AN IMMUNOLOGICAL STUDY OF CHICKEN SERUM ALBUMIN AND RELATED ORGAN ANTIGENS

Thesis by

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ABSTRACT

1. The percentage of the proteins in the plasma of New Hampshire Red chickens was found to increase from 2.0g to 5.8g per 100 ml., as the chickens matured.
2. The albumin/globulin ratio decreased from 0.75 to 0.25 with increasing age of the chickens.
3. The mobility of serum albumin in veronal buffer at pH 8.6, $u = 0.1$ was found to be $6.8 \pm 0.11 \times 10^{-5}$ $cm^2/volt/sec$.
4. More than 95% of the soluble proteins from livers and kidneys migrated with mobilities different from serum albumin.
5. Precipitin tests suggested that the soluble proteins of the liver include approximately one-half of one per cent homologous serum albumin, whereas the kidney does not include any serologically detectable amounts of serum albumin.
6. No measurable cross reactions occurred between rabbit anti-chicken serum albumin and the soluble proteins from chicken livers and kidneys.
7. More serum albumin was detected after the incubation of liver slices in KCl metabolite than before incubation. This suggested that a net synthesis may have occurred.
8. More serum albumin was detected in the soluble proteins within the slices after incubation in KCl metabolite to which different amounts of antiserum were added than in the controls where normal rabbit serum was present. This suggested that the antiserum inhibited the release of serum albumin from the cells.
9. The significance of these findings has been discussed.

INTRODUCTION AND REVIEW

Considering the physiological importance of the plasma proteins, their easy accessibility for study, and the number and variety of studies that have been devoted to them, one might expect that the organs involved in the fabrication of these proteins would by now have been identified with certainty. In 1939, Luck stated:

> "Indeed few problems in physiology can be in a more confused state than those concerning the origin of the plasma proteins. The field is rich in experiment but, as yet, the role of any single organ has not become clear. A priori, we might well regard the liver as an important site of formation, but experiments designed to test this hypothesis have led to conflicting results" (1).

During the years since the above statement, many data have been collected through the widespread use of the newer techniques of electrophoresis, ultracentrifugation and radio-active tracers. Progress has been rapid, and one may be optimistic enough to say that the problem is nearing a solution.

Whipple and his associates may be credited with much of the progress since 1939. (See general reviews by Madden and Whipple, 2, 3). They are responsible for the thesis of a dynamic equilibrium existing between tissue and plasma protein. A most eloquent exposition of this concept was given by Cannon (4). It implies the presence in the tissues of a large "protein pool" which is in equilibrium with the plasma protein. Depending on the demands made, tissue protein emerges from the cell to replenish the plasma protein, or the reverse can occur. The protein entering the cell is supposed to be changed in its identity, but without a fundamental cleavage into amino acids (5, 6, 7). Normal dogs were kept in nitrogen balance by oral feeding of carbohydrates and parenteral administration of homologous plasma protein. Phlorhizinized

dogs (8) were also maintained in nitrogen balance by intravenous feeding of plasma protein. No excess nitrogen or sugar was found in the urine, in contrast to the increases observed there after feeding the proteins by mouth. This suggested that the plasma protein underwent only a slight catabolic change "into large aggregates of amino acids" which were then recast into suitable cell protein.

If the breakdown of the protein were not too extensive, it is conceivable that some semblance of its intact antigenic nature might characterize its fragments. An antiserum, specific for a native circulating protein, could be employed to look for such antigenically related molecules within the cells of tissues suspected of being involved in plasma protein synthesis. An unequivocal finding of the antigen in a particular tissue would support the hypothesis of plasma protein formation there. If a positive result were obtained, what fraction or fractions of the cell would it be associated with: nuclei, mitochondria, microsomes or soluble protein? The answer to this question could conceivably add to our knowledge of protein synthesis. Furthermore, a specific antiserum would be of considerable value in analyzing the products obtained from tissue-slice experiments.

With these objectives in mind, rabbits were immunized against purified chicken serum albumin and the resulting antisera used to study the tissue distribution of materials related to this plasma protein.

The superiority of an immunological approach to the problem over a chemical one resides in the specificity of the antiserum. Employed quantitatively, it permits one to estimate, with an accuracy of about 5%, small amounts of antigen in mixtures without prior chemical fractionation. The method has been used successfully many times to analyze

biological fluids for a particular constituent (9, 10, 11, 12, 13). (See Kendall (14) for a review of the method and its applications).

At present, probably more is known about the site or sites of globulin synthesis than about any other plasma protein. For this reason, and for the reason that it has been sometimes difficult to study globulin and albumin synthesis separately, the globulins may well provide a point of departure for this discussion.

As soon as it was appreciated that antibodies were modified serum globulins, the research into the origin of antibodies took on new meaning. The immunologist had at hand a biologically active protein, and this biological activity, to a large measure, simplified the problem of detection. For reviews of the older literature, see Gay (15) and Jaffé (16).

Evidence favors the reticulo-endothelial system as the site of antibody globulin synthesis. The system is widespread in the body, including the reticulum and endothelial cells of the lymph nodes, spleen and bone marrow, the Kupffer cells of the liver and the endothelial cells of capillaries of the inner portion of the suprarenal cortex. In a wider sense, perivascular cells and mobile cells derived from the reticulo-endothelial system are also included.

Sabin (17) suggested that not all the tissues potentially capable of fabricating antibody need actually be involved in any particular instance of antibody response. Depending upon the route of injection, or infection, those portions of the reticulo-endothelial system first coming in contact with the antigen elaborate antibody. McMaster noted this phenomenon (18, 19) with regard to the regional lymph nodes draining the ears in mice and rabbits. Ehrich and Harris (20, 21)

reported that typhoid vaccine and sheep red blood cells injected into the hind foot pad of the rabbit stimulated local antibody production in the popliteal lymph node. Whether this was due to lymphocytes or plasma cells (22, 23) is still an open question. Hemolysin response to intravenous injection of sheep red blood cells is primarily of splenic origin (24, 25), although non-splenic sources also contribute. (See also De Gara, 26).

Evidence from another source is in general agreement with the above. Studies on the localization of injected antigen confirm that the reticulo-endothelial system is involved in antibody production (27, 28, 29, 30). (See Coons (31) for further references). These same tissues have been found to pick up antigen, and in some instances retain it for long periods of time. Pneumococcal polysaccharide has been detected in macrophages of the spleen, liver, and heart of mice which were injected six months previously (29). In vitro tracer studies with tissue slices obtained from rabbits immunized with formalin killed type VIII pneumococci (32) and bovine or human γ-globulin (33), suggest that the spleen is more active than the liver or the kidney in producing antibodies. Although the data are in accord with other findings, in vitro studies must be interpreted with caution. Conditions necessary for splenic elaboration of antibody may be different from those necessary to achieve similar results with other tissues. The situation is further complicated in vivo, since an hormonal mechanism may be involved in the release of antibody and probably of normal globulin as well. This controversial subject will not be discussed. The reader is referred to a review by White and Dougherty (34) and to two other papers, Fischel (35) and Eisen (36), in which both sides of the problem are presented.

Whereas globulin synthesis may occur in many different tissues, each site contributing to the total, albumin formation is attributed principally to the liver. Much of the evidence in favor of the liver comes from clinical studies. Marked changes in the plasma proteins are observed in patients with hepatic cirrhosis and hepatitis. Consistently, these diseases are associated with a hypoalbuminemia. Albumin/globulin ratios are lowered in every instance. Nevertheless, some discrepancies have been noted, particularly where hypoalbuminemia exists with no evident impairment of hepatic function. For a comprehensive review of the plasma proteins in disease, see Gutman (37).

The problem has also been attacked repeatedly from a nutritional standpoint. Proteins, protein hydrolysates and amino acids have been fed orally or administered parenterally to dogs with Eck fistulas, or made hypoproteinemic by plasmapheresis or doubly depleted by removal of both plasma and red cells (38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48). The information obtained from these studies has suggested that the liver plays a dominant role in plasma protein synthesis. Furthermore, the different digests and amino acid mixtures were found to be suitable as blood substitutes. It was observed further that different diets favored the regeneration of particular plasma proteins. For example, casein hydrolysate stimulated the production of both albumin and globulins, whereas lactalbumin favored the regeneration of albumin. Other studies wherein radioactive homologous plasma proteins were administered parenterally (5, 6) suggested that only partial catabolism occurred, followed by reassembly of the large fragments to form specific cell and tissue protein. Studies by Dent and Schilling (49) lend support to this hypothesis, while others by Abdou and Tarver do not (50, 51).

More detailed information concerning the role of proteins and protein hydrolysates in plasma protein formation can be found in two reviews (52, 53).

Perhaps one of the best studies relating the liver with plasma production was that of Miller et al. (54). Rat livers were isolated and perfused with oxygenated blood to which lysine-ϵ-C^{14} was added. Aliquots of plasma were removed at intervals up to seven hours after the addition of substrate and the samples fractionated and analyzed for radioactivity. The results showed the liver capable of synthesizing practically all the albumin and fibrinogen and probably more than 80% of the globulin proteins. As the authors pointed out, it would be of considerable significance to know whether γ-globulin was being elaborated. It would also be of significance to know whether the new plasma proteins were antigenically identical to the normal circulating proteins. Unfortunately, a serological analysis was not included.

Roberts and White (55) incubated minced rat livers in rat serum and at the end of three hours the medium was recovered by centrifugation. Electrophoretic analysis of the serum before and after incubation showed that the proteins released were mainly "α_1 and α_2" globulin. It is likely that the authors were observing tissue protein released from broken cells in the mince rather than a synthesis of plasma protein from intact cells (56, 57). Other studies by the same authors on partial hepatectomy and complete evisceration suggest that albumin falls off very rapidly in the plasma of operated animals, whereas the globulins vary little. Such experiments have not always been confirmed. Munro and Avery (58) found no marked changes in the relative concentrations of the different electrophoretic components of the plasma of

hepatectomized dogs. Cheng (59) made an exhaustive study on rabbits and rats subjected to a number of treatments known to cause liver damage. No significant changes in the plasma proteins were observed.

Tissue-slice experiments by Hoch-Ligeti (60) suggested that an albumin-like component appeared in the medium after incubation of liver slices. Information obtained from unperfused organs, however, can be misleading (see experimental section and discussion in this thesis) and should be interpreted with caution. Peters and Anfinsen (61) incubated chicken liver slices with $Na_2C^{14}O_3$. The proteins with the highest specific activities were found in the medium. Alcohol fractionation of the medium showed the greatest activity in the fraction precipitated by 33% to 43% ethyl alcohol. Since this was the optimum concentration of alcohol for serum albumin precipitation, it suggested that the "albumin" incorporated more of the C^{14} than any other protein. The 33% to 43% fraction was shown by electrophoretic and ultracentrifugal studies to be a mixture of proteins. Nevertheless, the mixture did contain a component with the physical properties of serum albumin. Further, rabbit anti-whole chicken serum precipitated 80% to 90% of the total counts in this fraction. The experiments suggested that serum albumin was produced by the liver slices and that this protein rapidly incorporated C^{14}.

It was mentioned earlier that globulin release may be under hormonal regulation. The same is probably true for albumin release (62, 63, 64). What other interrelationships exist in vivo are unknown, but one is impressed with the probability that whole series of interlocked reactions are involved, making the problem all the more difficult to solve. It has been encouraging, nevertheless, to review the great

progress that has been made in recent years.

METHOD

I. Preparation of Organ Extracts

1. Extraction of soluble protein

New Hampshire Red chickens of both sexes were used throughout these experiments. Organ extracts were obtained from young birds whose average ages were from one to two months.

A bird was placed under deep ether anesthesia and the entire sternum and most of the ribs were removed. After severing the postcaval and the two precaval veins, the left ventricle was cut and canulated. Locke's solution*, warmed to 38° C was perfused through the animal. A volume of fluid in milliliters approximately two to three times the body weight in grams was employed. The liver and kidneys were removed and placed in ice cold M/15 phosphate buffer at pH 7.8. From this point on, all operations were carried out either in an ice bath or in the cold room (+2° to +4° C). The same buffer was used throughout the entire procedure.

