ELECTROPHORETIC STUDIES OF BOVINE SERUMS WITH RESPECT TO *BRUCELLA*

by

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Electrophoretic Studies of the Proteins of Bovine Serums With Respect to *Brucella*

ELECTROPHORETIC STUDIES OF THE PROTEINS OF BOVINE SERUMS WITH RESPECT TO *BRUCELLA*[*]

It has long been known that when animals or humans are injected, or become infected with *Brucella*, there follows an appearance of demonstrable antibodies, often in high titer, in the blood serum. The role played by these antibodies in shortening the duration of the disease or in the destruction of the invading organisms is as yet unknown. So far no *in vitro* method has been devised which would indicate antibacterial activity on the part of *Brucella* antiserums. There have been obtained, however, in certain experiments by Huddleson and Pennell (19) data which suggest that a properly prepared antiserum plays an important role in the neutralization of the toxic components of the bacterial cell.

It was, therefore, the original purpose of this study to develop a purified and concentrated *Brucella* antiserum. A general but not exhaustive investigation included the employment of the more common methods of serum purification, such as the use of various salts like the sulfates of ammonium (1,11,12,15,16), and sodium (14,17,21), the chlorides of zinc, antimony, and aluminum (7,19); of various organic solvents like alcohol (9), acetone (46), and ether (32); of various procedures like heating of the serum; or the effect of certain enzymes (36,38,39,40), under controlled conditions of concentration, pH and temperature. Most of these methods proved fruitless in the first instance, or secondly,

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a growing realization concerning the ignorance of the nature of the antibody sought made even the appreciation of any progress difficult to appraise.

At about this time, fortunately, the highly improved electrophoresis apparatus of Tiselius (47) was beginning to come to the general attention of scientific workers in America. Its versatility and applicability has made it one of the greatest tools that the biochemist possesses in attacking the enigma of the proteins. It seems natural that the blood plasma proteins should have been subjected to electrophoresis preferentially since antibodies are found primarily in the blood. Studies on horse serum (4,5,48,50,51,52,53,54) were followed immediately by electrophoretic observations of the serums of the human (5,20,21,28,30), the guinea pig (45), the rabbit (45,49,50,51,52), and the bovine (4,45).

In general antibodies have been found to be associated with the γ-globulin (49,51,53). However, in one case (54) antipneumococcal antibody was found as a new component, T^1, in horse serum shortly after beginning hyperimmunization. Antitoxin appears entirely as a separate component, T, having a mobility ca.2.0 x 10^{-5} cm^2, $sec.^{-1}$, $volt^{-1}$ (55).

The present electrophoretic studies were undertaken in order to obtain a clear conception of the fundamental differences that exist between the proteins in normal bovine serum and those in serums from animals which had been either treated or exposed to live Brucella cells, or to some fraction therefrom; to identify the constituent of the serum with which Brucella antibody was associated; to learn something about the physico-chemical nature of the antibody; and with this information to develop a suitable method of purifying and concentrating Brucella antiserum.

Since little is known about the protein changes that occur in the serum of the bovine from birth to maturity or under various conditions,

it was considered advisable to begin the electrophoretic studies on blood serums from new-born calves taken before the ingestion of colostrum from normal and infected animals and to follow the changes that occurred after the ingestion of colostrum. This procedure was likewise followed in certain instances in adult animals before and after injection with Brucella.

MATERIALS AND METHODS

Serum Samples:- The blood specimens were drawn from the animals, permitted to clot at room temperature, and then stored overnight at 3°C. On the following day the serum was removed after centrifugation. It was dialyzed against a preliminary volume of buffer in the cold room for one day before being transferred to a two liter volume of buffer and allowed to dialyze for at least one more day before being placed in the electrophoresis cell. After dialysis the serum sample was adjusted to a protein concentration of one per cent by the refractometric method. Another centrifugation was usually necessary to insure complete clarity. The letter "C" before the number of a serum indicates calf, while the small letter after the number represents the order in which the blood specimen was drawn.

Buffer:- The composition of the buffer used throughout this study was 0.05 M diethylbarbituric acid, 0.025 M NaOH, and 0.075 M NaCl per liter which gives a pH of about 7.9 and an ionic strength of 0.1.

Protein Determination:- Total protein was determined by the differential refractometric method of Siebenmann (42,43). For adjustment of the serum samples to one per cent, the graphic method was used based upon a chart prepared from a serum of known protein concentration and dialyzed against buffer under comparable conditions.

Agglutination Titration:- All serum specimens were titrated with a smooth culture of Brucella abortus diluted to a turbidity of 1 McFarland scale in salt solution. The first serum dilution was 1:20 except in the case of new born calves when the first dilution was 1:5. Readings were made after incubation for 48 hours at 37°C.

Electrophoresis:- In these experiments the modified Tiselius apparatus described by Longsworth and MacInnes (26) was used. Photographs of the migrating boundaries were taken by the "schlieren scanning method" (24,30) of Longsworth. With the exception of a few initial runs the double length section of the electrophoresis cell (29) was employed in order to avoid the obscuring effect in the middle of the field by the horizontal glass plates of the original two section apparatus. The electrophoretic runs were carried out at 0.5°C. and at a potential gradient of 5 to 6 volts per centimeter for about two hours. The current was measured potentiometrically. Conductivity measurements were made on both the buffer and protein solution with a Shedlovsky cell (41) in a Dewar vessel containing crushed ice and water. The pH measurements were made at 25° with a glass electrode. Mobilities and areas of the diagrams were determined from the descending boundaries in the manner described by Longsworth, Shedlovsky, and MacInnes (26,27,30). The arrows indicating the direction of migration just below all electrophoretic diagrams illustrated in this paper are preceded by the letters "d" and "a" which represent the descending and ascending boundaries respectively.

EXPERIMENTAL AND DISCUSSION

A. Electrophoretic studies of normal calf serums before and after ingestion of colostrum.

Howe (18) had observed that the serum of the calf at birth contained neither euglobulin nor pseudoglobulin I in appreciable quantities, but that the ingestion of colostrum by the animal at any time within two days after birth resulted in the rapid appearance of these protein fractions in the blood. Earle (8) found a similar situation with regard to young foals, kids, lambs and pigs. This author advises that any program for feeding new-born animals should include either homologous colostrum, or some substitute from which the new-born animals may absorb either the protective substances ordinarily supplied in the colostrum, or their equivalent. According to Little and Orcutt (23) the blood of the new-born calf, with rare exceptions, is free from agglutinins towards Brucella until the animal has suckled and taken in the colostrum even when the blood and colostrum of the dam have a high agglutinin content.

It seemed logical to begin our electrophoretic studies first upon the serum of calves in order to observe the serum protein pattern at birth, and to follow the changes that occurred after the ingestion of colostrum and as the animal approached maturity. Table I reveals the results of this investigation. All calves except numbers 200, 101 and 100 were born to dams in herds free from brucellosis. The customary practice in this herd was to permit new-born calves to ingest only one "belly-full" of colostrum, before being separated from their dams and then fed milk from a pail. The outstanding characteristic of calf serum obtained before the ingestion of colostrum was the high concentration of

α-globulin which might almost equal or even exceed the concentration of albumin. On the other hand, γ-globulin was distinguished by its extremely small peak or complete absence. The usual positive error contributed by the ϵ-boundary was easily avoided in this series by virtue of the nature of the diagrams. In the case of calf 100 from an infected dam, the plasma was observed as well as the serum in order to determine the effect of fibrinogen. Fig. 1b of the plasma shows that the fibrinogen has completely masked the low γ-peak which was just discernable in the serum of the same blood specimen (Fig.1a). A second specimen (Fig.1c) of blood was drawn from calf 100 six days after birth and after it had ingested colostrum. An agglutination titer of 1:80 was now disclosed. The high α-peak had decreased from 44.2 to 30.4 per cent of the total area while the γ-peak has risen from 2.3 to 20.7 per cent. The albumin as well as the total protein had increased and the area of the β-peak had now doubled. Twenty-two days later the diagram (Fig.1d) began to approach that of the adult cow. The agglutination titer had increased to 1:160 but the γ-peak had diminished to 10.3 per cent. The α- and β-boundaries continued to be quite pronounced and the albumin was attaining its normal concentration. It might be noted that there was no correlation between agglutination titer and γ-globulin concentration. An explanation will be offered later in this paper.

The remaining specimens seem to lead to the generalization that the ingestion of colostrum by calves from normal, or more properly cows free from brucellosis caused an increase in the γ-peak from a few per cent to about 30 per cent, and a doubling of the β-peak, while the inverse held true for the abnormally high α-peak. The albumin appeared to decrease with respect to the total area; yet examination of the normal increase in total protein, as the calf grew older day by day, showed that the albumin likewise was increasing when expressed in grams per 100 cc. of serum. This

relationship between the various components appeared to persist for at least three or four weeks. However, the second specimen, b, of calf 484 (Fig.1f) drawn 46 days after birth (Fig.1e) revealed that by this time the relative concentration of the serum components began to approximate those of the normal heifer. Probably because of the absence as yet of some sort of antigenic stimulation the γ-peak, 9.7 per cent, was still quite low.

Close examination of Table I shows that rapid and profound changes occurred in the serum protein components of the calf after the ingestion of colostrum. It was proposed to follow these alterations at suitable intervals in a calf born of a normal dam. Calf CR2 was obtained from the same brucellosis-free herd previously mentioned. This calf enjoyed the remarkable distinction of being the first recorded offspring of a cow with a rumen fistula. In addition, the dam of this calf being a member of a group upon which fat metabolism studies were being made was given 5 to 6 gms. of hexyl blue 10 days before calving. Feeling that this fact might have materially affected the calf, another calf CA22, was also taken from the same herd. The protein distribution of the serum of calf CR2 obtained at birth as calculated from electrophoretic analysis, Table II, compared favorably with similar cases in Table I. The electrophoretic diagram of a sample obtained four hours after the ingestion of colostrum could be almost superimposed upon the first one indicating that very little absorption of globulin from colostrum occurred in that short interval. Twenty-four hours later a measurable shift in relative protein concentration could be demonstrated. The albumin and α-peaks began to diminish at the expense of the remaining globulins. However, in terms of grams per 100 cc. the albumin concentra-

TABLE I

Relative Electrophoretic Mobilities and Concentrations[1] of the Protein Components of Calf Serums Drawn Before and After the Ingestion of Colostrum

Calf Number	Age When Blood Drawn	Agglutination Titer	pH	Mobilities (U x 10^5)				Gm/100cc.		Concentrations Per Cent Total Area				Composition				Fig. No.
				A	α	β	γ	T. P.	Alb.	A	α	β	γ	A/G	α/A	β/A	γ/A	
200a	At birth		7.53	6.2	4.8	3.2	1.8			50.8	40.5	7.2	1.6	1.03	0.80	0.14	0.03	
101a	At birth	Negative	7.90	6.3	4.7	3.3		4.65		49.7	45.6	4.7	0.0	0.99	0.92	0.09	0.00	
100a	At birth	Negative	7.83	6.1	4.5	2.7	1.8	3.25	1.43	45.7	45.5	6.6	2.3	0.84	1.00	0.14	0.14	1a
100a[2]	At birth	Negative	7.83	6.0	4.5	3.0	ϕ 2.0	3.25	1.29	41.0	44.2	5.7	ϕ 9.0	0.82	1.08	0.14	0.00	1b
100b	6 days	1-80	7.73	5.8	4.3	2.7	1.5	4.84	1.76	37.6	30.4	11.2	20.7	0.60	0.81	0.30	0.55	1c
100c	22 days	1-160	7.90	5.5	4.3	2.3	1.3	5.91	2.69	45.6	29.4	14.6	10.3	0.84	0.64	0.32	0.23	1d
C483a	At birth	Negative	7.81	5.3	3.9	2.5	1.4	4.25	2.12	51.8	33.7	10.5	4.0	1.08	0.65	0.20	0.08	
C483b	9 days	Negative	7.82	6.3	4.7	2.7	1.9	7.65	2.31	31.4	20.3	15.3	33.0	0.46	0.65	0.49	1.05	
C281a	At birth	Negative	7.87	6.2	4.8	3.0	1.7	4.74	2.12	49.3	38.9	8.1	3.8	0.97	0.79	0.16	0.08	
C281b	10 days	Negative	7.82	6.6	5.0	2.6	1.7	6.83	2.27	34.0	17.8	20.4	27.8	0.52	0.52	0.60	0.82	
73a	At birth	Negative	7.86	6.2	4.7	3.0	1.9	3.60	1.88	52.3	39.3	6.8	1.6	1.10	0.75	0.13	0.03	
73b	15 days	Negative	7.82	6.6	4.7	3.1	1.9	6.99	2.62	38.3	18.7	17.1	25.9	0.62	0.49	0.45	0.67	
484a	At birth	Negative	7.85	6.2	4.9	3.2		4.10	2.29	57.1	36.8	6.1	0.0	1.33	0.64	0.11	0.00	1e
484b	46 days	Negative	7.78_{II}	7.4	5.5	3.7	2.3			54.0	24.2	11.9	9.7	1.18	0.45	0.22	0.18	1f

[1] The ϵ-boundary is not included in these estimations.

