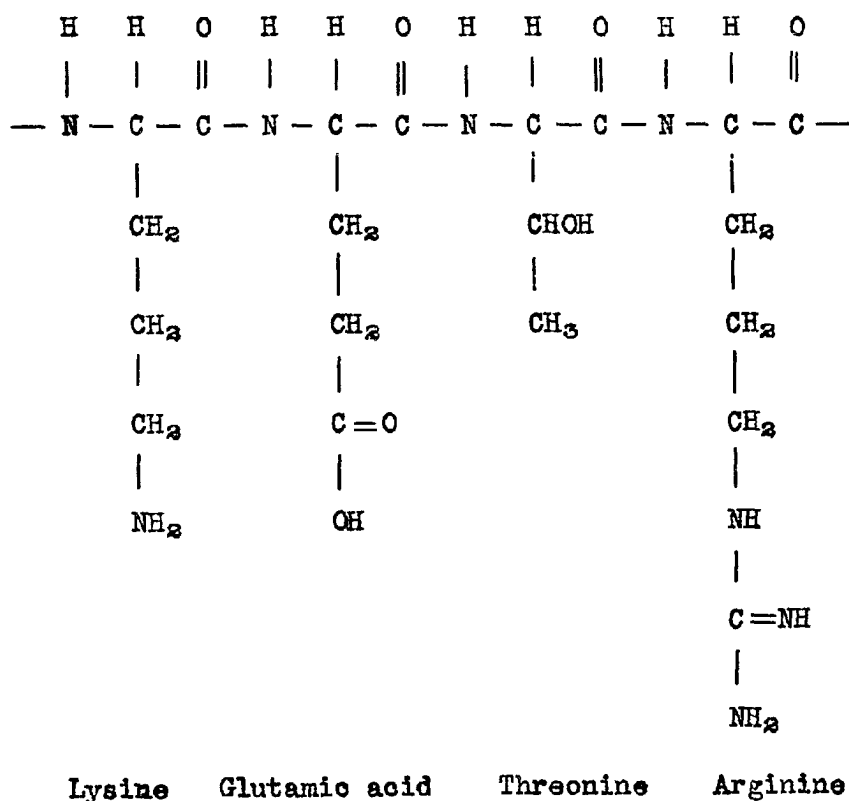
In this form it is entirely possible that the sulfadiazine anion unites with the positively charged lysine and arginine through the amino groups.

Bell and Roblin (1) present data to support the hypothesis that the sulfadiazine anion exists in solution, as shown above. Kumler et al., (10, 11, and 12) claim that the active form in solution, based on the resonance theory, is:



On this basis, it would mean that the positive amino group binds to the negative oxygen.

Amino acids can be looked upon as small molecular weight proteins as far as charge effects are concerned (18). Reactions of proteins are, in effect, reactions of the side groupings of the protein. Proteins are made up of amino acids linked to one another through the alpha carboxyl group and the alpha amino group (5). A hypothetical polypeptide with the side groups is given below:



Because of the presence of "free" amino active points in protein molecules, there is a strong indication that the binding of the sulfadiazine anion to the protein occurs through these amino groups.

The ε-amino groups from lysine residues, and the guanidinium groups from arginine residues are considered as carriers of positive charges at reactions acid to pH 8. The imidazole groups of histidine,

and any α -amino groups present in the molecule, carry positive charges when acid to pH 6, and are uncharged when alkaline to pH 8.5 or 9 (2). Thus, under the conditions which the various experiments were conducted, reaction between proteins and the sulfadiazine anion occurred mainly through the ϵ -amino groups from lysine and the guanidinium groups from arginine.