The organs were trimmed of surrounding fat and connective tissue while being dried slightly on filter paper. Blood vessels and connective tissue were removed by squeezing the organs in a sodium press through a fine wire mesh of approximately 1000 holes to the square inch. The mashed organs were weighed and the pulp transferred to 50 ml. centrifuge tubes. Buffer was added and the suspension centrifuged at 10,000 x g. for 5 minutes in a Sorvall angle centrifuge. The wash was discarded. Fresh buffer was added, the mash finely dispersed by a

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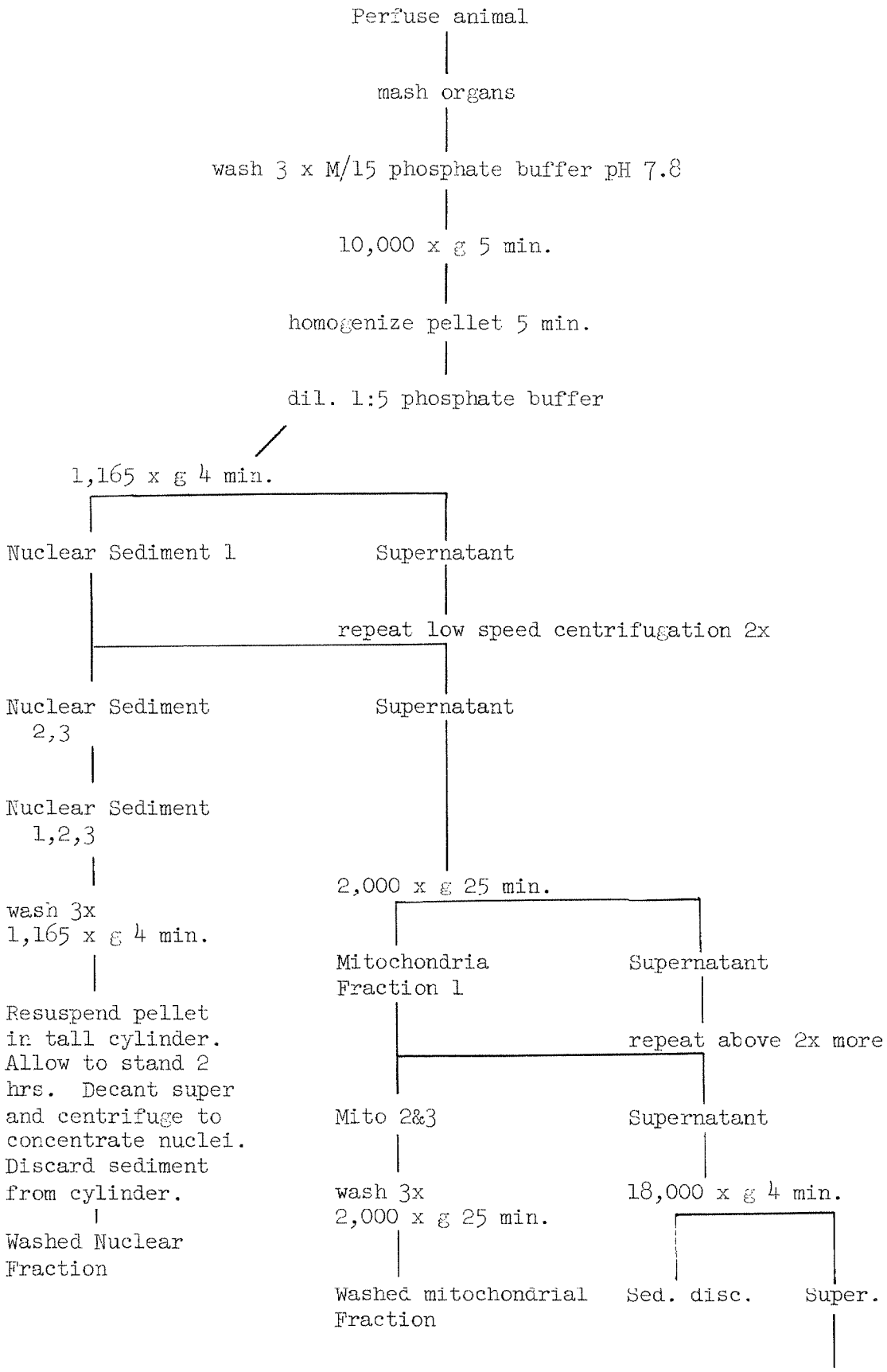
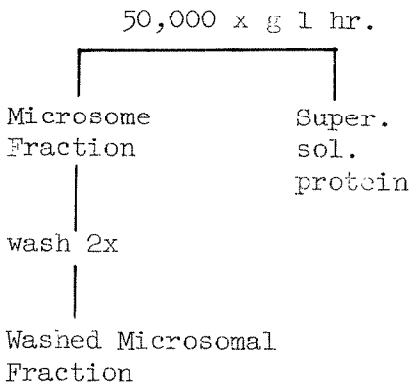
*A list of solutions will be found at the end of the methods section.

motor driven lucite pestle, and the suspension again centrifuged. In this manner the brei was washed from two to five times. The loss in nitrogen due to washing varied between one-third and one-half of the total. After the final washing, a volume of buffer approximately equal to the volume of the mash was added. The suspension, composed of intact cells and some of the heavier cell particles, was homogenized in a Potter-Elvehjem type homogenizer (65) for five minutes. The homogenates were diluted with buffer to a volume in milliliters approximately five times the mashed weight in grams for the liver and three times the weight in grams for the kidney. They were then centrifuged for 1 hour at 28,000 r.p.m. in a refrigerated Spinco preparative centrifuge. The r.c.f. in the middle of the tube was 50,000 x g. The supernatants were decanted and centrifuged again at the same r.p.m. for another hour. The final supernatants were dialyzed overnight against buffer. After dialysis, the solutions were pipetted in 1.1 ml. quantities into 1 dram shell vials, frozen and stored in a deep freeze. As needed, individual vials were thawed and used. Any unused solution was not refrozen, but was discarded. No flocculation was observed in these solutions for as long as two months.

2. Preparation of cell particles

Thoroughly washed cell particles were prepared by differential centrifugation. The procedure employed was based on the method of Claude (66). The following scheme describes the steps in such a preparation.


Perfuse animal
mash organs
wash 3 x M/15 phosphate buffer pH 7.8
10,000 x g 5 min.
homogenize pellet 5 min.
dil. 1:5 phosphate buffer
1,165 x g 4 min.
Nuclear Sediment 1
Supernatant
repeat low speed centrifugation 2x
Nuclear Sediment 2,3
Supernatant
Nuclear Sediment 1,2,3
wash 3x 1,165 x g 4 min.
Resuspend pellet in tall cylinder. Allow to stand 2 hrs. Decant super and centrifuge to concentrate nuclei. Discard sediment from cylinder.
Washed Nuclear Fraction
2,000 x g 25 min.
Mitochondria Fraction 1
Supernatant
repeat above 2x more
Mito 2&3
Supernatant
wash 3x 2,000 x g 25 min.
18,000 x g 4 min.
Washed mitochondrial Fraction
Sed. disc.
Super.



The washed particles were resuspended in the same buffer used in the preparation. Cell particles were used fresh or frozen in 1.1 ml. lots and stored in a deep freeze.

II. Fractionation of Serum Proteins

Serum albumin was prepared by the method of Adair and Robinson as given in (67) with one additional step. At the end of 5 reprecipitations the albumin fraction was dissolved in distilled water and dialyzed against distilled water to precipitate any water insoluble protein. Without this additional dialysis α-globulin continued to precipitate with the albumin, but it could be almost completely removed by this step. Salinity was restored by the addition of the calculated amount of solid sodium chloride, and the pH was adjusted to neutrality with 0.1 N NaOH. The preparations were kept in the deep freeze. After storage for as much as three months and repeated freezing and thawing, the solutions remained clear. No obvious denaturation occurred.

Blood was withdrawn from chickens by cardiac puncture. An 18 or 20 gauge needle an inch and a half long was employed. The bird was wrapped in a towel and held securely on its back by the left hand of an assistant. The head of the chicken was permitted to fall off the edge

of the table and was gently held in that position by the assistant's right hand. The triangular shaped area bordered by the clavicles and the crop was swabbed with alcohol. The crop was pushed to one side and the point of the needle inserted so that it passed between the clavicles and the coracoids into the heart. The path of the needle was parallel to the table and the long axis of the bird. It entered the heart without encountering any muscular or bony obstruction. A 50 ml. syringe was generally used with mature birds, and as much as 40 ml. has been withdrawn without anesthetic and with no ill effects to the animal. Nevertheless, the mortality to be expected with this procedure is quite high. Although no extensive data have been accumulated, deaths were in the neighborhood of 10%.

When serum was desired the blood was transferred to dry 40 ml. glass centrifuge tubes and immediately stirred with a thin hardwood applicator. Stirring was continued until after the clotting reaction was completed. In this manner between 50% and 60% of the original blood volume was recovered as serum. A negligible amount of lysis occurred. If the serum was allowed to clot without interference, approximately 1/2 of this yield was obtained. When plasma was needed, whole blood was mixed with 1/2 its volume of isotonic citrate solution. The clot and cells from serum or plasma were removed by centrifugation.

III. Electrophoresis

The electrophoretic runs were made in veronal buffer at pH 8.6 and 0.1 ionic strength as described by Longsworth (68). The protein solutions were dialyzed against three changes of buffer effecting a

final dilution of the original salt concentration of 1/20,000. Dialysis was performed in the cold room.

A Perkin-Elmer electrophoresis apparatus employing a modified Longsworth scanning method was used. Runs were made at +1° C in a 2 ml. open cell. Areas were calculated as suggested by More and White as the average of the upper and lower diagrams (69). Ascending patterns were used for both area and mobility calculations since a more accurate base line could be established with this pattern than could be done with the descending one.

IV. Tissue Slice Experiments

The bird was sacrificed by a sharp blow on the head. It was perfused with KCl metabolite in the manner already described. The organs were removed into ice cold KCl metabolite. Slices were cut with a modified Stadie slicer. The sections were placed in a 400 ml. beaker containing approximately 300 ml. of ice cold KCl metabolite. They were washed 3 times with 300 ml. quantities of ice cold KCl by swirling, allowing the sections to settle, and aspirating off the supernatant. The slices were distributed into duplicate 20 ml. reaction beakers. These beakers contained 1.0 ml. of 0.2 M $NaHCO_3$, 0.4 ml. of 0.2 M $CaCl_2$ and 3.0 ml. of KCl metabolite in the controls. The experimental beakers contained the same amounts of HCO_3^- and Ca^{++}, but in addition, different concentrations of normal rabbit serum or rabbit antiserum were added. The final volumes were brought to 4.4 ml. by adding the appropriate amount of KCl metabolite. The beakers were incubated at 38° C under an atmosphere of 95% O_2 and 5% CO_2 in a Dubnoff Metabolic Shaking Incubator for 2 hours. At the end of the

incubation time, the pH of the medium was recorded. Media from duplicate samples were pooled and made up to a final volume of 10.0 ml. Slices from duplicate beakers were also pooled, washed twice with KCl metabolite and homogenized. The crude homogenates were made up to a volume of 10.0 ml. The media and homogenates were centrifuged at 28,000 r.p.m. for 1 hour in the Spinco preparative centrifuge. The supernatants were decanted and tested for serum albumin. The zero time control consisted of slices which were not incubated but were ground up and treated in the same way as the experimental ones. All samples were analyzed for total protein, total soluble protein, and total protein in the medium by trichloracetic acid precipitation.

V. Protein and Nitrogen Analysis

Protein solutions were precipitated with an equal volume of 16% trichloracetic acid at room temperature. The precipitates were allowed to stand over night. The following day they were washed three times with 8% T.C.A. and the pellets quantitatively transferred to tared beakers. The precipitates were dried in an oven at 104° C until they maintained a constant weight. Nitrogen was determined with Nessler's reagent prepared according to the formula of Koch and McMeekin as found in (70).