[2] Plasma specimen. ϕ represents fibrinogen.

TABLE II

Electrophoretic Mobilities and Concentration[1] of the Components of Serums Drawn at Intervals from an Infected and Two Non-infected Calves

Calf Number	Age When Blood Drawn	Agglutination Titer	pH	Mobilities ($U \times 10^5$)				Gm/100cc.		Concentrations Per Cent Total Area				Composition				Fig. No.
				A	α	β	γ	T.P.	Alb.	A	α	β	γ	A/G	α/A	β/A	γ/A	
CR2	At birth	Negative	7.81	5.3	3.7	2.0	1.2	3.65	1.90	52.1	38.9	7.8	1.2	1.09	0.75	0.08	0.01	
"	4 hours	Negative	7.81	5.8	4.3	2.7	1.6	3.76	2.07	55.0	38.9	5.3	0.8	1.22	0.71	0.10	0.02	
"	28 hours	Negative	7.81	5.8	4.2	2.7	1.8	3.75	1.90	50.7	36.1	9.9	3.3	1.03	0.71	0.20	0.07	
"	7 days	Negative	7.81	5.7	4.1	2.7	1.9	4.12	1.91	46.4	35.8	13.0	4.8	0.86	0.77	0.28	0.10	
"	14 days	Negative	7.78	5.9	4.4	2.8	1.7	4.13	1.86	45.1	30.6	18.3	5.9	0.82	0.68	0.41	0.13	
CA22	At birth	Negative	7.82	6.2	4.6	3.2		5.84	2.96	50.3	38.8	10.9	0.0	1.01	0.77	0.21	0.0	2a
"	4 hours	Negative	7.82	6.2	4.6	3.1	1.8	4.54	2.02	44.6	29.7	12.0	13.8	0.80	0.67	0.27	0.31	2b
"	28 hours	Negative	7.82	6.1	4.7	3.3	1.8	6.04	2.16	35.7	19.1	10.7	34.5	0.56	0.53	0.30	0.97	2c
"	7 days	Negative	7.83	5.5	4.1	2.3	1.2	5.94	2.11	35.5	23.3	14.4	26.8	0.55	0.66	0.41	0.76	
"	16 days	Negative	7.83	5.9	4.7	2.9	1.7	5.80	2.40	41.4	23.0	15.4	20.2	0.71	0.56	0.37	0.49	2d
C876	At birth[2]	1:10,000	7.78	5.7	4.1	2.5	1.3	6.59	1.17	17.7	32.2	12.1	38.0	0.21	1.82	0.69	2.15	3a
"	28 hours	1:10,000	7.78	5.6	4.1	2.6	1.4	8.53	1.20	14.1	23.8	14.1	48.1	0.16	1.69	1.00	3.41	3b
"	7 days	1:20,000	7.78	5.9	4.5	2.9	1.6	6.88	1.63	23.7	22.6	16.8	36.9	0.31	0.95	0.71	1.56	3c
"	14 days	1:10,000	7.78	5.9	4.4	2.9	1.7	6.99	2.55	36.5	15.3	17.0	31.3	0.57	0.42	0.47	0.86	3d

[1] Correction was made for the ϵ-boundary in these calculations.

[2] There is cause for doubting that no colostrum had been ingested before withdrawal of the first blood sample because the calf was born during the night.

tion continued surprisingly constant even over a two week period while the total protein increased slowly. At least in this case the increase in total protein was accounted for entirely by the two slower moving globulins. The degree of absorption was unusually slow over the entire period and even the total protein had increased only to 4.13 from 3.65 gm per 100 cc. The significance in this connection of the hexyl blue dye is problematical. The agglutination titration was negative throughout.

Calf CA22, (Fig.2a) normal in appearance and from a normal dam, showed enhancement of the γ-peak (Fig.2b) to 13.8 per cent within four hours after the ingestion of colostrum (Table II.). Within 24 hours the γ-globulin (Fig.2c) increased to a maximum of 34.5 per cent, and the α-globulin decreased from its original concentration of 38.8 to 19.1 per cent. Calf CR2, on the other hand, showed practically no absorption of globulin even after two weeks. In a week the α- and β-globulin concentration of the serum of calf CA22 was almost normal; the γ-globulin after its initial rapid increase was now leveling off, and in 16 days (Fig.2d) all components were distributed in the manner usually found in young heifers.

B. Electrophoretic patterns of serum drawn at intervals from a Brucella infected calf.

Immediately after completing the study of the serum changes in the calves, numbers CA22 and CR2, born of normal dams, an opportunity appeared to follow parallel changes in a calf born of an infected dam. Calf 876, a full-term calf, was from an infected dam that had been exposed to Brucella four months before parturition. It was unfortunate that this calf was born during the night; that it may have nursed some-

time before it was discovered lying by its dam's side the next morning was quite likely. Nonetheless, the first blood sample (Fig.3a) that was obtained immediately proved to have the lowest albumin and the highest γ-globulin concentration of any calf serum studied. The high total protein, 6.59 gm. per 100 cc. (Table II) along with the agglutination titration of 1:10,000, further supported the suspicion that colostrum had been previously ingested. The colostrum agglutination titer was 1:40,000. By the next day during which colostrum was voluntarily ingested the total protein rose to 8.53 gm. per 100 cc. while the albumin remained unchanged. The α-globulin which had its greatest concentration immediately at birth had already decreased from 32.2 per cent of the total area to 23.8 (Fig.3b); albumin had reached a minimum of 14.1 while the γ-globulin had attained a maximum of 48.1 per cent. Within a week the albumin had begun to rise slowly in spite of a reduction of total protein to 6.88 gm. per 100 cc. (Fig.3c). The gradual diminution of α-globulin continued while that of the γ-globulin was more abrupt; yet the titration had climbed to 1:20,000. In two weeks the albumin peak had almost attained its customary prominence as the various peaks now assumed the outline of the general bovine electrophoretic pattern (Fig.3d). It might be noted that the β-globulin separated from the massive γ-globulin with difficulty. The separation between these two boundaries was improved when neither was in great excess. The titration continued at 1:10,000

The mobilities of the various boundaries were uniform albeit averaging slightly less than those of adult cows.

C. Electrophoretic patterns of heifer serums after vaccination with the U. S. B. A. I. Brucella Strain 19.

The literature is replete with experimental data based upon Strain 19. As far as can be determined no one had followed the response of the serum proteins to the action of strain 19 vaccination. The first two cases of Table III were about a year old when vaccinated; another year later blood was withdrawn for electrophoretic analysis. The patterns resembled those of the vaccinated heifers of Table IV. The distribution of the serum proteins was approximately normal as could be demonstrated from the figures of the concentration or composition column of Table III; however, the total protein of calf C73 was somewhat higher than would be expected in a calf of its age.

In order to determine the immediate effect of an injection of 6 cc. of Brucella strain 19 vaccine, two calfs, A98 and B94, about five months of age were selected (Figs.4a and 4d). Calf A98 showed no agglutination in a 1:20 dilution when vaccinated, but calf B94 possessed a titer of 1:320; the total serum protein in each was about the same. Eighteen days after the injection both showed an increase in agglutination titer, and an enhancement in all globulins with the γ-peak showing the most marked change (Figs.4b and 4e). The albumin, of course, was reduced. By the end of 25 days the agglutination titer in each had fallen to 1:20 along with a decrease of the γ-peak, while the albumin rose to its normal concentration of better than 50 per cent (Fig.4c and 4f). The composition column clearly presents the relatively rapid return of the serum proteins to a normal distribution. Here in this study, as well as in the previous ones, the change in the γ-peak played the most

TABLE III

Electrophoretic Mobilities and Concentrations[1] of Serum Components of Calves Vaccinated with Strain 19.

Number	Birth	Vaccinated	Blood Drawn	Aggl. Titer	pH	Mobilities (U x 10^5)				Gm/100cc.		Concentrations Per Cent Total Area				Composition				Fig. No.
						A	α	β	γ	T.P.	Alb.	A	α	β	γ	A/G	α/A	β/A	γ/A	
R73a	9/39	9/21/40	10/25/41	1:40	7.86	6.0	4.4	3.0	1.6	7.76	3.62	46.6	17.1	7.7	28.6	0.87	0.37	0.23	0.61	
P81	9/39	7/14/40	10/25/41	1:320	7.99	5.9	4.3	2.9	1.4	7.07	3.61	51.0	20.2	8.1	20.7	1.04	0.40	0.16	0.41	
A98a	6/10/41	10/25/41	10/25/41	Neg.	7.99	6.4	4.5	3.3	1.9	6.43	3.68	57.2	19.1	5.3	18.3	1.34	0.33	0.09	0.32	4a
A98b			11/11/41	1:640	7.86	6.1	4.5	3.2	1.8	6.01	2.78	46.3	22.6	7.7	23.3	0.86	0.49	0.17	0.50	4b
A98c			1/19/42	1:20	7.86	5.3	3.8	2.5	1.3	6.73	3.56	52.9	14.6	10.6	18.2	1.12	0.28	0.20	0.41	4c
B94a	5/13/41	10/25/41	10/25/41	1:320	7.98	5.8	3.9	2.7	1.3	6.58	3.88	58.9	15.0	6.0	20.1	1.43	0.26	0.10	0.34	4d
B94b			11/11/41	1:160	7.86	6.1	4.5	3.2	1.7	6.35	3.10	48.8	17.0	8.0	26.1	0.95	0.35	0.16	0.54	4e
B94c			1/19/42	1:20	7.86	6.0	4.3	3.1	1.6	6.11	3.16	51.8	13.2	9.5	22.1	1.08	0.26	0.18	0.49	4f

[1] Correction was not made for the ϵ-boundary in these estimations.

important role. Vaccination with strain 19 caused only a mild and temporary shift in the equilibrium of the serum proteins. In less than a month serum agglutinins had practically disappeared and a more or less normal serum protein distribution was restored. Fig. 4 shows that, unlike calf serum patterns after the ingestion of colostrum, the β-boundary did separate easily from the γ-peak under the present conditions. The descending patterns (Figs.4b and 4e) of calves A98b and B94b of the first serum samples drawn after injection showed a double-peaked α-globulin. This phenomenon was not observed in the patterns obtained previous to vaccination or those obtained several weeks subsequent to injection.

D. Electrophoretic patterns of a general series of bovine serums.

Several workers (4,45) have performed electrophoretic studies upon bovine serum. In general most of the specimens were purported to come from so-called normal cows. In Table IV there are collected data based on electrophoretic analyses from a number of animals under various conditions. Since our chief interest in this entire paper was brucellosis, all serum specimens were titrated for Brucella agglutinins. Obviously, it is not intended to consider an animal normal if Brucella agglutinins were absent. It must be remembered that antigens other than Brucella might affect the distribution and amount of serum proteins. To make such exhaustive tests in order to prove the normalcy of the animal would have enlarged the scope of this study far beyond the facilities available.