When serum proteins are surrounded by an alkaline medium, albumin has a greater negative charge than γ -globulin. The isoelectric points of albumin and γ -globulin are 4.45 and 6.02 respectively, and thus, the pH of the alkaline medium is further removed from the isoelectric point of the albumin than it is from the isoelectric point of the γ -globulin. α - and β -globulin isoelectric points are 4.98 and 5.28 respectively, and, therefore, have intermediate negativity in reference to albumin and γ -globulin. Conversely, of the serum proteins in an alkaline medium, γ -globulin has the greatest positive charge, followed by β -globulin, α -globulin and finally albumin (14). The net charge of the serum proteins is negative, but positive charges do reside in the molecules, such as in the lysine and arginine residues.

The electrophoretic patterns of the precipitation of sulfadiazine in the presence of serum between pH 9.2 and 7.0 show that sulfadiazine preferentially binds with γ -globulin. The graphs of the serum protein components of the filtrates, Fig. 6 and Fig. 17, illustrate that γ -globulin is readily removed during the precipitation of the sulfadiazine. α -, β -globulins and albumin are also removed, but not nearly as much as γ -globulin. The sulfadiazine anion combines with the positively charged portions of the serum

proteins. Since γ -globulin has the greatest positive charge, it binds with sulfadiazine more readily. With α -, β -globulins and albumin serum components, which have a smaller degree of positive charges, there is less binding with the sulfadiazine anion.

In the experiments performed by Davis, human serum was separated into fractions with varying quantities of $(\text{NH}_4)_2 \text{SO}_4$. An electrophoretic analysis was determined for each fraction. These fractions were dialyzed against a phosphate buffer solution which contained sulfathiazole. After complete equilibration the drug concentration was found to be greater in the cellophane bag containing the protein solution. He found that the drug concentration paralleled the albumin concentration. These experiments were carried out at pH 7.4. From this he concluded that sulfathiazole was bound to the albumin. He also found that there was practically no binding to γ -globulin.

It was found in this laboratory that when whole serum is fractionated and the fractions dispersed in a suitable buffer, they do not seem to react the same as when they are present in the whole serum. For example, albumin in serum is surrounded by α -, β -, and γ -globulins. There are protein interactions between albumin and the globulins. The positive charges of albumin combine with the negative charges of the globulins, and vice versa. The globulins also interact among themselves. Thus fractionated serum does not include these protein interactions and results obtained with fractions do not give the true picture.

With precipitation at pH 4.0, the electrophoretic patterns illustrate that β - and γ -globulin remained in the filtrate and albumin and α -globulin were removed with the precipitate. It was

also demonstrated that a serum protein component, which has an electrophoretic mobility between β -, and α -globulins and was designated as β -globulin, was removed from a mixture of serum and sodium sulfadiazine at pH 9.2. On the basis of the charges carried by the various serum proteins, these facts are difficult to interpret. Apparently the charges on the proteins are not the only factors that are involved in the binding of the proteins to sulfadiazine anions.

SUMMARY

Serum proteins isolated by acid precipitation of sulfadiazine between pH 9.2 and 7.0 in the presence of Brucella anti-serum were found to be bacteriocidal against Brucella abortus in the presence of fresh normal rabbit serum to serve as complement and traces of sodium sulfadiazine.

The above serum proteins were identified by electrophoretic analysis and found to consist of globulins, mainly γ -globulin. A small amount of albumin was also present. The sulfadiazine anion combined with the positively charged positions with the serum protein molecule. In the precipitation of sulfadiazine in the presence of serum at pH 4.0, an electrophoretic study revealed that the β -, and γ -globulins remained in the filtrate, and the albumin and α -globulins were removed with the precipitate.

The crystals formed in a solution of serum and sodium sulfadiazine at pH 9.2 and placed at 4°C., contained serum protein, which was found to be almost pure β -globulin as determined by electrophoretic analysis.

At pH 7.7 sulfadiazine reacted with 1 molar solutions of lysine, arginine, n-butyl amine but did not react appreciably with acetic acid, glutamic acid, urea, tyrosine, histidine, threonine, and glycine. The sulfadiazine was found to react with positively charged areas in the amino acids, such as the ϵ -amino group of lysine and the guanidinium group of arginine.

ACKNOWLEDGMENT

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