VI. Antiserum Analysis

1. Precipitin tests

Precipitin tests were performed in 15 x 100 mm. centrifuge tubes. The combined solutions were made up to a total volume of 2.0 ml., unless otherwise stated. The stoppered tubes were incubated

in a water bath at 37° C for 1 hour and then refrigerated for 48 hours. At the end of this interval the precipitates were centrifuged in the cold room and washed three times with ice cold saline. Three ml. of saline were used for each washing. After the final wash, 0.5 ml. of concentrated low nitrogen H_2SO_4 was added to each tube and the precipitates digested over a low flame for 1 1/2 hours. After clearing the brown solutions with superoxol, the contents of each tube were quantitatively transferred with distilled water into 50 ml. Folin tubes. One ml. of 5 N NaOH and enough distilled water were added to each tube to bring the volume to 35 ml. 15 ml. of Nessler's reagent was added, the solution stirred and read in a Klett-Summerson colorimeter with a number 42 blue filter (400-465 mu) twenty minutes later. The data were analyzed by the method of Heidelberger and Kendall (71). (See Kendall (72) for a comprehensive treatment of the equations).

2. Complement fixation tests

Complement fixation was carried out according to the procedures given by Kabot and Mayer in Experimental Immunochemistry (67). The 100% lytic end point was used.

VII. Immunization and Bleeding Schedule

Six rabbits were immunized against chicken serum albumin. Small concentrations of protein were injected on alternate days. The first dose of antigen was given subcutaneously, and the succeeding ones administered intravenously. Six to nine injections were given before trial bleedings were begun. Reimmunization was accomplished by means of one subcutaneous, one intraperitoneal and one intravenous injection. The

animals received a total of between 15 and 70 mg. of protein. Two rabbits were immunized against alum precipitated serum albumin. The remaining four received the native protein.

Trial bleedings were made daily beginning the second day after the last injection. A small sample of blood, usually one to two ml., was obtained from a cut in the marginal ear vein. The serum was tested by adding it to different concentrations of antigen in 4 mm. capillary tubes and recording the flocculation times with a stop watch. The most rapid flocculation times were observed to occur with sera from trial bleedings taken between the third and fifth day after the last injection. By the sixth day, the titers had fallen off. Routinely, the animals were bled by cardiac puncture on the fourth and fifth days. A total of 100 ml. of blood was obtained from each rabbit in the two days of bleeding. Complement was inactivated by incubating the sera at 56° C for 20 minutes.

Solutions Used

Phosphate Buffer

Na_2HPO_4	9.474 g./L.
KH_2PO_4	9.078 g./L.

Volume of Na_2HPO_4	Volume of KH_2PO_4	pH of mixture
9.25	0.75	7.8

Locke's Solution

NaCl	9.0 g.
KCl	0.42 g.

Locke's Solution (Cont.)

$CaCl_2$	0.24 g.
$NaHCO_3$	0.20 g.
H_2O	1 Liter

Veronal Buffer

Barbital	22.083 g.
NaOH	4.000 g.
H_2O	to 1 Liter
pH at 20° C	8.6

KCl Metabolite

KCl	9.0 g.
Glutamic acid	0.735 g.
$MgCl_2 \cdot 6H_2O$	0.203 g.
K_2HPO_4	0.174 g.
H_2O	1 Liter

Adjust pH to 7.8 with 10 N KOH

Citrate Solution

Sodium citrate	20.0 g.
NaCl	5.0 g.
H_2O	1 Liter

EXPERIMENTAL

I. Electrophoretic Analysis of Chicken Plasma and Purified Serum Albumin

Chicken plasma was analyzed electrophoretically for two reasons, (a) its pattern would serve as a reference for those obtained from the soluble organ extracts, (b) a value for the mobility of the albumin component could be determined.

Deutsch (73) had reported that serum albumin in White Leghorn hens had a mobility different from that found in roosters. It was important to ascertain whether this phenomenon also occurred in New Hampshire Reds. Accordingly, runs were made on plasma of both sexes of New Hampshire Reds. No differences in the serum albumin mobilities of male and female birds were demonstrated.

Qualitative and quantitative changes were observed, however, in these patterns as the birds matured. Furthermore, the changes were associated with an increase in total plasma protein. These observations confirm a study by Brandt et al. (74) in which a more extensive analysis was undertaken.

Figures 1 and 2 show the patterns obtained with young and adult plasma of the two sexes. The most obvious change with maturity is the relative increase in the total globulins in comparison to the albumin. According to Brandt, this is an absolute increment as well. A fast moving component (F) migrating ahead of the albumin can be observed in the adult female pattern. It is most pronounced on the ascending side. This component, obvious only in laying hens, may be associated with egg production. Roepke and Bushnell (75) were able to demonstrate a

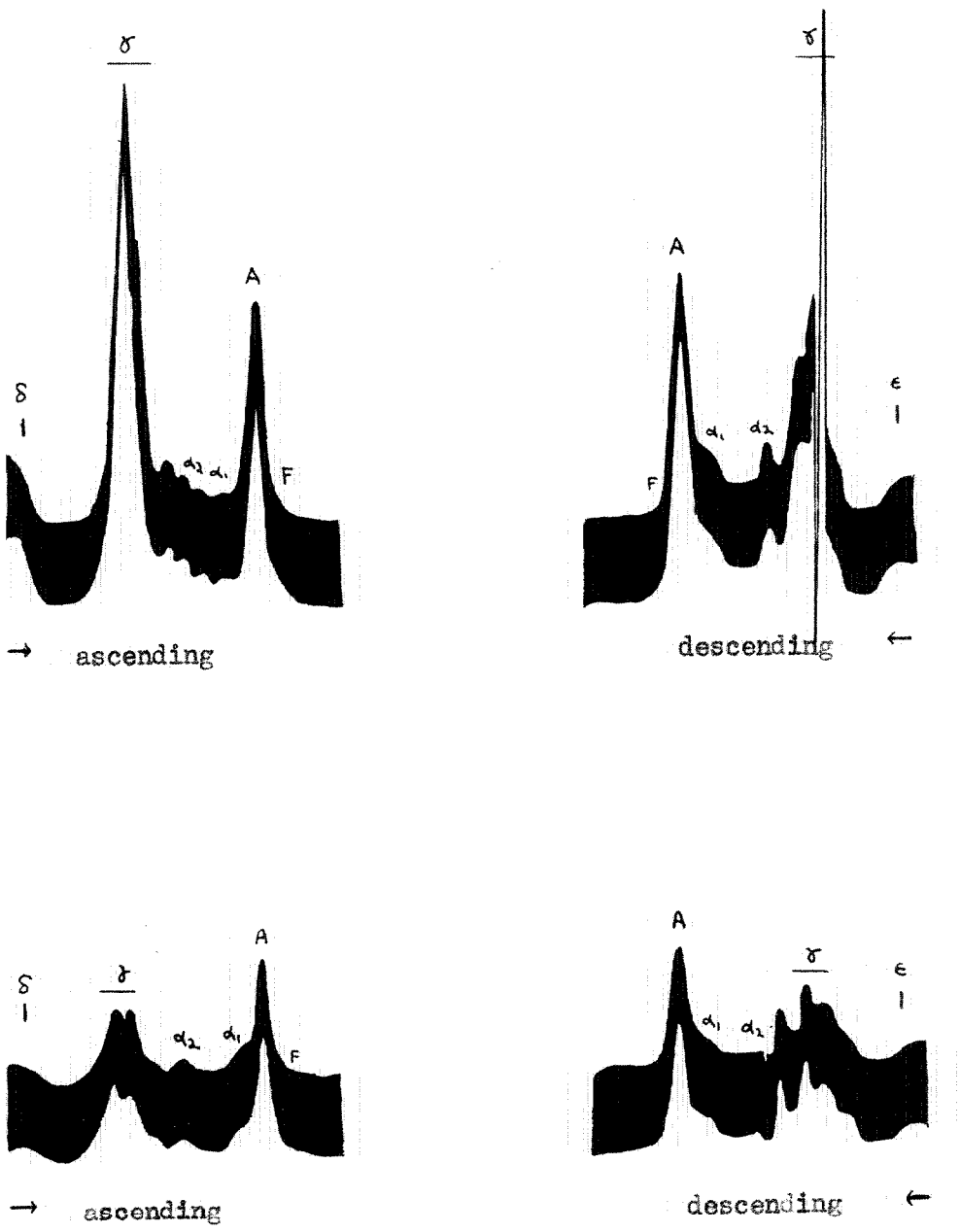


Fig. 1. Electrophoretic patterns of hen plasma in veronal buffer, pH 8.6, 0.1 u, after 7,200 sec. Upper, mature hen, protein conc. 2.9%. Lower, young hen, protein conc. 1.5%.

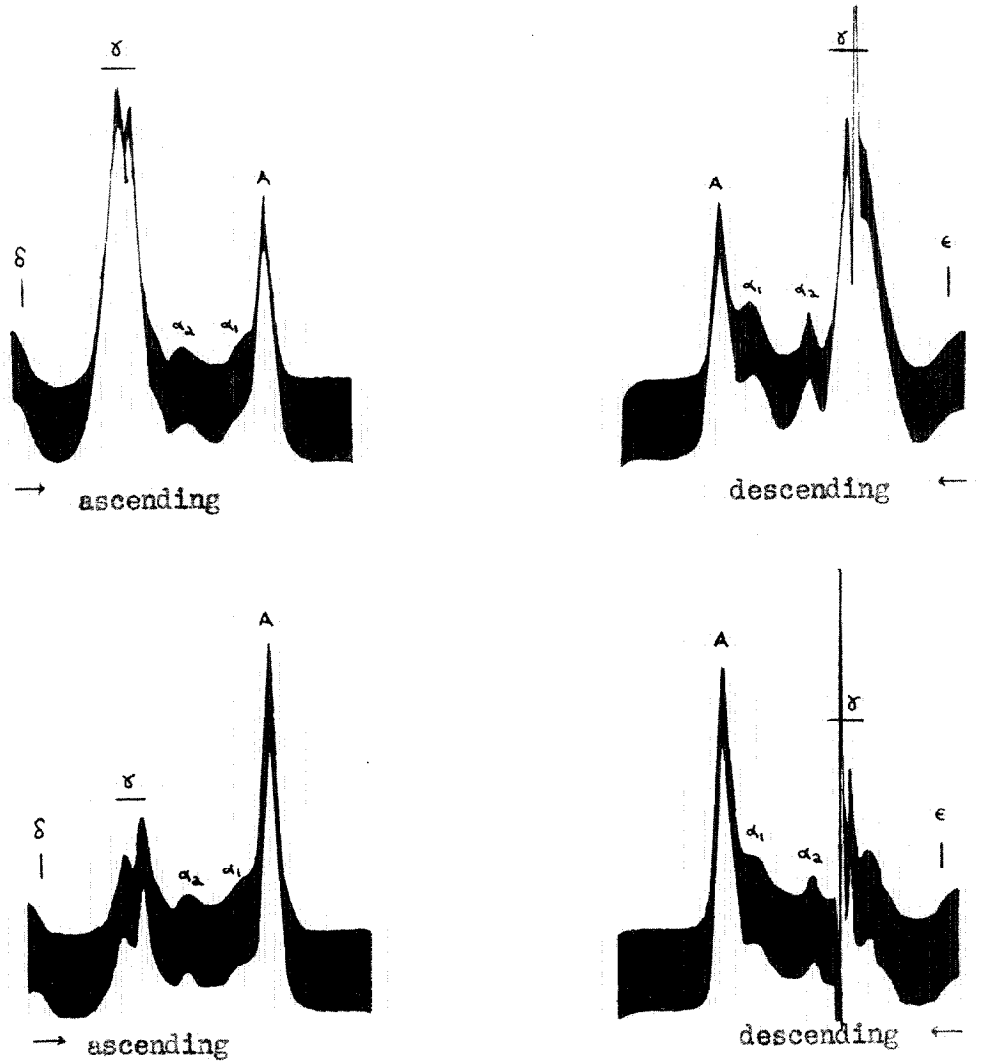


Fig. 2. Electrophoretic patterns of rooster plasma in veronal buffer, pH 8.6, 0.1 u, after 7,200 sec. Upper, mature male, protein conc. 2.9%. Lower, young male, protein conc. 1.5%.

difference between antisera produced against laying female serum and adult male serum in their reactions with vitellin. Anti-female sera precipitated vitellin whereas anti-male sera did not. Whether these reactions are due to antibodies against the F component is not known.