This series represents a compilation of analyses gathered over a year's time not only from an experimental herd, but from animals throughout the state of Michigan. This study was undertaken to determine

TABLE IV

Relative Electrophoretic Mobilities and Concentrations[1] of Protein Components of Bovine Serums

Cow Number	Remarks	Aggl. Titer	pH	Mobilities (U x 10^5)				Gm/100cc.		Concentrations Per Cent Total Area				Composition				Fig.
				A	α	β	γ	T.P.	Alb.	A	α	β	γ	A/G	α/A	β/A	γ/A	No.
297	Pregnant	Negative	7.80	6.2	4.4	2.8	1.6			40.9	20.6	8.6	29.9	0.69	0.50	0.21	0.73	5a
478	Pregnant	Negative	7.75	5.3	3.7	2.3	1.1	5.64	2.72	48.2	15.2	8.3	28.3	0.93	0.31	0.17	0.59	
755297	Non-pregnant	Negative	7.93	6.2	4.7	3.1	1.9	6.82	3.20	46.9	20.2	10.0	22.9	0.88	0.20	0.10	0.49	
716511a	Non-pregnant	Negative	7.81	5.7	4.0	2.6	1.5	5.81	2.88	49.5	21.9	10.0	18.5	0.98	0.44	0.20	0.37	5b
716511b	Suspicious,non-preg.	1:80	7.99	6.2	4.4	3.0	1.6	5.28	2.21	41.9	21.3	9.3	27.4	0.72	0.51	0.22	0.65	5c
A23	Age 5, cyst ovaries	1:80	7.86	6.1	4.4	2.8	1.4	9.38	2.75	29.3	21.8	7.4	41.5	0.41	0.74	0.25	1.41	5d
881a	Suspicious	1:20	7.82	6.9	4.9	3.2	1.8	7.68	3.02	39.3	14.3	9.7	36.7	0.65	0.36	0.25	0.93	
887*	Pregnant	1:40	7.75	5.7	4.2	3.1	1.6	5.90	2.71	46.0	12.9	8.8	32.3	0.85	0.28	0.09	0.70	
478b*	Pregnant	1:160	7.77	6.1	4.4	2.8	1.7	6.68	2.95	44.1	21.8	10.3	23.7	0.79	0.49	0.23	0.54	
478c*	Pregnant	1:40	7.82	6.6	4.9	3.2	1.8	6.43	3.23	50.2	21.5	8.7	19.6	1.01	0.43	0.17	0.39	
530873a*	Pregnant	1:1280	7.82	6.5	4.6	3.0	1.8	7.13	3.17	44.4	16.8	8.2	30.6	0.80	0.38	0.18	0.69	
480a*	Pregnant	1:1280	7.88	5.8	4.1	2.6	1.5	6.94	2.93	42.2	17.4	10.0	30.3	0.73	0.41	0.24	0.72	
650640a*	Pregnant	1:1280	7.85	6.5	4.8	3.3	1.8	6.25	2.74	43.9	16.1	8.6	31.4	0.78	0.37	0.20	0.71	6a
340D6120	Exp.via eye,preg.	1:2560	7.80	6.1	4.5	3.5	1.6	6.44	2.31	35.8	17.2	3.3	43.7	0.56	0.48	0.09	1.22	6b
883	Exp.via eye,preg.	Negative	7.81	5.9	4.2	3.0	1.3	6,60	2.63	39.8	21.9	6.2	32.2	0.66	0.55	0.16	0.81	6c
T36a	Infected naturally	1:20,000	7.85	6.4	4.2	2.9	1.7	7.86	3.41	43.4	16.1	5.7	34.8	0.77	0.37	0.13	0.80	
143993b	Infected naturally	1:40,000	7.86	6.6	4.8	3.2	1.6	9.20	3.25	35.3	14.5	7.4	42.6	0.55	0.41	0.21	1.21	6d
201a	Infected naturally	1:40,000	7.85	6.3	4.6	3.1	1.7	7.94	3.27	41.2	17.0	8.5	33.1	0.63	0.41	0.21	0.81	

[1] These estimations do not include a correction for the ϵ-boundary.

* These heifers were injected with a water-soluble crushed cell fraction from Brucella.

the pattern and mobilities of the serum protein constituents from a large number of cows, and to determine if the relative concentrations of the constituents were significantly altered by: (a) the presence of pregnancy; (b) a low agglutinin titer; (c) the treatment with a water-soluble extract from smooth Brucella abortus cells; (d) artifical exposure to a virulent culture of Brucella abortus by way of the conjunctiva; or (e) the presence of natural infection accompanied by an abnormally high agglutination titer.

Svensson (45) stated that the most characteristic property of cow serum was the large γ-peak, and that the β-globulin was small and difficult to separate from this γ-globulin. While he noted that his observations were based on only a few samples, Table IV shows that in general his conclusions were correct. However, illustrated examples in Figs. 5 and 6 show that with the exception of those serum samples where the γ-globulin content was high, the β-globulin did separate quite appreciably from the γ-boundary. The first four samples (Table IV) were taken from young, supposedly normal heifers. The most unusual of this group was cow 716511 (Fig. 5b) with the lowest γ-globulin concentration of any studied. This animal was in a herd free from brucellosis. A second blood sample obtained almost two months after the first showed a marked change (Fig.5c); the albumin concentration dropped from 49.5 to 41.7 per cent and the γ-globulin increased from 18.5 to 27.4 per cent, while the agglutination titer changed from negative to 1:80. The various albumin-globulin ratios of the next column accentuate this change. Cow A23 was 5 years old and pregnant; she came from an experimental herd that had been free of brucellosis for over ten years. The γ-peak (Fig.5d) and total protein were very high. Her previous history showed a temporary

indisposition due to cystic ovaries from which she had recovered about two months before this blood specimen was taken. Cow 881 (Fig.7a) was assumed to be normal and for that reason a pH-mobility relationship (to be discussed later in this paper) was run along with a serum (cow 143993b) having a titer of 1:40,000. A γ-globulin concentration of 36.7 per cent, which was much higher than any of the first four specimens in Table IV, indicated that the freedom from previous infection of some sort was open to question.

The next group included pregnant heifers that had been injected with 5 and 10 mg. of a water-soluble crushed cell fraction from Brucella at an interval of one week. Blood samples were taken about ten days after the last injection. Cows 530873, 480 and 650640 (Fig. 6a) were injected on or near the same days. Table IV shows that the agglutination titers and relative concentrations of the protein components are almost identical in spite of differences in total protein. Sample b of cow 478 was taken six weeks, and sample c, eight weeks after the last injection. It was noted that the total protein and the agglutination titer diminished as well as the γ-globulin while the albumin rose. This animal was exposed by way of the eye to Brucella three months after treatment and calved normally; yet Brucella organisms were isolated from the milk.

Artifical exposure of cow 340D6120 (Fig. 6b) to Brucella by way of the eye caused a tremendous increase in the γ-peak, the highest in this particular series; yet the agglutination titer was only 1:2560. Cow 883 (Fig.6c) displayed remarkable individuality by failing to develop agglutinins and calving normally after exposure by way of the eye to 6,000,000 live organisms. The serum of this animal had remained consistently negative to the agglutination test.

In contrast, the last three members of the series acquired infection naturally. Their agglutination titers (1:40,000) and total protein concentration (8 to 9 gm. per 100 cc.) were extraordinarily high. In the case of cow 143993b (Fig. 6d), albumin accounted for only 35.3 per cent while γ-globulin comprised 42.6 per cent of the total protein; β-globulin was about average.

Looking at the series in its totality it is seen that under the given conditions of electrophoresis the mobility for each protein component was far from constant; however, it did fall within a certain range. Tiselius and others (6,49) have pointed out that the mobility of a given protein, besides depending upon the pH and ionic strength of the buffer solution, is also a function of its own concentration as well as that of other constituents because of the constant interaction therewith. If the list given may be considered a fair representation of bovine serum, the average mobility for albumin, α-, β-, and γ-globulin is 6.2, 4.4, 3.0, 1.6 respectively. Closer inspection of the patterns will reveal that the classification by Tiselius of the serum proteins is an oversimplification. All too often the α- and β-boundaries appear with two or more peaks. The significance of these added subdivisions has been appreciated but not investigated particularly in this paper.

Again, being arbitrary and ignoring the individual characteristics of the serum specimens, the average relative concentration in per cent of each component with respect to total protein is 42.4 for A, 18.3 for α, 8.3 for β, and 31.1 for γ. A ratio greater than 0.8 for γ/A would seem to be significant immunologically.

E. Mobility - pH relationship of the proteins in a serum containing Brucella agglutinins in a high titer and in one containing no agglutinins.

Knowing that the pH of a buffer could affect the rate of electrophoretic separation of protein components of different isoelectric points, a series of buffers of different pH were prepared in the hope that antibody might be separated. If it be assumed that a serum of very high agglutination titer would contain an appreciable quantity of antibody, then a concomitant electrophoretic run with a negative, or so-called normal serum would be expected to produce diagrams sufficiently different to distinguish the antibody. Another object of this study was to learn if the pH-mobility curve and the isoelectric points of the four components differed significantly in a specific antiserum and a negative serum.

Tiselius (49) determined the pH-mobility curve of horse serum both on isolated components and on whole serum. He found the isoelectric point of albumin, α-, β-, and γ-globulin to be 4.64, 5.06, 5.12 and 6.0 respectively. Cohn (5) also has discussed the importance of these values with regard to the separation and purification of the serum proteins.

The choice of buffers for the wide p^H range presented a problem because of the desire to use uni-univalent buffers. Michaelis (34) showed that a buffer system from p^H 2.62 to 9.64 could be prepared by using proper concentrations of the following: sodium barbiturate, sodium acetate, sodium chloride, and hydrochloric acid. Accordingly, buffer solutions all of 0.1 ionic strength and desired p^H were prepared.

Cow 143993 was naturally infected; her serum contained the high total protein of 9.20 gm. per 100 cc. and showed an agglutination titer of 1:40,000. The serum of cow 881, supposedly normal, contained a total

protein of 7.68 gm. per 100 cc. and showed a titer of 1:20 which is rather common because most cow serums at times show at least a slight reaction to Brucella. Figs. 7a and 7b illustrate the striking similarity between the diagrams of the two serums.

At pH 7.82 cow 881 showed the more complex α-globulin. At other pH values the difference, if any, between the two samples was slight. One unusual phenomenon, common to both, was the appearance in the ascending boundary at pH 6.14 of a small peak migrating just ahead of the tall albumin peak (Fig. 7c). With increasing active acidity and a closer approach to their isoelectric points, the degree of separation between the globulins became increasingly difficult to distinguish. The most grotesque diagram and the one with the greatest dissimilarity between the ascending and descending boundaries was the one obtained at pH 3.53 (Fig.7f).

After dialysis an appreciable amount of precipitate was noticed in the samples at pH 6.14, 5.18 and 4.65; the serum at pH 3.65 was slightly turbid and possessed a more pronounced yellow tinge than the others. Table V records the results of the agglutination test upon the dialyzed antiserums after adjustment to a protein concentration of one per cent. At pH values of 5.18 and 4.65 the serum showed complete agglutination at one dilution less than the others, and the one at pH 3.53 showed a prozone phenomenon.

The relative areas of each component at the several pH values is also recorded in Table V. The inconsistent values obtained indicate the obvious impossibility of attaining correct determinations by electrophoretic analysis if the boundaries are not sufficiently separated from each other. This fact was always noted when the working pH value was close to the isoelectric point of the proteins involved.

TABLE V

Some Physico-Chemical and Concentration Characteristics of Two Bovine Serums at Different pH Values

Cow Serum	Aggl. Titer[1]	pH	Potential Gradient v/cm	Time, Seconds	Mobilities (U x 10^5)				Concentrations Per Cent Total Area				Fig. No.
					A	α	β	γ	A	α	β	γ	
143993b	1:5000 T	7.81	4.61	8,200	-6.16	-4.43	-3.23	-1.92	33.8	11.7	9.6	44.8	7b
	1:5000 T	6.14	4.92	10,300	-3.43	-2.44	-1.18	-0.12	43.2	8.1	9.9	38.8	7c
	1:2500	5.18	5.30	10,800	-2.06	-0.49	+0.51	+1.13	42.2	6.7	18.7	32.4	7d
	1:2500	4.65	4.47	11,300	-0.51	+0.75	+1.30	+2.23	40.7	11.5	35.5	13.2	7e
	1:5000	3.53	4.44	9,200	+3.40	+4.84	+4.84	+4.84	40.4	.	59.6	.	7f
881a	Negative	7.82	5.38	8,400	-6.86	-4.94	-3.24	-1.79	39.3	14.3	9.7	36.7	7a
	Negative	6.14	4.76	10,800	-3.67	-2.51	-1.54	-0.38	48.2	8.3	9.0	34.4	
	Negative	5.18	5.27	10,000	-2.40	-0.26	+0.15	+1.04	39.4	20.6	21.1	18.9	
	Negative	4.65	4.55	10,400	-0.24	+1.36	+1.64	+2.49	44.9	20.5	23.9	10.7	
	Negative	3.53	4.48	9,100	+4.02	.	+5.56		41.5		58.5		

[1] Titration was done after adjustment of the solutions to one per cent protein.

The mobility values as ordinates are plotted against pH values as abscissas in Fig. 8a for cow 143993 and in Fig. 8b for cow 881. Considering the difficulty in obtaining these values on whole serum, the isoelectric points found for the protein components of the two serums compared favorably with themselves and with those found by Tiselius (49) for horse serum.

Over the range of pH investigated no antibody boundary was differentiated. It was unfortunate that serum 881 had an unusually large γ-peak in spite of a negligible titer. At about this time an investigation upon the absorption of an antiserum of high titer was completed. It was found that agglutinin antibody was associated with the γ-globulin (Section G). In the light of this fact it is understandable why serum 881 with a γ-peak almost as large as that of serum 143993 produced patterns insignificantly different. It must be remembered that the increased production of γ-globulin is not the function of any particular antigen alone. Van der Scheer, Wyckoff, and Clarke (53) found that pneumococcus, meningococcus and pasteurella hyperimmune serums from horses contained increased amounts of γ-globulin.

F. Electrophoretic diagrams of a horse and a cow serum after injection with a water-soluble crushed cell fraction from Brucella.