Table 1 gives a summary of the electrophoretic data obtained.

TABLE 1

Electrophoretic Analysis of Chicken Plasma

	Mobilities x 10^{-5} cm^2/volt/sec.					% Alb. of total protein	mg. Total protein ml.
	Globulins						
	γ^*	α_2	α_1	Alb.	F		
male mature	2.8	4.6	6.1	6.9		20	58
male young 2 mo.	2.7	4.2	5.7	6.6		43	30
female mature	2.8	4.3	5.9	6.9	7.6	24	49
female young 6 wks.	2.7	4.6	6.3	7.0		37	32

$^*\gamma = \gamma + \phi + \beta$

Mobility and area measurements were made on three times enlargements, a representative diagram of which appears in Figure 3. The protein concentrations and per cent albumin are similar to those found by Sanders, et al. (76) and the mobilities compare favorably with those obtained by Deutsch and Goodloe with White Leghorns (73).

Figure 4 shows the pattern obtained from a purified preparation of serum albumin. The mean mobility from seven preparations was 6.8 x 10^{-5} cm^2/volt/sec. with a standard deviation of $\pm$ 0.11. Figure 5 shows an enlargement from which the mobility and area measurements were made. The albumin was never obtained in crystalline form, although Laskowski had reported crystallizing chicken serum albumin (77).

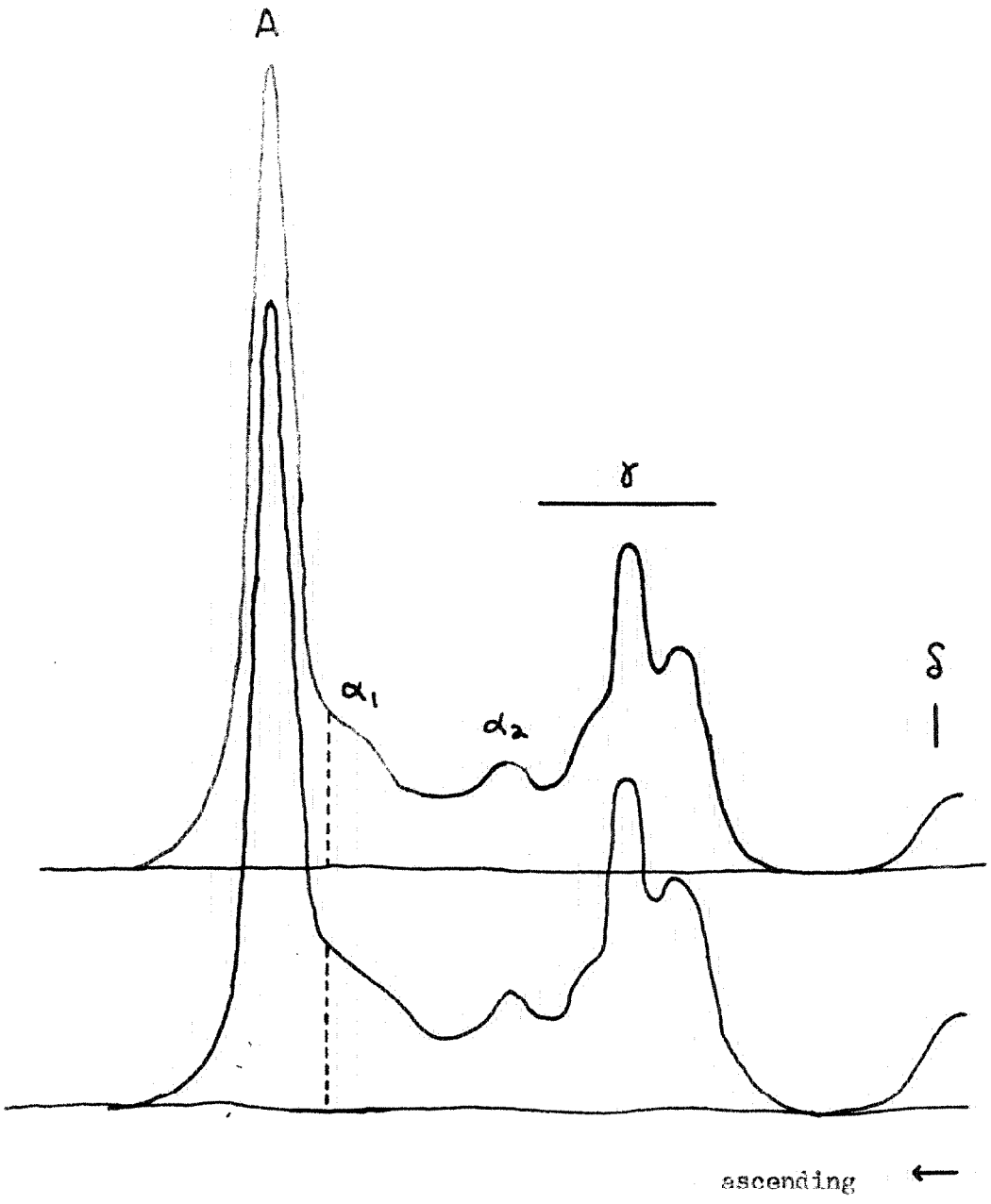


Fig. 3. Ascending electrophoretic pattern of young male plasma in veronal buffer, pH 8.6, 0.1 u, after 7,200 sec. Enlarged 3x.

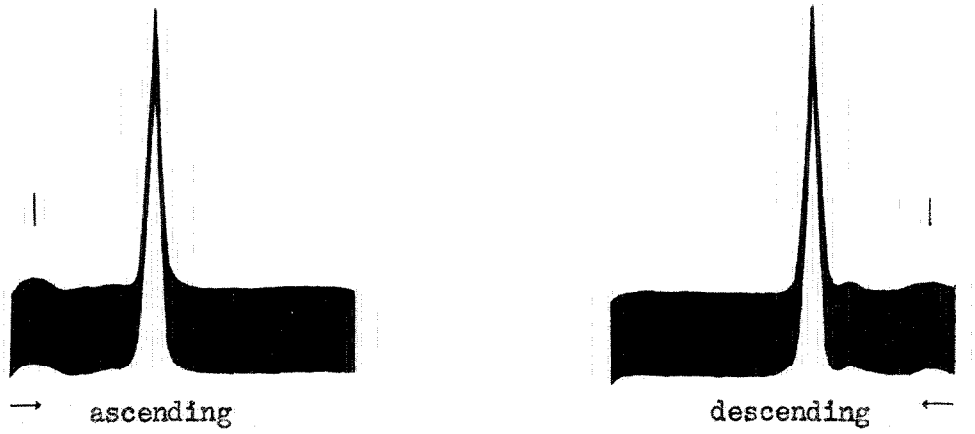


Fig. 4. Electrophoretic pattern of 5x reprecipitated serum albumin in veronal buffer, pH 8.6, 0.1 u, after 3,600 sec. Protein conc. 0.5%.

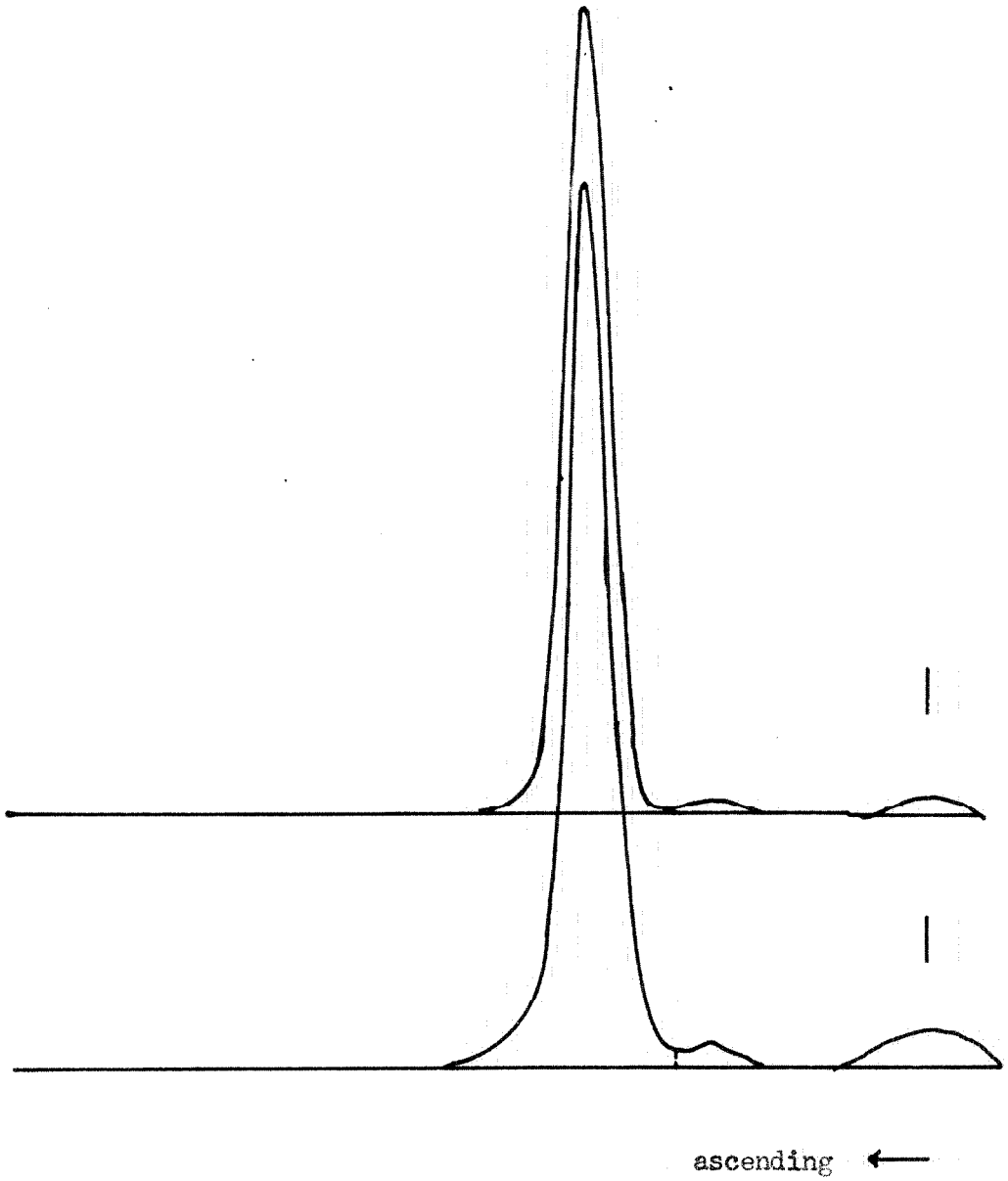


Fig. 5. Electrophoretic pattern of 5x reprecipitated serum albumin in veronal buffer, pH 8.6, 0.1 u, after 3,600 sec. Protein conc. 0.5%. Enlarged 3x.

II. Electrophoretic Analysis of Liver and Kidney Soluble Extracts

Although considerable effort has been directed to the study of enzymes and enzyme systems extractable from organs such as the liver, few detailed studies have been reported concerning the physicochemical properties of the soluble protein extracts (78, 79, 80, 81, 82, 83, 84). For fuller discussions of the problem the reader is referred to two reviews (85, 86).

The present study does not attempt a complete characterization of the soluble organ extracts. The extracts were not fractionated. No stability studies of the different components were made. Only one solvent was used for extraction, and electrophoretic runs were made in only one buffer and at one pH. For the results of different treatments see the references cited.