A water-soluble crushed cell fraction* has been prepared in this laboratory which has successfully immunized guinea pigs against Brucella. Having found that this fraction stimulated the production of antibody

* The manner of preparation and results of this fraction with guinea pigs is to appear in a forthcoming publication.

against Brucella, an experiment was planned to inject both a horse and a cow at intervals with this fraction and to follow the effect upon the serum proteins electrophoretically, and if possible to determine the location of the antibody in the electrophoretic pattern.

Electrophoretic patterns were made and the agglutination titers determined on both animals before the first injection of material. Whereas the cow was negative, the horse showed an agglutination titer of 1:80, indicating possible previous infection with Brucella. The original patterns taken before injection are illustrated in Figs. 9a and 10a. The next sample from the cow was not taken until it had received three injections each of 5 mg. of material at two day intervals. A sharpening of the γ-peak occurred as it rose from 22.9 to 25.2 per cent of the total area, Table VI. An agglutination titer of 1:1280 was now noted. A third bleeding was made just prior to the fourth injection. Although the total protein was returning to the original concentration after an initial drop at the beginning of the experiment, the γ-peak decreased slightly while the agglutination titer continued to rise to 1:5000 (Fig. 9b). Two more injections were given and the fourth bleeding was performed one week after the third. The total protein, γ-globulin, and agglutination titer (1:10,000). all attained a maximum figure. Another bleeding was made two days later without a further injection. The same three factors all showed a slight decline (Fig. 9c). A sixth and last injection was made at which time, 21 days after the first, the agglutination titer was 1:5000. The animal was permitted to rest six weeks before a final serum specimen was examined (Fig. 9d). The total protein was practically restored to the original concentration but the agglutination titer was now only 1:640. The γ-peak

TABLE VI

Response of Serum Proteins[1] of Cow 755297 Injected With a Water-Soluble Crushed Cell Fraction of Brucella

Injection No.	Injection Date	Blood Drawn	Aggl. Titer	pH	Mobilities (U x 10^5) A	α	β	γ	Protein T.P.	Protein Alb.	Concentrations Per Cent Total Area A	α	β	γ	Composition A/G	α/A	β/A	γ/A	Fig. No.
		4/21/41	Negative	7.93	6.2	4.7	3.1	1.9	6.82	3.20	46.9	20.2	10.0	22.9	0.88	0.20	0.10	0.49	9a
1	4/24/41																		
2	4/26/41	4/29/41	1:1280	7.81	6.1	4.5	3.2	2.1	6.39	3.09	48.4	18.1	8.3	25.2	0.94	0.37	0.17	0.52	
3	4/28/41	5/1/41	1:5000	7.81					6.62	3.12	47.1	20.4	9.1	23.4	0.89	0.43	0.19	0.50	9b
4	5/1/41																		
5	5/6/41	5/8/41	1:10,000	7.80					7.04	3.18	45.1	18.3	8.2	28.5	0.82	0.41	0.18	0.63	
		5/10/41[2]	1:5000	7.80	6.3	4.5	3.0	1.8	6.59	3.19	48.4	14.1	10.2	27.1	0.94	0.29	0.21	0.56	9c
6	5/15/41	5/15/41	1:5000						6.47										
		6/26/41	1:640	7.81	5.8	4.2	2.9	1.8	6.92	2.85	41.2	20.6	8.7	29.4	0.70	0.50	0.21	0.71	9d

[1] The ε-boundary is included in these estimations.

[2] Three liters of blood were withdrawn.

had maintained its increased area and even gained a little more. The albumin pattern had lost about 5 per cent of the total area.

The horse reacted locally and systemically to all injections each of 5 mg. of material given about 3 days apart. A blood specimen (Fig. 10b) obtained 10 days after the first injection displayed a rise of total protein from 6.03 to 7.66 gm. per 100 cc; since the concentration of albumin remained constant, 2.36 to 2.35, the entire increase was due to globulin alone. Further injections were made and their effect is recorded in Table VII. The highest agglutination titer obtained was 1:5000 with a γ/A ratio of 1.38. The blood specimen (Fig. 10c) analyzed after the fourth injection showed that, based upon proportion of total protein, all fractions had decreased except the γ-globulin. Letting albumin equal 100, α-globulin had fallen from 36 to 31, β-from 42 to 40, but the γ-globulin rose from 77 to 138. Regardless of the mode of expression, the γ-peak alone showed a consistent enhancement.

The total protein showed an increasingly accumulative effect with each succeeding injection, an observation that has been commonly known in immunization processes. The total protein rose from 6.03 to 8.60 gm. per 100 cc. after six injections over a period of 33 days. An examination of the albumin concentration over this period revealed the striking fact that with the exception of a small drop in the interim, and in spite of a 30 per cent increase in total protein, the circulating albumin per unit volume of serum maintained practically a constant concentration, about 2.85 gm. per 100 cc. Even after a rest of almost seven weeks during which time the total protein concentration dropped to 7.13 and the agglutination titer to 1:640, the circulating albumin was 2.92. (Fig. 10d). This observation would seem to indicate that albumin played no direct part in the immune

TABLE VII

Response of Serum Proteins[1] of a Horse Injected With a Water-Soluble Crushed Cell Fraction of Brucella

Injection No.	Injection Date	Blood Drawn	Aggl. Titer	pH	Mobilities (U x 10^5) A	α	β	γ	Protein T.P.	Alb.	Concentrations Per Cent Total Area A	α	β	γ	Composition A/G	α/A	β/A	γ/A	Fig. No.
		4/4/41	1:80	7.83	6.0	4.0	3.4	1.6	6.03	2.36	39.2	14.0	16.6	30.1	0.64	0.36	0.42	0.77	10a
1	4/5/41	4/8/41	1:2560	7.80	5.9	4.2	3.2	1.1	7.23	2.89	40.0	15.4	13.5	31.1	0.67	0.39	0.34	0.78	
2	4/8/41	4/15/41	1:1280	7.81	5.9	4.3	2.9	1.6	7.66	2.35	30.7	19.5	16.5	33.2	0.44	0.63	0.54	1.08	10b
3	4/15/41	4/18/41	1:5000	7.86					7.70	2.41	31.3	13.4	12.2	43.1	0.46	0.43	0.39	1.38	
4	4/21/41	4/24/41	1:1280	7.86					7.94	2.57	32.4	10.0	12.8	44.8	0.48	0.31	0.40	1.38	10c
		5/1/41	1:2560	7.80					7.81	2.72	34.8	10.9	10.7	43.6	0.53	0.31	0.31	1.25	
5	5/1/41																		
6	5/6/41	5/8/41	1:2560	7.80	5.7	3.9	3.2	0.9	8.60	2.86	33.3	11.2	12.3	43.3	0.50	0.34	0.37	1.30	
		6/26/41	1:640	7.81	5.9	4.0	2.9	1.6	7.13	2.92	40.9	10.9	11.5	36.5	0.69	0.27	0.28	0.89	10d

[1] The ε-boundary is included in these estimations.

process and that there was no intraconversion of albumin and globulin. The increase in the γ-boundary, for instance, had come from protein newly produced from antigenic stimulation. Cessation of this stimulation resulted in a marked decrease in total protein which could be attributed mostly to the globulin fractions. The albumin, therefore, concerned primarily with the maintaining of the proper osmotic equilibrium appeared to play the role of a disinterested bystander during the process of antibody formation.

Comparing the reaction of the two animals, the most salient fact was the tremendous response of the γ-peak of the horse which attained a maximum of 44.8 per cent of the total area from an initial 30.1 per cent, while the cow increased only to 28.5 from an initial 22.9 per cent. The γ/A ratio sharply expresses this fact: the horse climbed from 0.77 to 1.38 while the cow slowly and vacillatingly rose to 0.63 from 0.49. In this particular case the extraordinary difference in species response might be accounted for partly to some prior sensitization of the horse to Brucella. The agglutination test detected no pronounced difference in agglutinin production of the two experimental animals. In fact after the long rest period both returned to the same titer of 1:640. With regard to increase of total protein, the cow failed to produce any consistent response but fluctuated above and below its initial concentration, finally stopping approximately at its normal figure.

G. Electrophoretic studies of Brucella antiserums after agglutinin absorption.

The preceding studies strongly pointed to the γ-fraction of the serum as the one associated with Brucella antibody. Unlike anti-pneumococcus

horse serum (35) where over 30 per cent of the total globulin may be accounted for by antibody, and in which the disproportionate enhancement of the γ-peak during hyperimmunization immediately designated this fraction as the sole associate of pneumococcus agglutinin, no such striking pictorial representation was found with cow Brucella antiserum. After a long and patient search two antiserums of unusually high titer (1:40,000) were discovered. Believing that an appreciable portion of the area of the Longsworth diagram was attributable to antibody, a process of absorption was developed to remove it. A dense suspension of a 48 hour growth of smooth Brucella abortus culture was prepared, washed, and then suspended in 50 cc. of phenol-salt solution. Five cc. of this suspension was added to 10 cc. of antiserum. After thorough agitation, the tube was placed at 4°C. for 48 hours with further occasional shaking. The agglutinated cells were centrifuged off, and 5 cc. more of the suspension was added, and the treatment repeated. After a dilution of 2.5 times, the equivalent amount of packed cells was added to prevent further dilution. In a typical experiment, a series of eight absorptions was necessary to remove the agglutinins entirely. The first two absorptions removed about 50 per cent, and the fifth removed 90 per cent of the agglutinins.

Table VIII records the results of absorption upon the two serums of high agglutination titer. Considering the error in electrophoretic analysis, it is evident that the antibody is associated solely with the γ-peak. The electrophoretic runs of antiserum 201a were so carefully controlled that the two diagrams (Figs.11a and 11b), obtained before and after absorption, coincide exactly except at that portion of the γ-peak with which the antibody was associated. In antiserum 201a, the antibody

TABLE VIII

Effect of Agglutinin Absorption Upon the Protein Components[1] of Brucella Antiserum

Serum Specimen	Absorption	Aggl. Titer	pH	Mobilities (U x 10^5)				Total Protein	Proportion of Total Protein in Per Cent				Composition				Fig. No.
				A	α	β	γ		A	α	β	γ	A/G	α/A	β/A	γ/A	
201a	Before	1:40,000 P	7.85	6.3	4.6	3.1	1.7	7.94	41.2	17.0	8.5	33.1	0.63	0.41	0.21	0.81	11a
	After	Negative	7.85	6.2	4.6	3.3	1.9		45.6	17.8	7.9	28.5	0.84	0.39	0.18	0.48	11b
143993b	Before	1:40,000 P	7.86	6.6	4.8	3.2	1.6	9.20	35.3	14.5	7.4	42.6	0.55	0.41	0.21	1.21	11c
	After	Negative	7.86	6.7	4.8	3.2	1.6		39.3	15.4	7.3	37.9	0.65	0.39	0.19	0.96	11d

[1] These estimations do not include a correction for the ϵ-boundary.

accounted for 4.16 per cent of the total protein or 7.8 per cent of the total globulin. In antiserum 143993b (Figs. 11c and 11d), the antibody accounted for 4.7 of the total protein, or 7.7 per cent of the total globulin. These values are correlative with the agglutination titer of the two antiserums.

Having demonstrated conclusively the location of Brucella antibody in the antiserum pattern, some of the uncertainties of the preceding studies can now be explained. If it is remembered that γ-globulin may be enhanced by any one of several antigens, and that after initial stimulation the subsequent increase need not be proportional to the number or amount of antigen injected, it can now be surmised that in the preceding study (Section F), the cow pattern showed no progressive or startling increase in the γ-peak because its γ-peak was initially high possibly from some prior antigenic stimulation. The previous history of this heifer was not known completely.

In sections A and B of this paper mention was made that no direct correlation existed between the Brucella agglutination titer and area of the γ-peak. Now it is clear that Brucella antibody is only a small part of the γ-peak and that the remainder of the γ-globulin may be normal, or antibody globulin due to some antigen other than Brucella; and hence not detectable in a Brucella antibody titration procedure.

That the γ-globulin is not homogeneous is evident from the diffuse and spreading character of its peak. When more functional tests are made upon this fraction, its complexity will be realized. Pillemer et al. (37) recently have found that one of the components of complement composing only 0.6 per cent of the total protein has a mobility of about 2.9×10^{-5}.

Ordinarily this component is masked in the γ-peak. The supposition that the γ-fraction may contain other functional units traveling with a similar mobility is entirely a reasonable one.

H. Electrophoretic studies of Brucella antiserum of high titer fractionated with ammonium sulfate.

The problem in the preparation of purified and concentrated serums is inseparably concerned with the distribution of the antibodies among the various protein fractions of antiserum. In general, antibody produced by bacterial exotoxin and virus is located in the pseudoglobulin fraction or can be precipitated with this fraction (34-50% saturated $(NH_4)_2SO_4$). The antibody produced by bacteria (agglutinins, precipitins, opsonins) is located in the euglobulin fraction. Tetanus antitoxin precipitates with the two fractions, and according to Klobusitzky (22) is irregularly distributed between them. For this reason wasteful and inefficient serum purification can be avoided only by the individual examination of every lot of serum.