Primarily, the electrophoretic analyses served as an independent assay for serum albumin in the different preparations. The amounts detected by this method were compared later with the immunological data.

Figure 6 shows the electrophoretic diagrams obtained from twice washed liver and kidney soluble extracts. (See methods). The similarity of the patterns, particularly in the ascending limb, is most striking. The two large, poorly resolved components labeled 2 and 3 in Figure 7 constitute approximately 90% of the total protein in each organ. The mobilities of the arbitrarily designated points are given in Table 2. Calculations were made from the enlarged Figure 7. The mobilities are considered no more than orders of magnitude. The slowest moving components may contain some free hemoglobin. The proteins migrating ahead of A may be nucleoprotein, since both

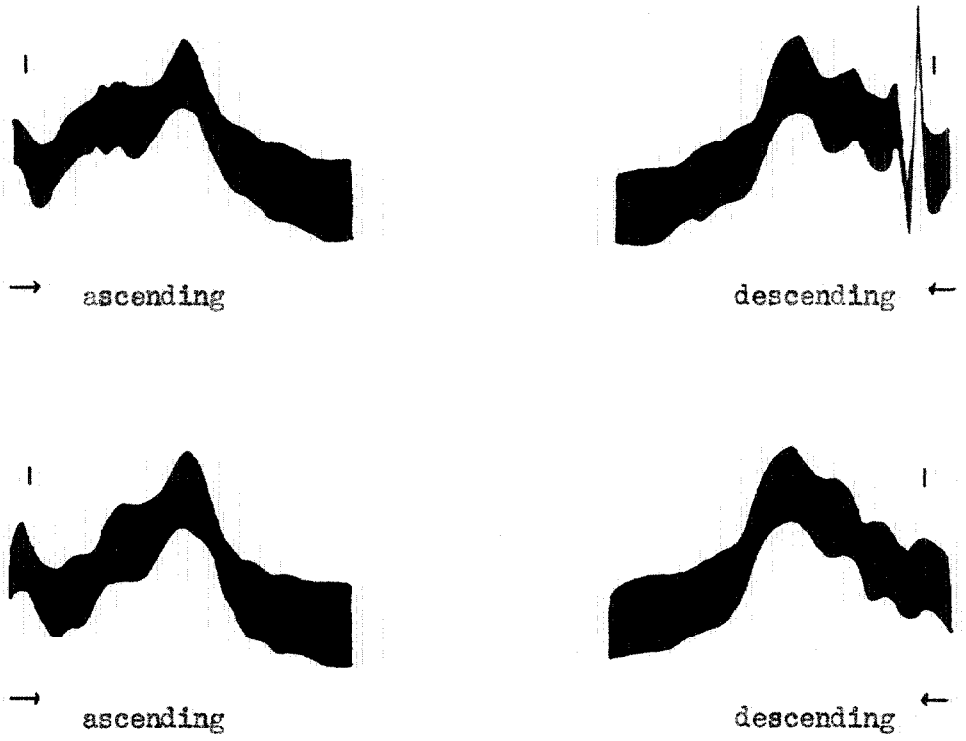


Fig. 6. Electrophoretic patterns of the soluble proteins obtained from chicken livers and kidneys in veronal buffer, pH 8.6, 0.1 u, after 7,200 sec. Upper, liver. Protein conc. 3.2%. Lower, kidney. Protein conc. 2.7%.

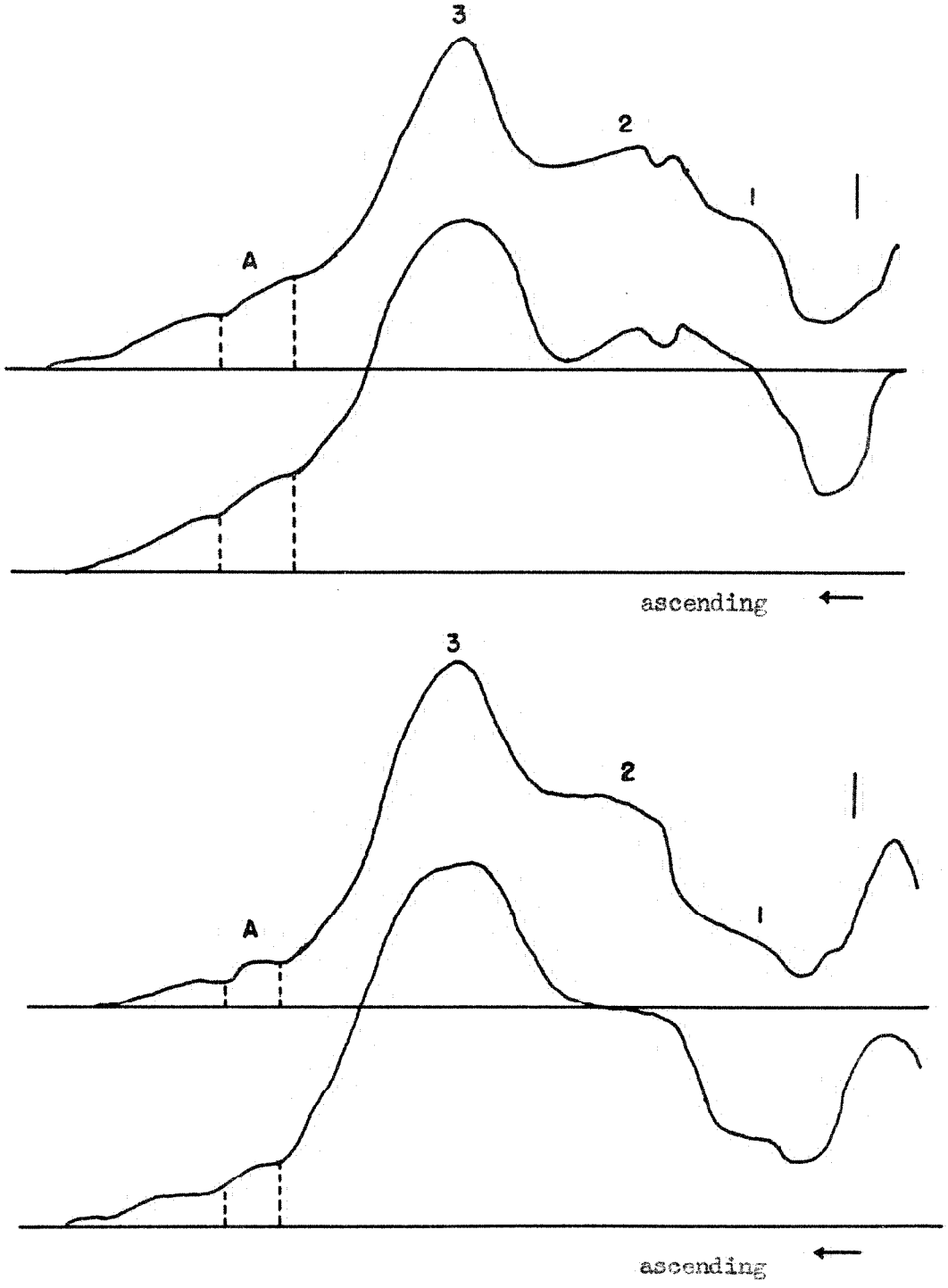


Fig. 7. Ascending electrophoretic patterns in veronal buffer, pH 8.6, 0.1 u, after 7,200 sec. Upper, liver. Lower, kidney. Enlarged 3x for mobility and area calculations.

extracts gave faint but positive Dische tests (87).

TABLE 2

Electrophoretic Analysis of Liver and Kidney Supernatant Fluids*

Arbitrary components	u x 10^{-5} cm^2/volt/sec.	% of Total	
		Liver	Kidney
1	1.5		
2	2.5		
3	4.5		
A	6.8	4.6	2.3

*See Fig. 7

The anomaly found on the descending side in the liver was consistent for the two chickens so examined. It was not observed in the kidneys of these same birds.

Component A was located by drawing a line through the patterns at the point where free chicken serum albumin would migrate. Since the line fell at places that suggested small peaks, the areas under these peaks were calculated. Such small concentrations of what might be serum albumin have also been found by electrophoretic analysis of rabbit and rat liver soluble protein (83, 84). This subject will be taken up in detail in later sections.

III. Antisera Analyses

An antiserum is described as specific if it reacts with its homologous antigen, but fails to do so with a variety of heterologous materials. This ability to discriminate among antigens is not an attribute

of every antiserum. Whether such an antiserum can actually be obtained is questionable. (See Landsteiner (88) for specificity studies with chemically defined haptens). Nevertheless, specific antisera were desired for this study.

The serum albumin was known from electrophoretic analysis to be contaminated. It also proved to be inhomogeneous by immunological criteria (67, 89, 90, 91, 92). With this in mind the total amount of antigen injected was small, and reimmunizations were kept at a minimum (93, 94, 95, 96).

Two sources of error were disregarded in the analyses of the precipitin reactions, (a) the concentration of the contaminant in the albumin preparations, and (b) the amount of complement remaining after heat inactivation which could have combined with the specific precipitates (97). Both errors were calculated as too small to be detected by the methods employed.

Tables 3-6 give the data obtained in the precipitin analysis of rabbit antisera CA1 course 1, CA1 course 2, CA5 and CA6. The data are graphically represented in Figures 8-15.

TABLE 3

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum†

Rabbit CA1 course 1 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Antibody N pptd. calc.* ug.	Tests on super.
6.8	87.2	80.4	11.8	79.4	B excess
11.4	128.0	117.0	10.2	118.0	B excess
16.0	158.0	142.0	8.9	144.0	No B; no Sa
22.8	187.0	164.0	7.2	165.0	No B; no Sa
34.0	128.0	(84.0)			Sa excess
56.8	55.8				Sa excess
79.6	46.4				Sa excess
114.0	46.0				Sa excess

†See Figures 8 and 9.

SaN = serum albumin nitrogen

BN = antibody nitrogen

B = antibody

Sa = serum albumin

*A plot of BN/SaN vs. SaN added throughout the region where all of the SaN added is precipitated may be described by the equation for a straight line.

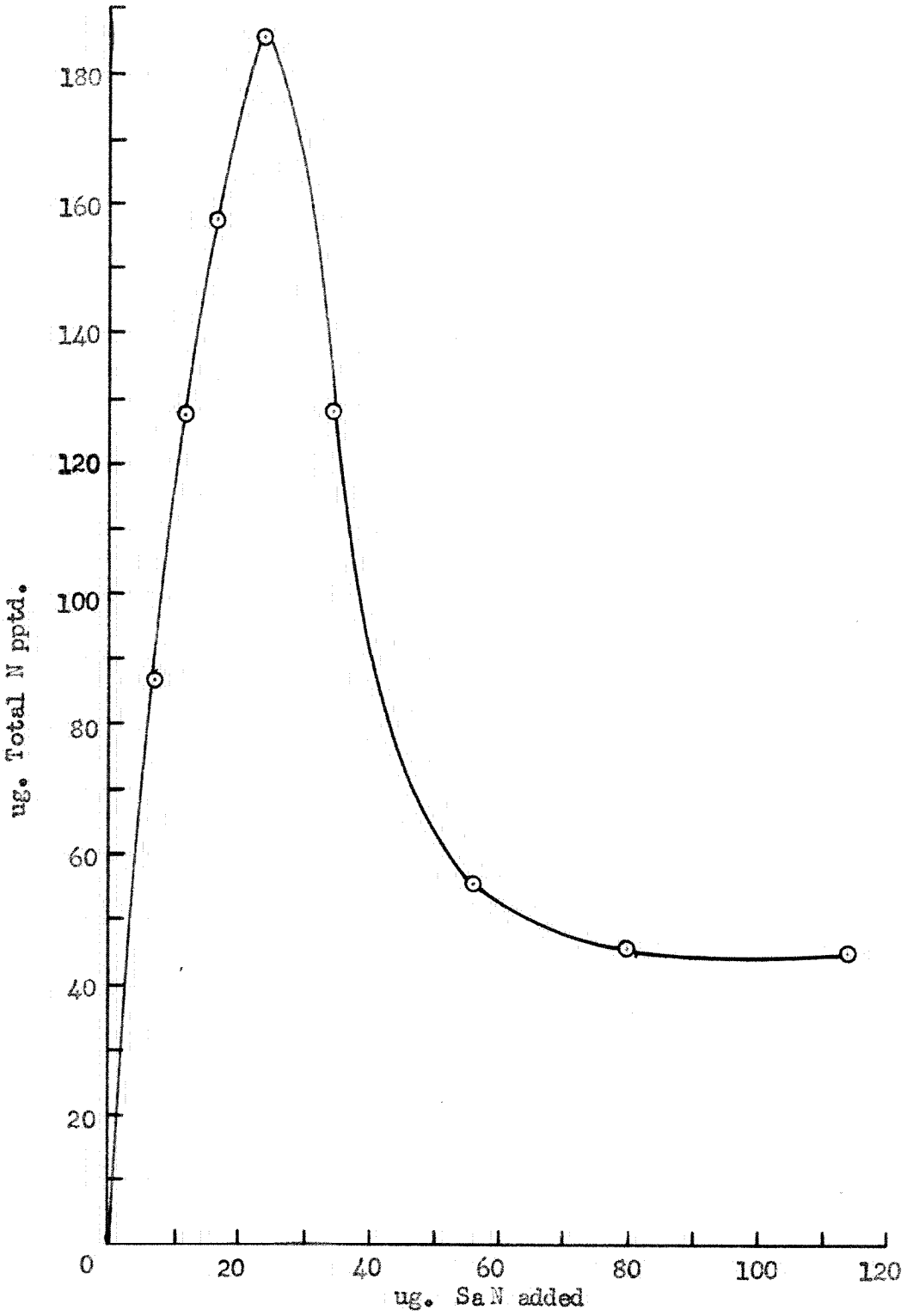


Fig. 8. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA1 course 1.