The use of ammonium sulfate in serum fractionation is classical, and yet modern. Gibson (12) in 1907 first used this salt to precipitate the entire immune globulin by half saturation and then subsequently extracted with sodium chloride. Banzhaf (1) subdivided immune serums into the euglobulin, pseudoglobulin, and albumin fractions. Other early workers like Chick and Martin (3) and Homer (15,16) later elaborated the entire procedure.

Tiselius (49) found that the serum protein subdivisions obtained with ammonium sulfate fractionation and other chemical procedure were not identical with the fractions separated by electrophoresis. The salt

fractions contained mixtures of the electrophoretic components. Cohn and coworkers (4) have followed the fractionation of horse serum with various saturations of ammonium sulfate. Svensson (45) more recently has followed electrophoretically the supernatants of serums from the horse, the cow, the swine and the rabbit precipitated with varying amounts of ammonium sulfate. In all these preceding studies the serum of average or normal animals was used; hence no attempt was made to follow the antibody content of the fractions.

Table IX presents the data obtained from both the precipitates and supernatants from a serum of high Brucella agglutinin content, and the resulting agglutination titers of the supernatants which were easily restored to the original volume. On account of the small amount of material, the agglutination titers of the precipitates can be approximated readily by difference.

The entire procedure and the preparation of the saturated ammonium sulfate was made at room temperature with the reaction adjusted near pH 6. The serum 201, 75 cc., was diluted once with saline since separations (4) appear to be slightly more effective if the proteins are less concentrated than normally found in serum. The diluted serum was divided into three lots of 50 cc. each and placed in graduates. A calculated amount of the saturated ammonium sulfate solution was dialyzed into serum through rotating cellophane membranes according to the method of McMeekin (31). Equilibrium was attained in about 24 hours. The precipitates after centrifugation were washed with small aliquots of ammonium sulfate of the concentration at which separation was effected. All fractions were dialyzed against water to remove the ammonium sulfate, diluted to a one per cent protein con-

TABLE IX

Composition[1] of the Supernatants and Precipitates of a High Titer Antiserum (201a) Fractionated With Varying Amounts of Ammonium Sulfate

Fraction	Ammonium Sulfate Saturation Per Cent	Protein Gm/100cc.	Per cent of Total Protein	Aggl. Titer	pH	Mobilities (U x 10^5)				Concentrations Per Cent Total Area				Composition				Fig. No.
						A	α	β	γ	A	α	β	γ	A/G	α/A	β/A	γ/A	
Whole serum[2]		3.97	100	1:20,000	7.85	6.3	4.6	3.1	1.7	41.2	17.0	8.5	33.1	0.63	0.41	0.21	0.81	12a
Supernatant	34	1.94	48.9	1:5,000	7.82	6.3	4.7	2.8	1.8	60.0	15.3	10.2	14.3	1.50	0.26	0.17	0.24	12b
"	40	1.50	37.8	1:320	7.82	6.2	4.7	2.8		76.4	13.2	10.3	0.0	3.24	0.17	0.14	0.0	12c
"	50	1.12	28.2	Negative	7.82					79.5	11.2	9.2	0.0	3.88	0.14	0.12	0.0	12d
Precipitate	34	2.02	51.0		7.82	6.6	4.4		1.7	2.1	21.1	0.0	76.6	0.02	9.64	0.0	34.93	13b
"	40	2.46	62.1		7.82	6.7	4.4		1.7	4.6	22.4	0.0	72.9	0.05	4.86	0.0	15.76	13c
"	50	2.84	71.7		7.82	6.5	4.4		1.7	9.5	23.7	0.0	66.7	0.11	2.49	0.0	7.0	13d

[1] Correction was made for the ε-boundary in these estimations.

[2] The serum was diluted once with physiological salt solution.

centration, and these were dialyzed against buffer to attain equilibrium for the usual electrophoretic study. The ϵ-boundary was not included in these estimations. In spite of the danger of irreversible changes occurring in the precipitates, they were investigated electrophoretically and included in Fig. 13. Contrary to an observation by Svensson (45) the diagrams of the precipitates show that an increasing amount of albumin, although small, is brought down with increasing amounts of salt. The diagrams of the supernatants (Fig. 12) show that considerable amounts of α- and β-globulins are still in solution at a salt saturation of 50 per cent. It has been found generally that albumin does not precipitate below 55 per cent saturation.

Further inspection of Table IX discloses the interesting facts that none of the β-globulin was precipitated at all salt concentrations used, and only a very small amount, if any of the α-globulin. Since the isoelectric point of the γ-fraction was found to be about 6, this experiment was tantamount to isoelectric - salt precipitation, It is not surprising, therefore, that the β- and most of the α-globulins remained in solution, at least up to the salt concentration employed. Blix, Tiselius, and Svensson (2) have shown that these components contain phospholipids, cholesterol and carbohydrates, and are extremely complex. Following the fractionation with the agglutination test, the association of Brucella agglutinins with the γ-peak was confirmed since with the complete removal of the γ-globulin the titration was negative.

I. Electrophoretic studies of Brucella antiserum fractionated with Ethanol-water mixtures at low temperature.

Mellanby (33) and Hardy and Gardiner (13) have fractionated serum by means of alcohol at low temperature. Felton (9) developed a method of

concentrating the antibody of pneumococcus antiserum with ethyl alcohol which is still a standard procedure in some biological laboratories. Cohn, Luetscher, Oncly, Armstrong, and Davis (4) more recently have refined considerably the technique of fractionating normal serum and plasma proteins by equilibration across membranes with ethanol-water mixtures of controlled pH, ionic strength and temperature.

The use of a differential organic solvent like ethyl alcohol for fractionating seemed to offer an advantage over that of ammonium sulfate. Denaturation may be prevented if ethanol-water mixtures are carefully added to proteins at a low temperature. Ferry and coworkers (10) have been able to maintain crystallized egg albumen in ethanol-water mixtures at -5°C. for over ten days. It seemed worthwhile, therefore, to determine whether antibody separation could be obtained from bovine antiserum by diffusing ethanol through a rotating cellophane membrane.

Four, 25 cc. amounts of serum from cow 143993b, having an agglutination titer of 1:40,000 were diluted once with saline. Appropriate concentrations of ethanol-water mixtures were prepared of which the concentration of alcohol by volume was determined from their specific gravity as measured with a Westphal balance. A membrane containing the calculated amount of ethanol-water was submerged into the diluted serum and gently rotated by means of an electric motor. The whole process was carried out in the cold room, and in addition, the serum container was kept in a cracked ice and water bath to insure maintenance of a temperature of 0°C.. Because of the high dilution in the two higher concentrations of ethanol, salt was added to restore approximately the original ionic strength of the serum.

After equilibration, the precipitates were centrifuged, washed twice and together with aliquots from the supernatants were put up in cellophane sacs and dialyzed in the cold against saline until most of the excess alcohol was removed. All samples were later dialyzed against the usual buffer for at least two days and adjusted to 1 per cent protein concentration in preparation for the Tiselius apparatus. The agglutination titer was determined on all samples after adjustment to 1 per cent protein concentration. Table X reveals that ethanol fractionation was not as clear cut as with ammonium sulfate. Under the conditions described 10 per cent ethanol caused an insignificant amount of precipitation, while 20 per cent ethanol precipitated about 38 per cent of the total protein (Table X). In this precipitate the γ-peak rose from 42.6 to 87.3 per cent of the total area carrying only one-half of the agglutinin content; and in the supernatant, albumin increased to 62.8 from 35.3 per cent, while the γ-globulin diminished to 11.2 from 41.6 per cent, but retaining about one quarter of the antibody.

Raising the ethanol concentration to 30 per cent caused only a slightly greater precipitation over that at 20 per cent, but the antibody brought down in the precipitate was almost doubled. In marked contrast to the effect of ammonium sulfate which did not precipitate out any β-globulin up to a salt saturation of 50 per cent, ethanol failed to precipitate any α-globulin up to a concentration of 30 per cent. This observation would seem to suggest a manner of separating α-globulin from β-, and β- from γ-globulin.

Bringing the concentration of ethyl alcohol to 40 per cent effected the greatest change upon the α-globulin which had hardly been touched during previous concentrations. Fig. 15d readily reveals how most of

TABLE X

Composition[1] of the Supernatants and Precipitates of a High Titer Antiserum (143993b) Fractionated With Varying Amounts of Ethanol at 0°C.

Fraction	Ethanol Per Cent by Volume	Protein Gm/100cc.	Per Cent of Total Protein	Aggl. Titer[2]	pH	Mobilities (U x 10^5)				Concentrations Per Cent Total Area				Composition				Fig. No.
						A	α	β	γ	A	α	β	γ	A/G	α/A	β/A	γ/A	
Whole serum[3]	0	4.10	100	1:5120	7.86	6.6	4.8	3.2	1.6	35.3	14.5	7.4	42.6	0.55	0.41	0.21	1.21	14a
Supernatant	10	4.08	99.5	1:5120	7.99	5.9	4.2	2.6	1.3	38.4	13.8	5.9	41.6	0.62	0.36	0.15	0.98	15a
"	20	2.54	61.9	1:1280	7.99	6.1	4.5	3.0	1.8	62.8	17.9	7.9	11.2	1.69	0.29	0.13	0.18	15b
"	30	2.04	49.7	1:1280	7.99	6.0	4.3	2.8	1.4	66.1	16.9	9.4	7.4	1.95	0.26	0.14	0.11	15c
"	40	1.43	34.8	1:2560	7.99	6.1	4.3	2.7	1.5	75.2	2.4	11.5	10.9	3.02	0.03	0.15	0.14	15d
Precipitate[4]	10	0.02	00.5															
"	20	1.56	38.1	1:2560	7.99	6.1		3.0	1.4	1.9	0.0	10.6	87.3	0.02		5.40	44.1	14b
"	30	2.06	50.3	1:5000	7.99	6.1		3.5	1.4	3.7	0.0	11.2	87.7	0.04		3.00	22.5	14c
"	40	2.67	65.2	1:2560	7.99	6.3	3.8		1.5	6.7	19.7		75.1	0.05	3.92		14.9	14d

[1] The ϵ-boundary is included in these estimations.

[2] Agglutinin titer was made after adjustment of the solution to one per cent protein.

[3] Antiserum diluted with one volume of physiological salt solution.

[4] Insufficient protein for an electrophoretic determination.

the α-peak was removed for the first time. Fig. 14d illustrating the accompanying precipitate, however, shows the probability of denaturation because instead of the two usually well-defined α- and β-peaks, the diagram shows a much skewed peak with a mobility in between that of the two globulins. The agglutination titers of both the precipitate and the supernatant now are almost equivalent, indicating that alcohol precipitation, even for the same component, was indiscriminate. Unlike ammonium sulfate which completely precipitated all of the γ-globulin at 40 per cent saturation, ethanol removed no component completely even at the highest concentration under the conditions mentioned (Fig. 15). It was also noted that in the precipitates there was a slight but direct increase of albumin with alcohol concentration (Fig. 14).

While neither the salt nor the alcohol method will preferentially detach the antibody from the γ-peak, a combination of the two methods would probably be effective in obtaining a γ-globulin entirely free of any of the other chief protein components of serum. Having prepared a pure solution of total γ-globulin, further study could be continued to separate the antibody from a major portion of the inert γ-constituent in an attempt ultimately to prepare a truly purified and concentrated antiserum.

SUMMARY

The electrophoretic patterns of a series of normal and brucellosis infected bovine serums have been obtained using the schlieren scanning method of Longsworth. The mobilities and concentrations of electrophoretically distinct protein components have been computed from these patterns. The mobilities of the serums fall into four well defined groups corresponding to albumin, α-, β-, and γ-globulins. The outstanding characteristic of the serum of a new-born calf was the extremely high concentration of α-globulin in contrast to a negligible, or almost complete absence of γ-globulin. Within four hours after the ingestion of colostrum by a normal calf the γ-peak might account for about 15 per cent of the total protein, while in an infected dalf the figure might rise to 30 or 40 per cent. Agglutinins were evidently absorbed also as measured by the agglutination procedure. By the end of two weeks all protein components were in the relative concentration usually found in young heifers. Vaccination of calves with U.S.B.A.I. Brucella strain 19 caused only a slight change in the normal serum distribution; even the slight increase in γ-globulin along with the agglutinin titer returned to normal within a month. No significant or permanent change could be demonstrated electrophoretically to account for the immunizing property of strain 19. The pattern of the vaccinated animal resembled the customary pattern of any young heifer.