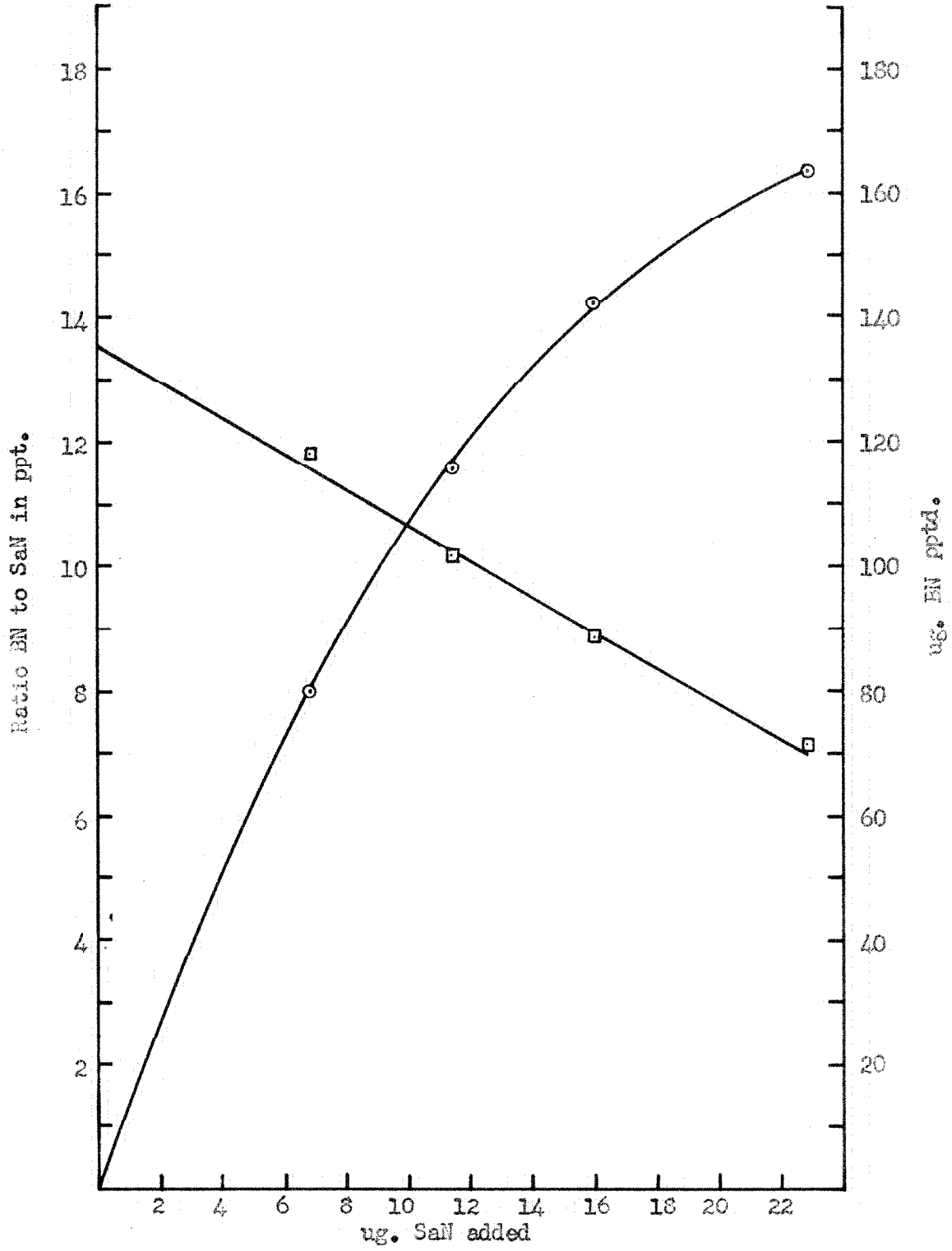


Fig. 9. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA1 course 1.

$$BN/SaN = a - b\ SaN \qquad (1)$$

where a = ordinate intercept

b = slope

Multiplying both sides of (1) by SaN gives an equation of quadratic form from which the amount of antibody nitrogen precipitated can be calculated.

$$BN = a\ SaN - b(SaN)^2 \qquad (2)$$

The constants a and b can be evaluated from the straight line in Figure 9. Substituting these values into equation (2) yields:

$$BN = 13.5\ SaN - 275\ (SaN)^2 \qquad (3)$$

SaN max. is calculated by differentiating equation (3) with respect to SaN and setting the first derivative equal to 0.

$$\frac{d\ (BN)}{d\ (SaN)} = 13.5 - 550\ SaN \qquad (4)$$

$$SaN = \frac{13.5}{550}$$

$$SaN\ max. = 0.024\ mg.$$

By substituting 0.024 mg. for SaN in equation (3) BN max. is calculated:

$$BN\ max. = 13.5\ (0.024) - 275\ (0.024)^2$$

$$BN\ max. = 0.166\ mg.$$

The Heidelberger and Kendall equation (71) is also of quadratic form.

$$BN = 2\ R\ x - \frac{R^2}{A}\ x^2 \qquad (5)$$

where BN = antibody nitrogen in mg.

A = maximum antibody nitrogen in mg. precipitated at the equivalence point.

R = ratio of A to mg. antigen nitrogen precipitated at the equivalence point.

x = antigen nitrogen in mg.

Equation (2) is made identical to equation (5) by letting $a = 2R$ and $b = \frac{R^2}{A}$.

TABLE 4

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum †

Rabbit CA1 course 2 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Antibody N pptd. calc.* ug.	Tests on super.
4.8	67.6	56.8	11.8	57.6	B excess
9.6	112.0	102.0	10.6	101.0	B excess
14.4	139.0	125.0	8.7	129.0	B excess
19.2	160.0	141.0	7.3	145.0	tr. B
24.0	59.4				Sa excess
48.0	38.4				Sa excess
96.0	38.4				Sa excess

*$BN = 13.5\ SaN - 313\ (SaN)^2$

BN max. = 146 ug./ml.

SaN max. = 21.6 ug./ml.

†See Figures 10 and 11.

The symbols in this table and in all of the subsequent tables are the same as those explained under Table 3.

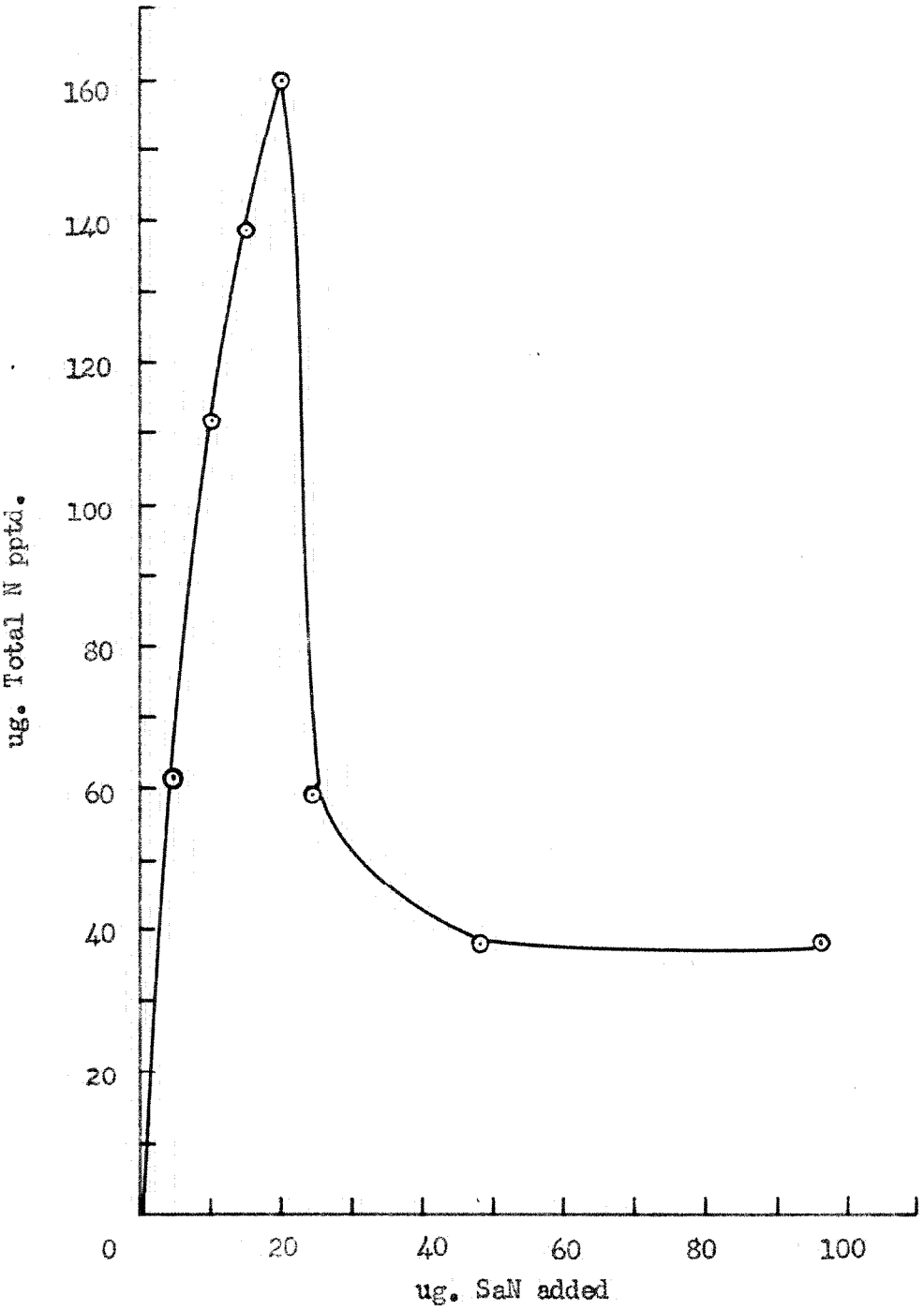


Fig. 10. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CAl course 2.