The average mobility from a large number of various bovine serums for albumin, α-, β- and γ-globulin was 6.2, 4.4, 3.0, and 1.6 respectively. From the same group the average relative concentration in per cent of each component with respect to total protein was found

to be 42.4 for albumin, 18.3 for α-, 8.3 for β-, and 31.1 for γ-globulin. A ratio greater than 0.8 for γ/A appeared to be significant immunologically. Using a uni-univalent system of buffers at different pH values and at a constant ionic strength of 0.1, the isoelectric point of the protein components of a bovine antibrucella serum was found to be 4.45, 4.98, 5.28 and 6.02 for albumin, α-, β-, and γ-globulin respectively.

Injection of a water-soluble crushed cell fraction at intervals into a horse and a cow caused an accumulating increase in total protein in both species. In contrast to the cow the horse showed a tremendous increase of the γ-globulin which attained a maximum area of 44.8 per cent from an initial 30.1 per cent while the cow increased only to 28.5 from an initial 22.9 per cent. Apparently, the albumin concentration per unit volume of blood remained constant during the whole period of hyperimmunization in spite of a rise of over 25 per cent in total protein in the case of the horse.

Absorption of an antiserum of high titer showed the γ-globulin to be the associate of Brucella antibody. Ammonium sulfate fractionation at a pH of 6 by diffusion through a rotating cellophane membrane succeeded in precipitating out all the γ-component including about 99 per cent of the antibody at a salt concentration of 40 per cent saturation. Ethanol introduced into antiserum in a similar manner but at 0°C. was less discriminate in the fractions precipitated. Under the conditions described, an alcohol concentration of 40 per cent by volume failed to precipitate more than 50 per cent of the antibody, while 30 per cent of the alcohol did succeed in precipitating over three quarters of the agglutinin content. A combination of the two protein precipitants seemed to offer the possibility of fractionating

serum into components classified by Tiselius. After obtaining total γ-globulin in this way, further experimentation might yield a method to further purify and concentrate Brucella antibody.

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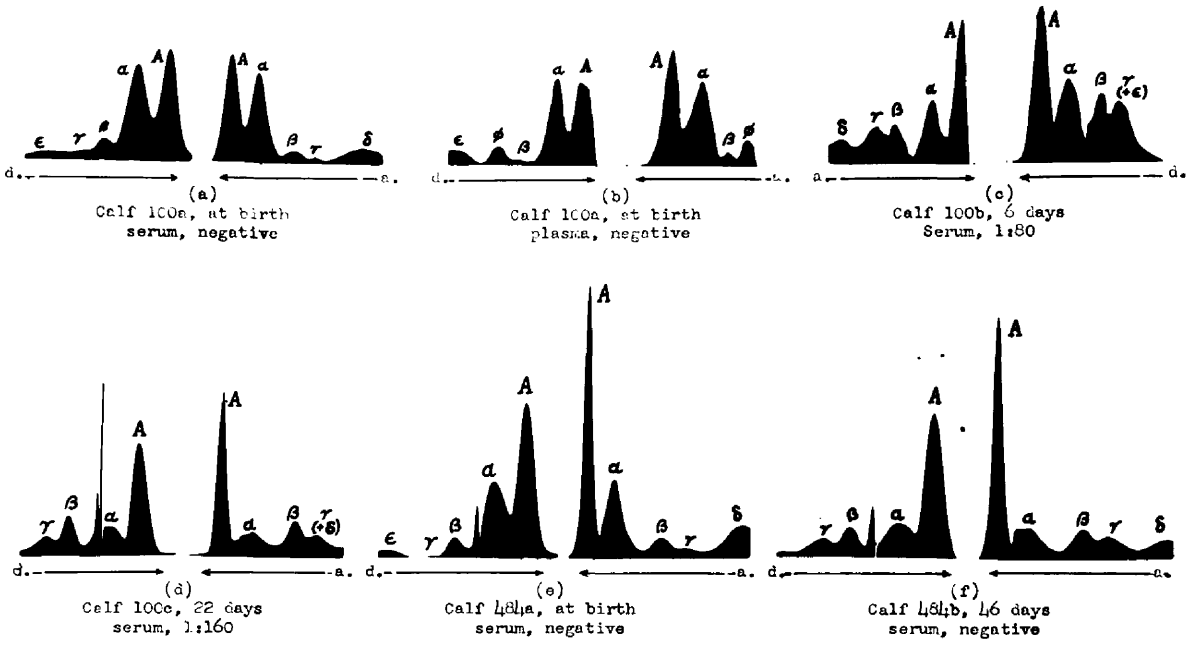


Fig. 1. Blood Specimens Drawn Before and After Ingestion of Colostrum with Age of Calf and Agglutination Titer.

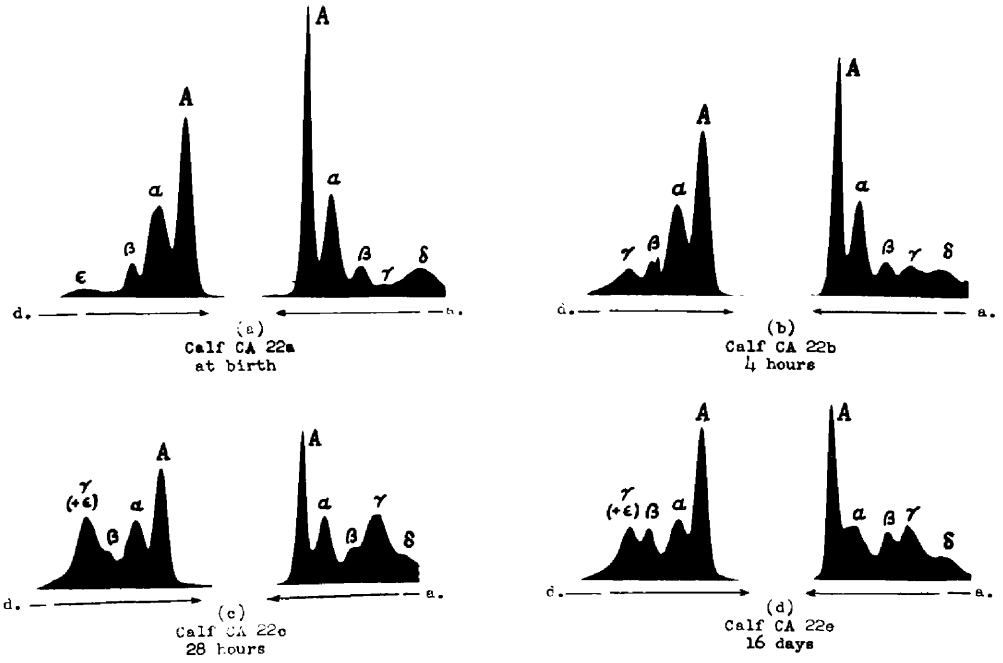


Fig. 2. Serum Specimens from a Normal Calf Drawn at Intervals with Age of Calf; Agglutinin Titer was Always Negative.

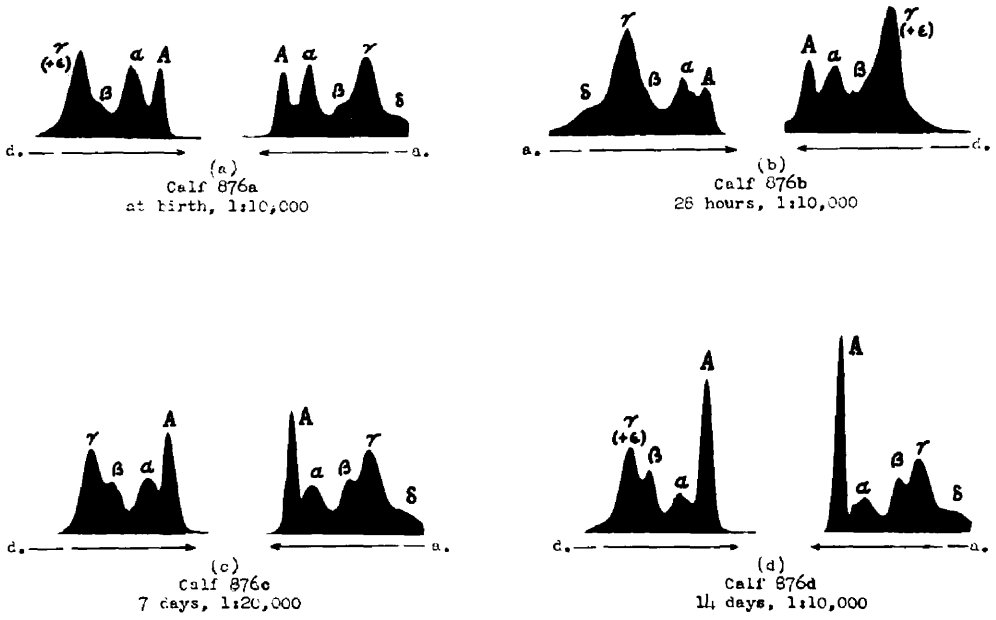


Fig. 3. Serum Specimens from an Infected Calf Drawn at Intervals with Age of Calf and Agglutination Titer.

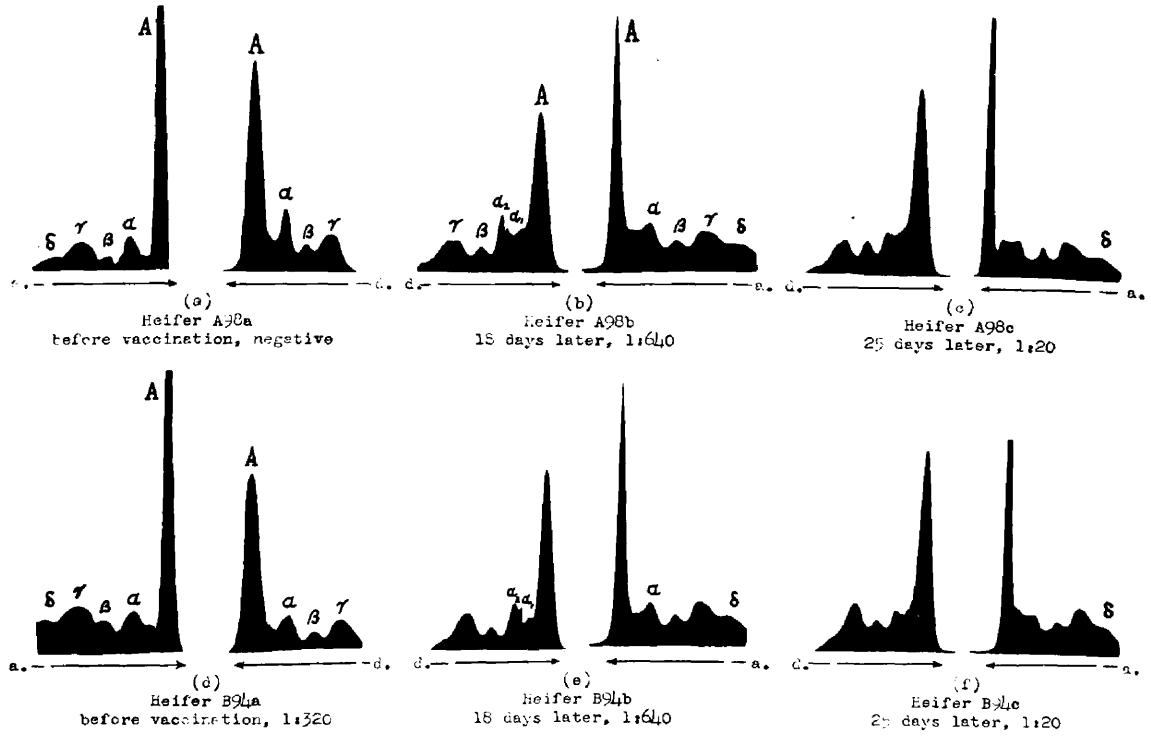
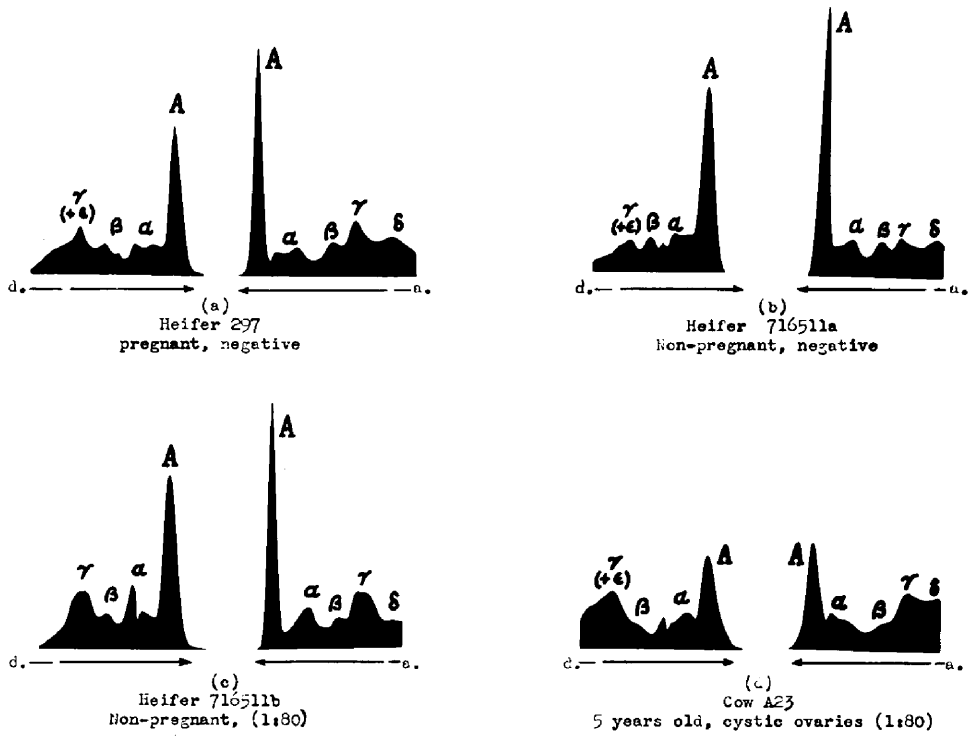


Fig. 4. Serum of Heifers Vaccinated with U.S.B.A.I. Brucella Strain 19.