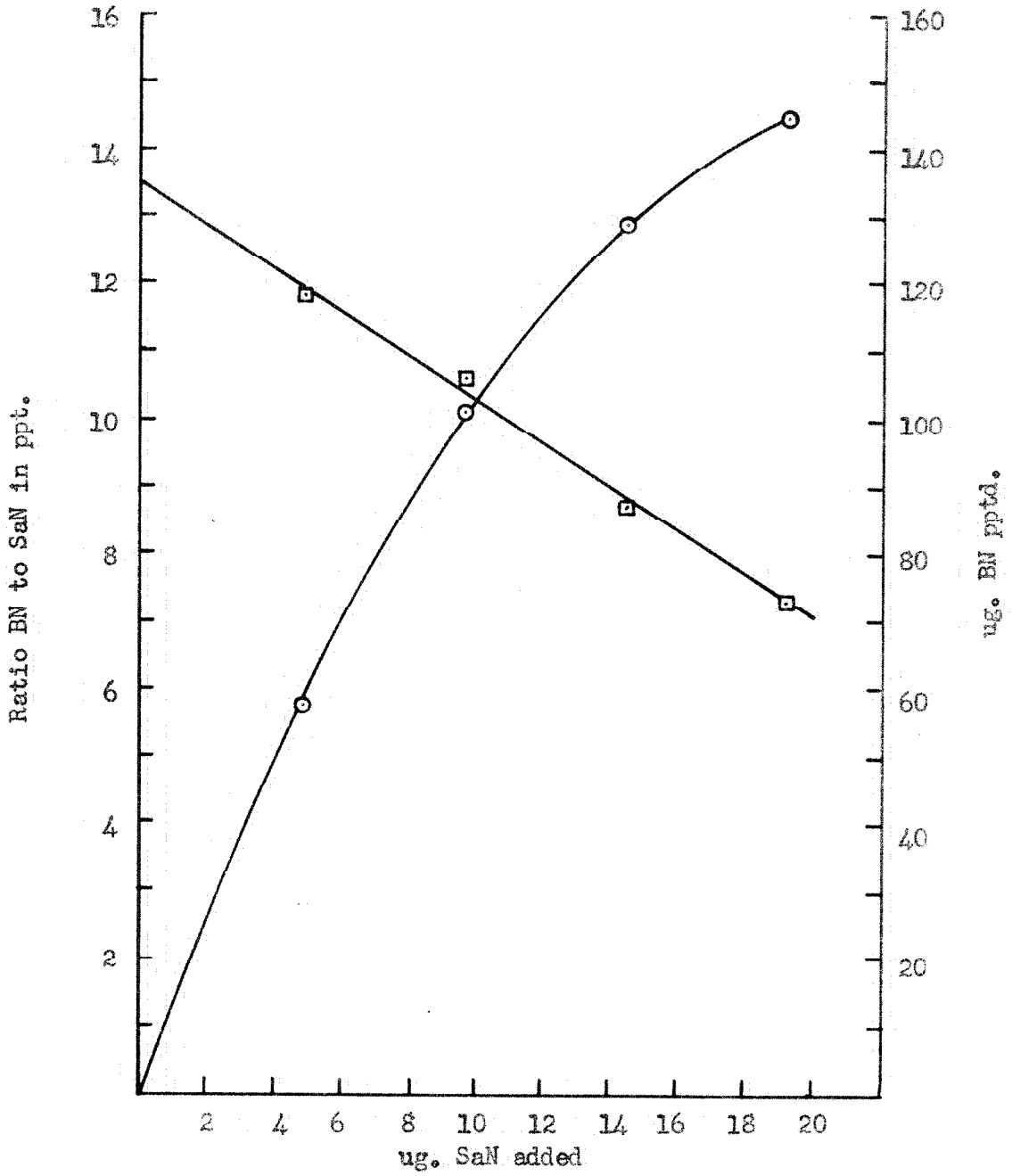


Fig. 11. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA1 course 2.

TABLE 5

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum†

Rabbit CA5 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Antibody N pptd. calc.* ug.	Tests on super.
4.9	59.6	54.7	11.4	58.0	B excess
7.4	86.4	79.0	10.7	79.2	B excess
12.2	122.0	110.0	9.0	109.0	B excess
17.0	135.0	118.0	6.9	119.0	No B; no Sa
24.4	139.0	(115.0)			tr. Sa
38.6	94.8				Sa excess
48.8	77.4				Sa excess
72.2	61.6				Sa excess
122.0	60.4				Sa excess

*$BN = 13.5\ (SaN) - 380\ (SaN)^2$

BN max. = 120 ug./ml.

SaN max. = 17.8 ug./ml.

†See Figures 12 and 13.

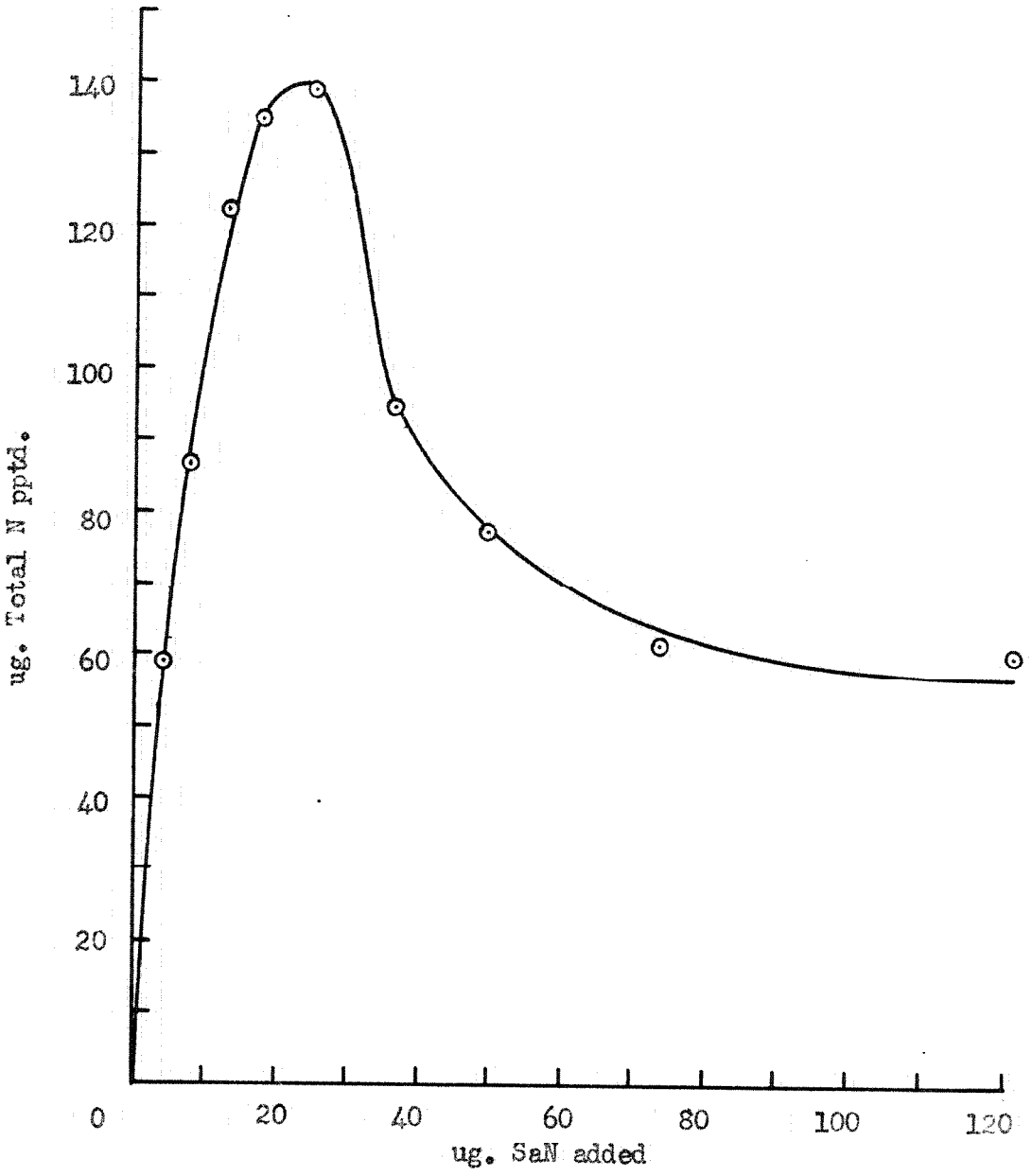


Fig. 12. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA5.

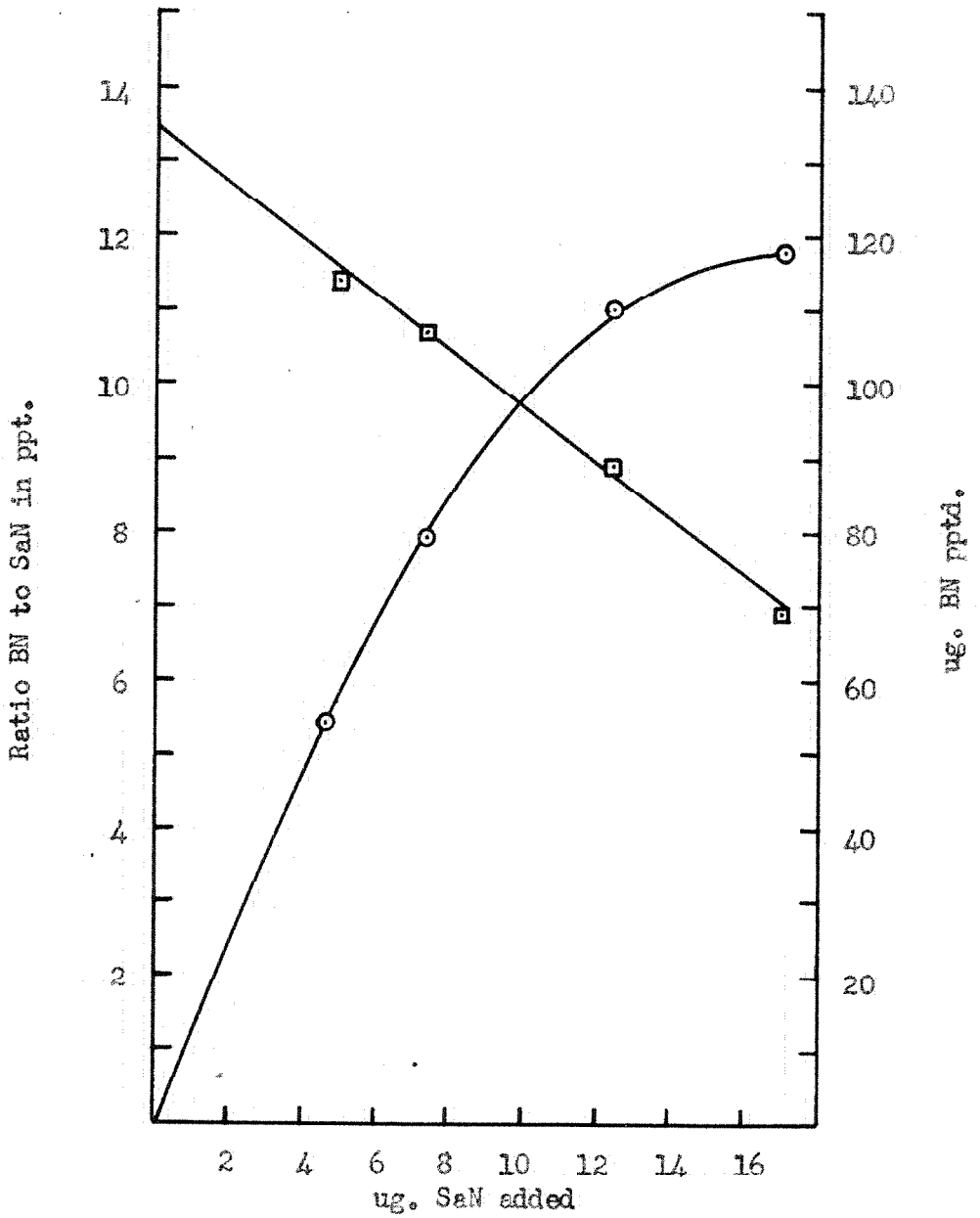


Fig. 13. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA5.