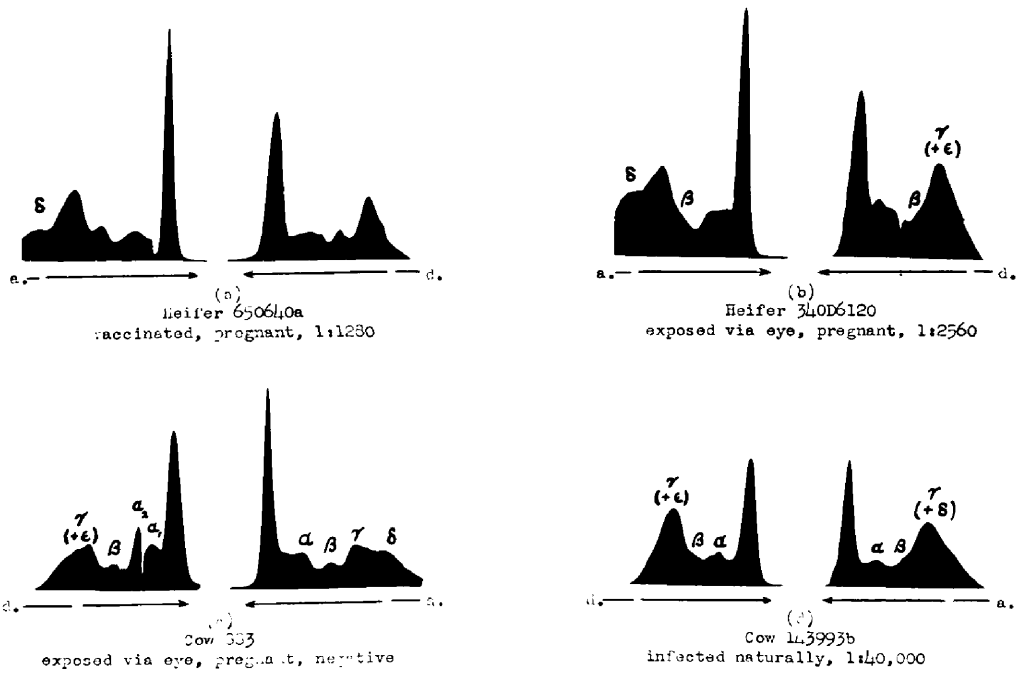
(a)
Heifer 297
pregnant, negative

(b)
Heifer 716511a
Non-pregnant, negative

(c)
Heifer 716511b
Non-pregnant, (1:80)

(d)
Cow A23
5 years old, cystic ovaries (1:80)

Fig. 5. A General Series of Bovine Serums with Remarks and Agglutination Titer.



(a)
Heifer 650640a
Vaccinated, pregnant, 1:1280

(b)
Heifer 340D6120
exposed via eye, pregnant, 1:2560

(c)
Cow 333
exposed via eye, pregnant, negative

(d)
Cow 1439936
infected naturally, 1:40,000

Fig. 6. A General Series of Bovine Serums with Remarks and Agglutination Titer.

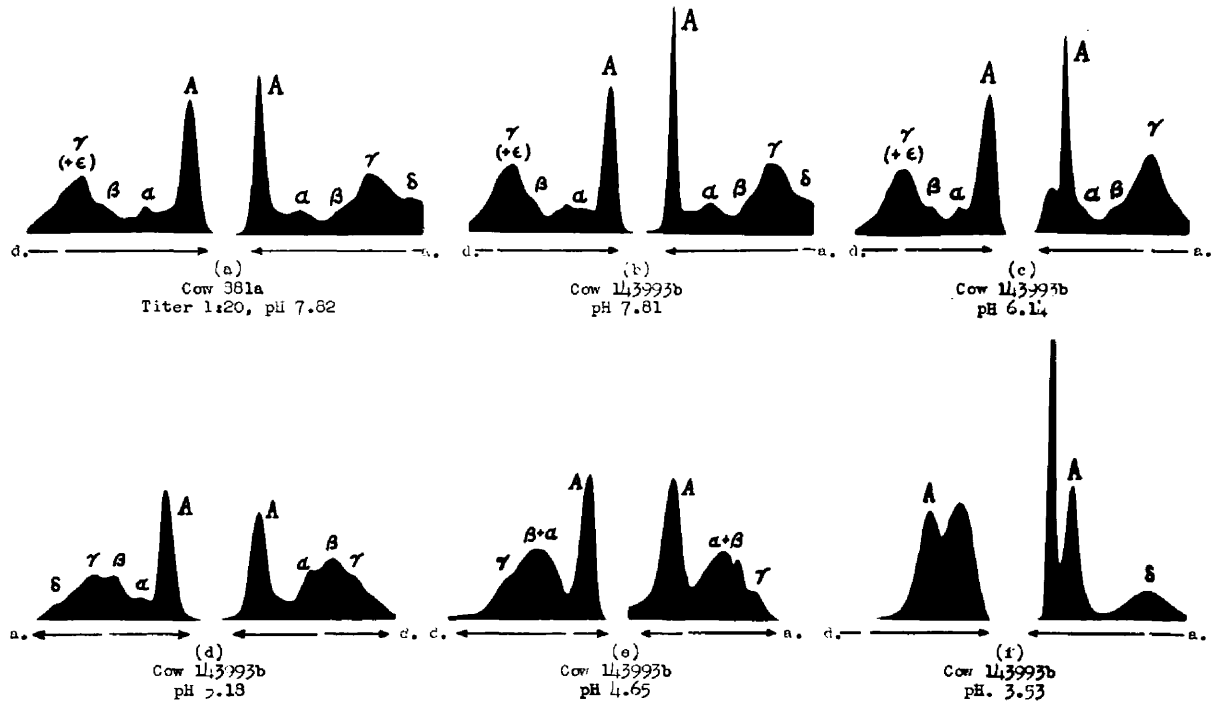


Fig. 7. Mobility - pH Relationship of the Serum Proteins of a Cow with an Agglutinin Titer of 1:40,000.

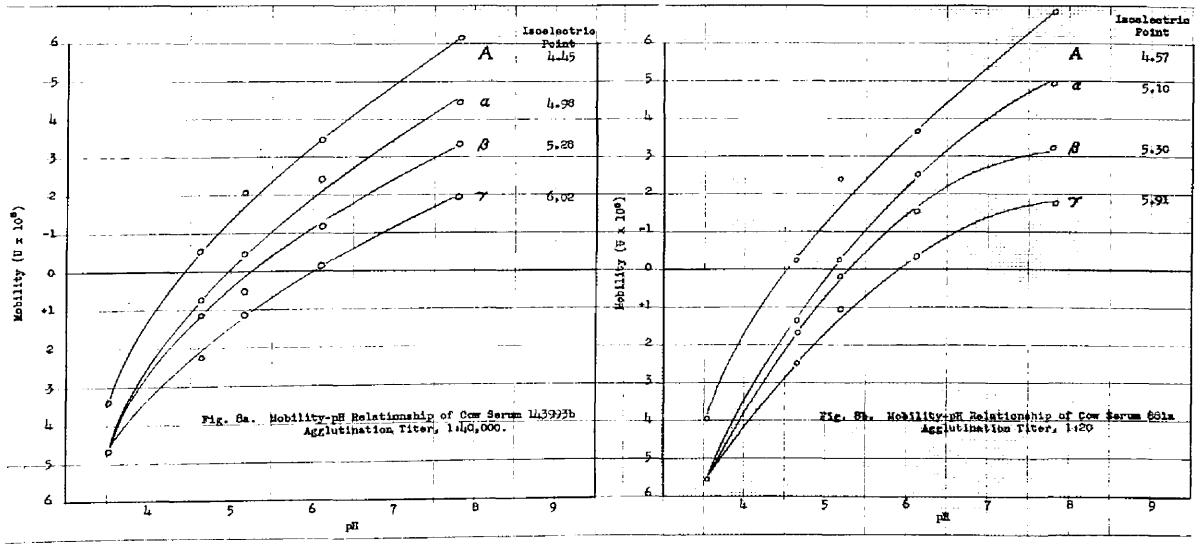


Fig. 8a. Mobility-pH Relationship of Cow Serum 143993b Agglutination Titer, 1:40,000.

Fig. 8b. Mobility-pH Relationship of Cow Serum 381a Agglutination Titer, 1:20

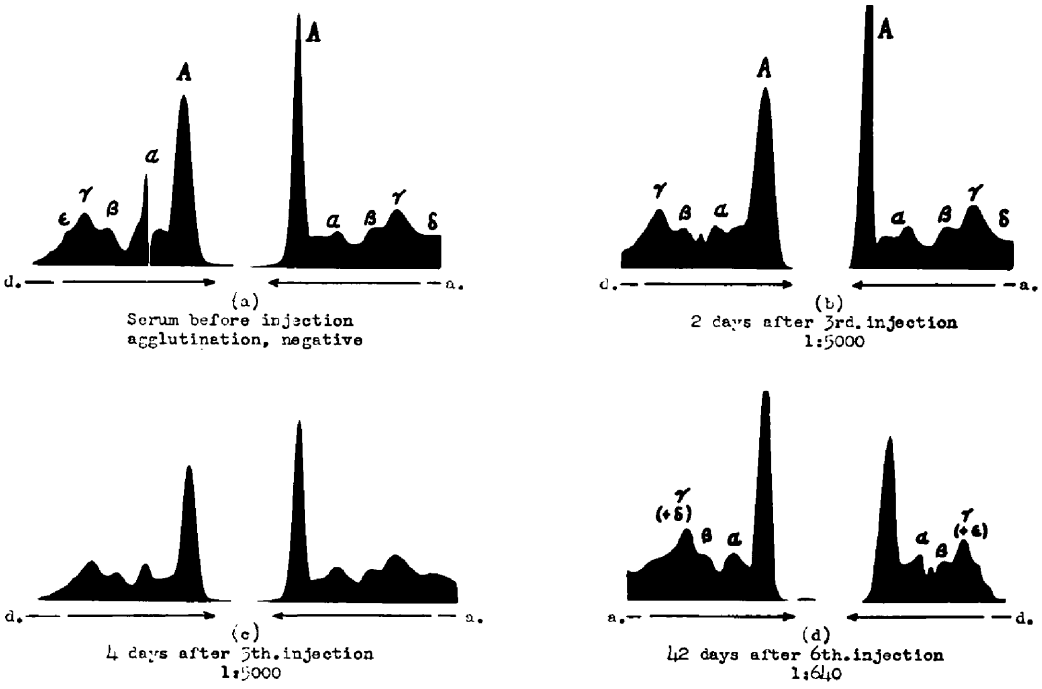


Fig. 9. Response of the Serum Proteins of Heifer 755297 to Injections of a Water-Soluble Crushed Cell Fraction of Brucella.

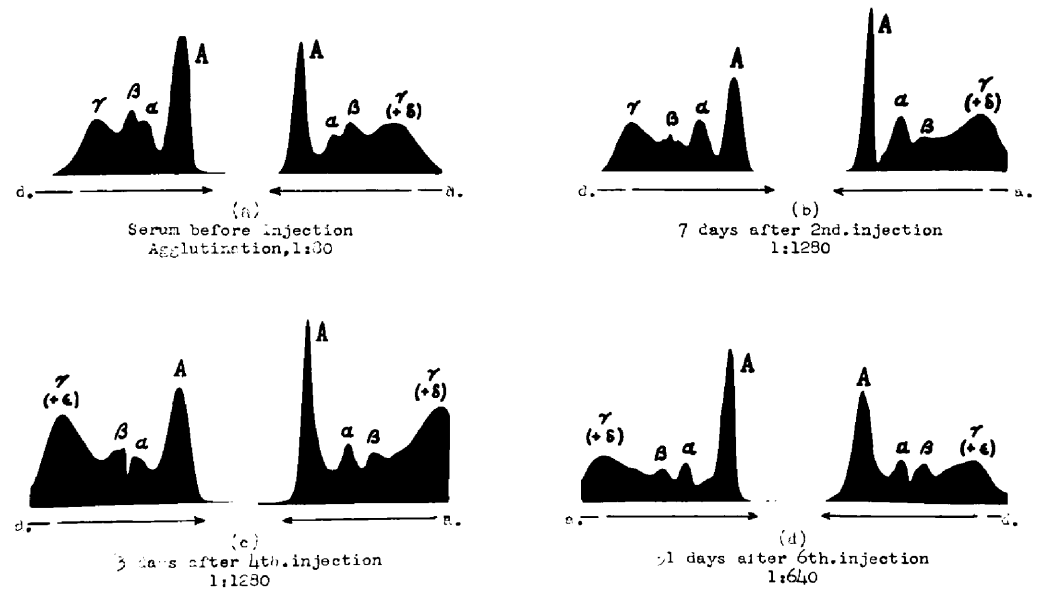


Fig. 10. Response of the Serum Proteins of a Horse to Injections of a Water-Soluble Crushed Cell Fraction of Brucella.

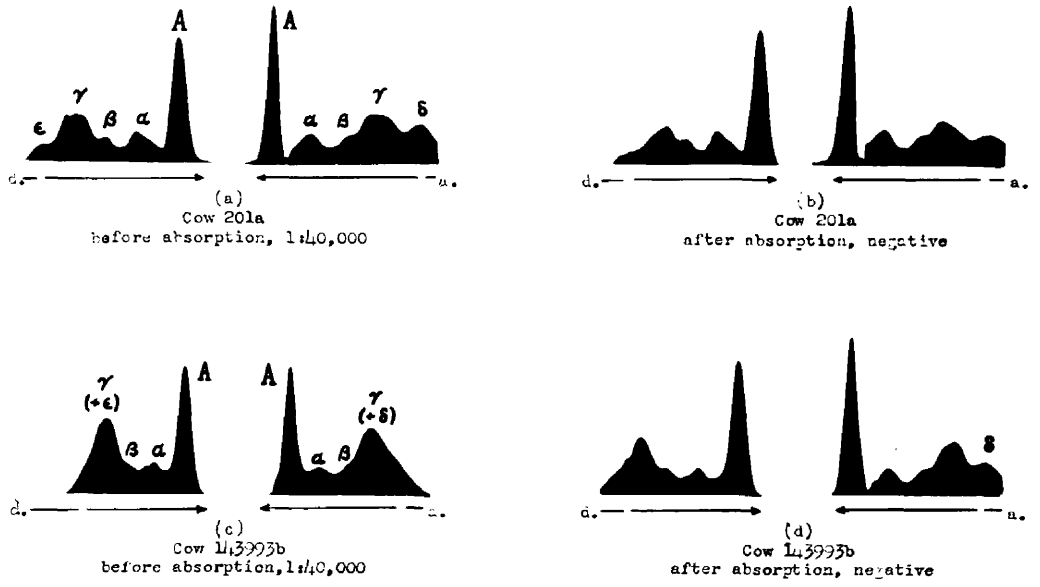


Fig. 11. Effect of Agglutinin Absorption upon the Protein Components of a Brucella Antiserum.

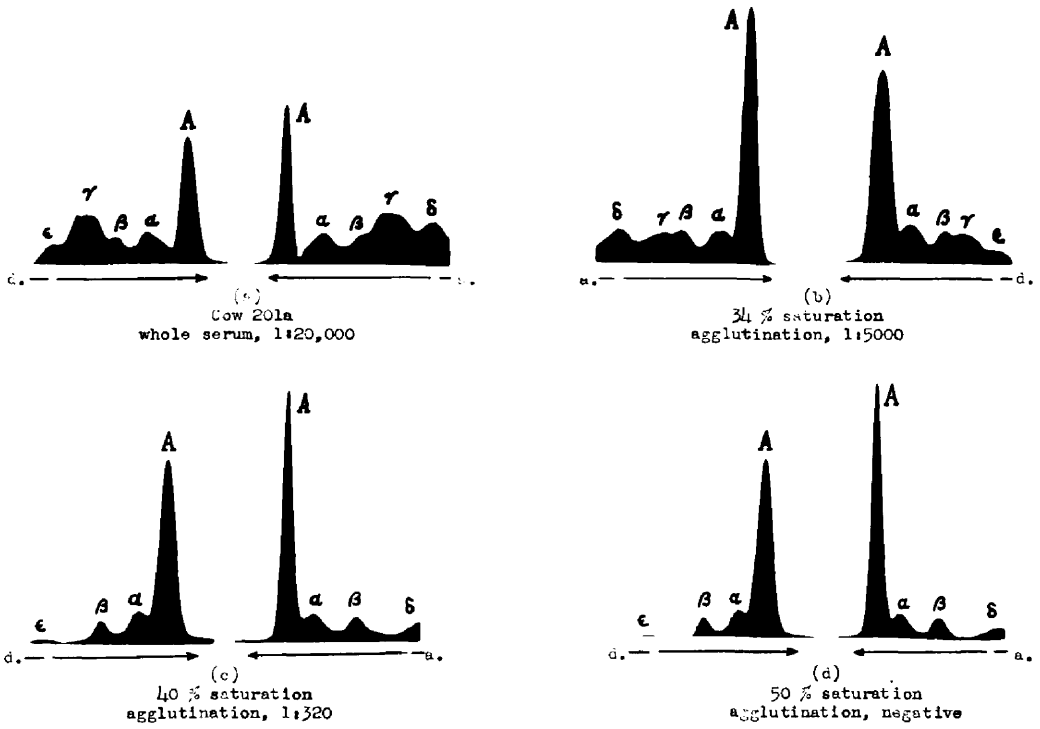


Fig. 12. Supernatants of a Brucella Antiserum Fractionated with Varying Amounts of Ammonium Sulfate.

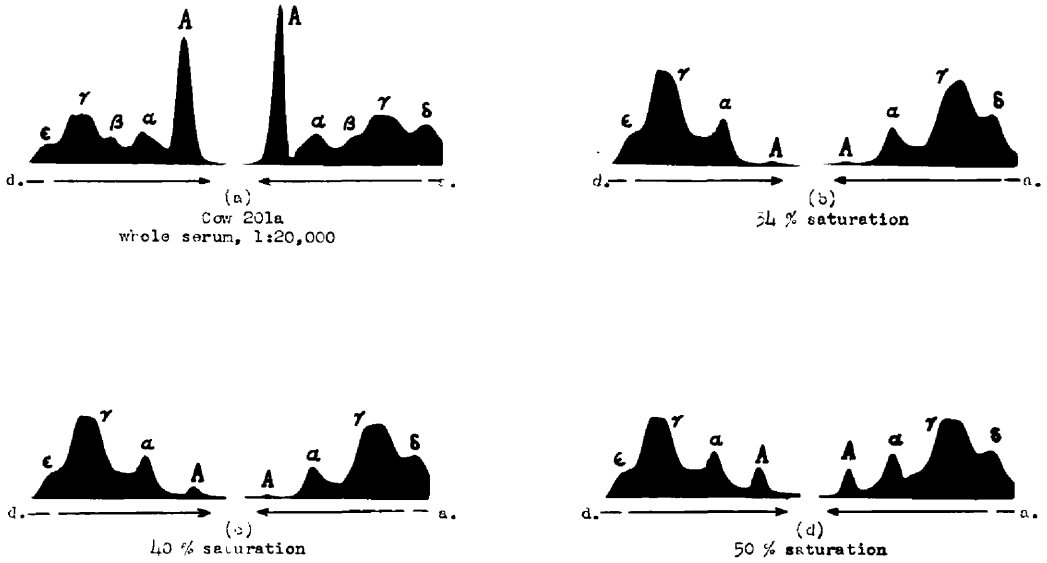


Fig. 13. Precipitates of a Brucella Antiserum Fractionated with Varying Amounts of Ammonium Sulfate.

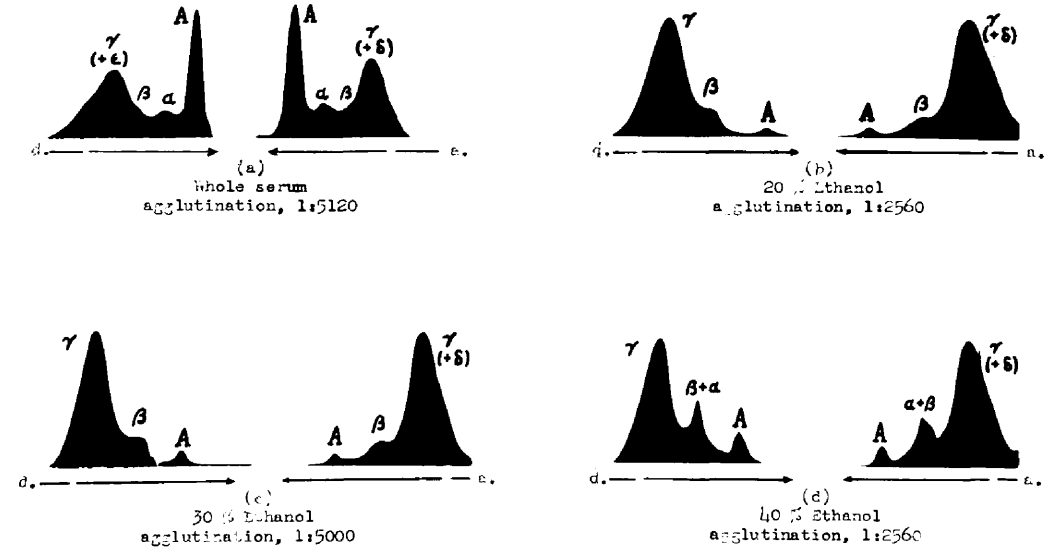


Fig. 14. Precipitates of a Brucella Antiserum Fractionated with Varying Amounts of Ethanol at 0°C.

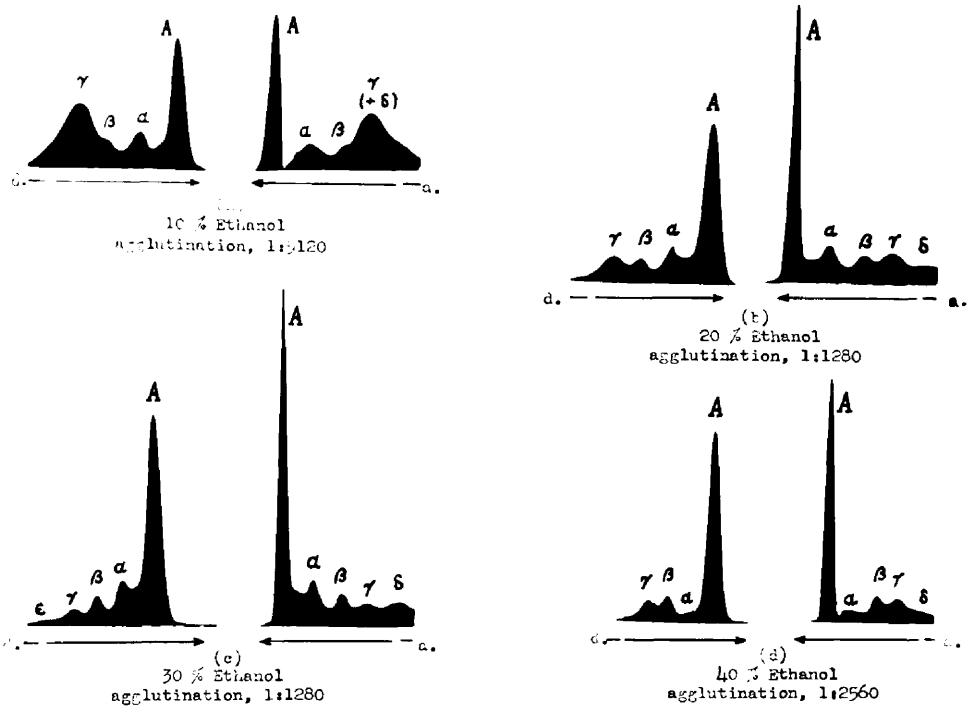


Fig. 15. Supernatants of a Brucella Antiserum Fractionated with Varying Amounts of Ethanol at 0°C.