TABLE 6

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum†

Rabbit CA6 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Antibody N pptd. calc.* ug.	Tests on super.
4.8	53.0	49.1	10.2	51.2	B excess
7.2	72.6	65.4	9.1	71.0	B excess
12.0	89.0	77.0	6.3	77.0	no B; no Sa
16.8	76.8	(60.0)			tr. Sa
24.0	56.2				Sa excess
36.0	48.8				Sa excess
48.0	41.4				Sa excess
72.0	35.8				Sa excess

*$BN = 13.5\ SaN - 590\ (SaN)^2$

BN max. = 77.4 ug./ml.

SaN max. = 11.4 ug./ml.

†See Figures 14 and 15.

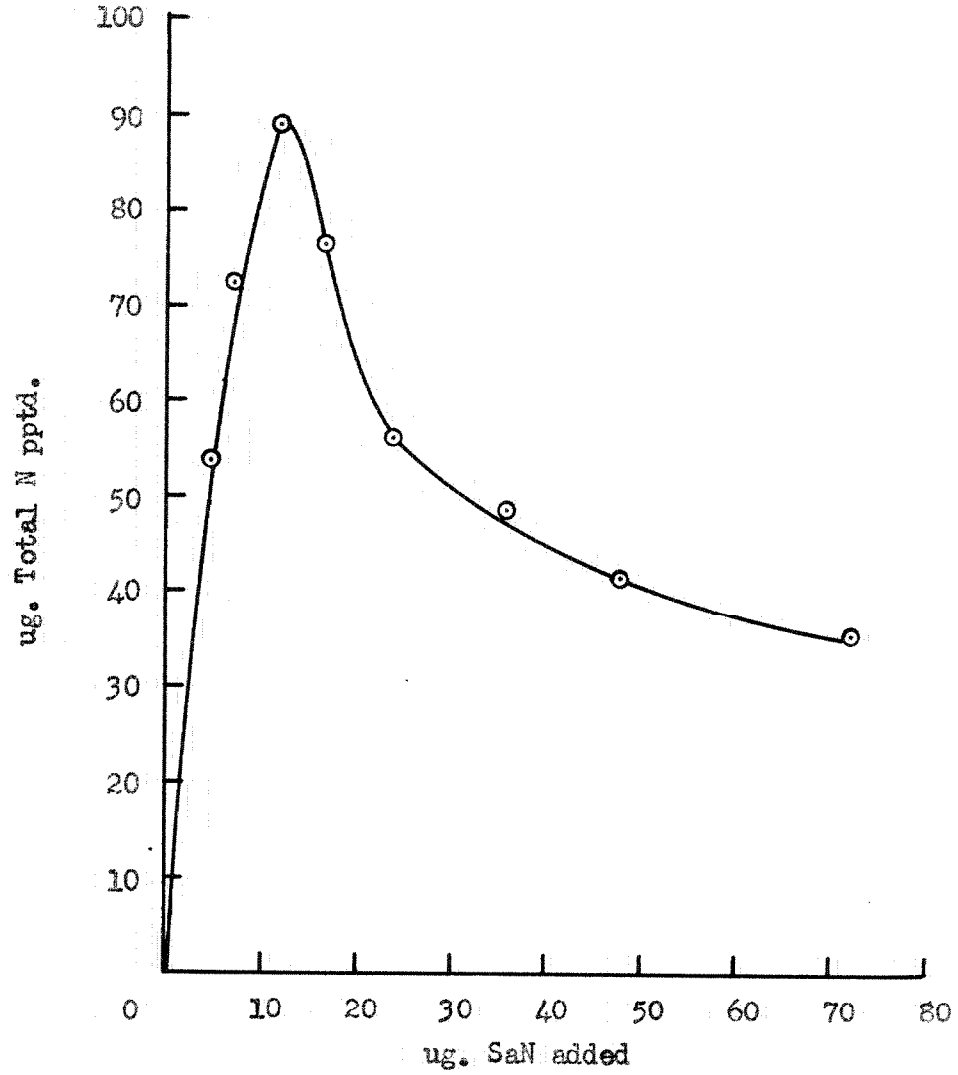


Fig. 14. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA6.

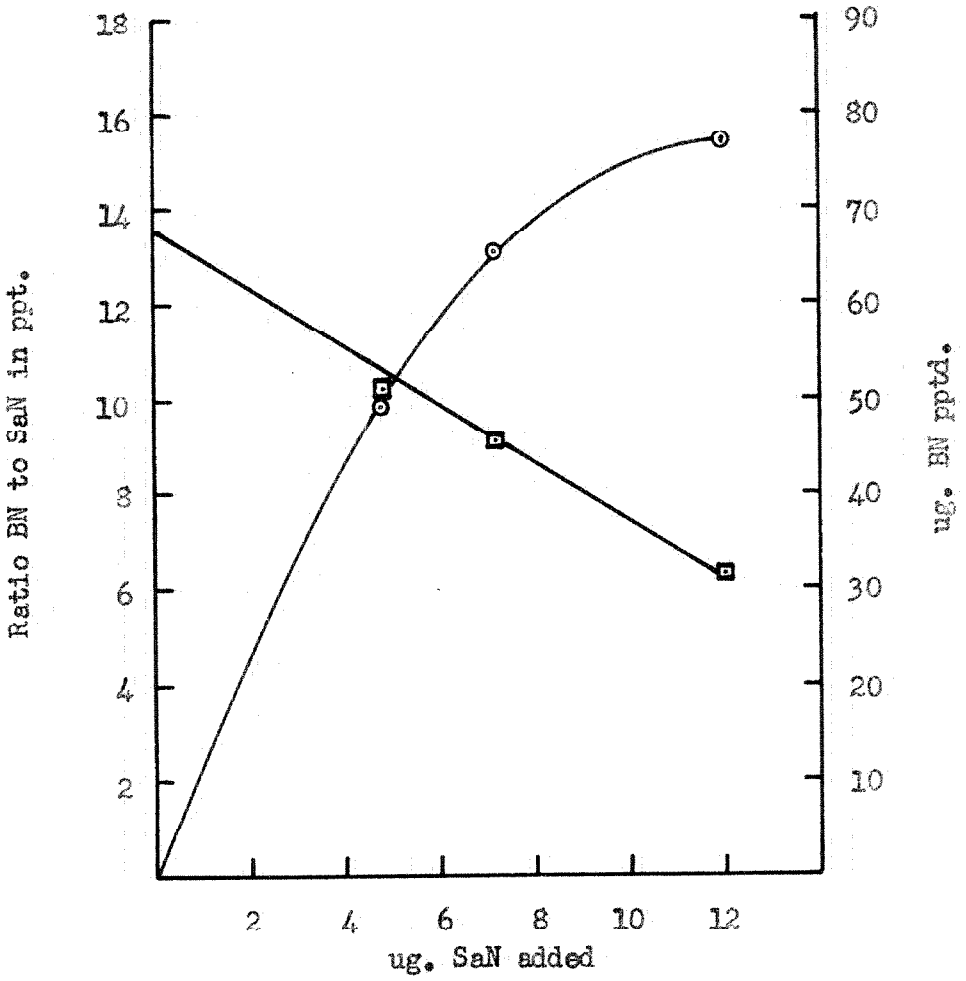


Fig. 15. Quantitative course of the precipitin reaction between chicken serum albumin and rabbit anti-chicken serum albumin. Serum CA6.

In general the curves were similar. Through the region of antibody excess, the quantitative relationships were the same. They all showed a steep rise until the equivalence point and a sharp fall with a gradual leveling out in antigen excess. Except for the differences in the degree of inhibition in extreme antigen excess, the sera were qualitatively alike, differing only in their quantitative relationships. Excess antibody and excess antigen were not detected together in any one supernatant. This suggested that a single antigen-antibody reaction took place. Further, it should be noted that the curves were easily subject to a quantitative analysis. The calculated amounts of antibody precipitated compared well with observed results. The titers were adequate but not high.

Four other antisera that were prepared but not used for further tests will be described briefly. Table 7 presents the results obtained from serum CA1 after the third course of immunization.

TABLE 7

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum

Rabbit CA1 course 3 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Tests on super.
5.2	84.8	79.6	15.3	B excess
7.8	109.0	101.0	12.9	B excess
13.0	148.0	135.0	10.4	B excess
18.0	170.0	152.0	8.4	B excess
25.8	198.0	172.0	6.7	no B; no Sa
38.8	250.0	210.0	5.4	no B; no Sa
51.6	130.0	(78.4)		Sa excess
77.4	67.4			Sa excess
129.0	57.0			Sa excess

Rabbit CA1 received 10 mg. of serum albumin protein in three small doses after a rest of almost two months since it was last bled. This antiserum was different from both the first and the second bleedings. The ratios in extreme antibody excess were higher than previously observed. Furthermore, a plot of SaN/BN vs. SaN added did not yield a straight line but rather a marked curve. No attempt was made to derive an equation for the curve.

Heidelberger and Kendall(98) observed an increase in the R values for rabbit anti-egg albumin on continued reimmunization. Changes in

the reactivity of the antibody to other haptenic groupings on the antigen molecule were offered as a partial explanation of the phenomenon. Accompanying such changes was usually a broadening of the equivalence zone and a loss in specificity. Accordingly, CA1 course 3 was not used in any of the tests.

Table 8 shows the data obtained with rabbit CA2.

TABLE 8

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum

Rabbit CA2 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Antibody N pptd. calc.* ug.	Tests on super.
7.7	51.0	43.3	5.6	45.0	B excess
11.8	69.4	57.6	4.9	60.4	B excess
13.8	79.2	65.4	4.7	66.2	B excess
19.7	101.0	81.3	4.1	78.0	tr. B
29.6	110.0	(80.4)			tr. Sa
39.4	114.0				tr. Sa
59.0	96.2				Sa excess
78.8	72.8				Sa excess
98.6	44.6				Sa excess

*$BN = 6.8\ SaN - 144\ (SaN)^2$

BN max. = 80.4 ug./ml.

SaN max. = 23.6 ug./ml.

The characteristics of this serum were different from all the other sera examined. The value for R was approximately one-half that previously found, and the total nitrogen precipitated continued to rise through a region of excess antigen calculated to be almost double the antigen nitrogen at the equivalence point.

Tables 9 and 10 present the data obtained from rabbits CA3 and CA4. Both rabbits received a total of 49 mg. of alum precipitated serum albumin according to the procedure given in (64).

TABLE 9

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum

Rabbit CA3 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Tests on super.
2.6	31.2	28.6	B excess
5.2	52.4	47.2	B excess
7.7	75.6	67.9	B excess
12.9	114.0	101.0	B excess
18.1	138.0	120.0	B excess
25.8	187.0	161.0	B excess
51.6	222.0	170.0	B and Sa
77.4	143.0	65.6	Sa excess
129.0	95.8		Sa excess

TABLE 10

Addition of Increasing Amounts of Serum Albumin to Constant Volumes of Antiserum

Rabbit CA4 (1.0 ml. serum)

Serum albumin N added ug.	Total N pptd. ug.	Antibody N by diff. ug.	Tests on super.
2.6	29.3	26.7	B excess
5.2	52.0	46.8	B excess
7.7	66.8	59.1	B excess
12.9	98.0	85.1	B excess
18.1	139.0	121.0	B excess
25.8	171.0	145.0	B excess
51.6	113.0	61.4	B and Sa
77.4	79.6		B and Sa
129.0	87.6		Sa

The presence in the same supernatant of both antigen and antibody suggested that more than one precipitating system was concerned. In all probability, the adjuvant enhanced the antigenicity of the small globulin contaminant to such an extent that both rabbits responded to it. For a detailed discussion on the action of adjuvants see Freund (99). Without absorption studies it cannot be estimated how much antibody is specific for the albumin and how much for the globulin contaminant. Minor antigenic impurities have been known to stimulate greater antibody response than the major component (100).

All the sera described were obtained from the animals between the third and fifth day after the last injection. Flocculation times based on trial bleedings suggested that the titers reached a peak during this interval and that by the sixth day they were falling off. Complex sera obtained from immunizing rabbits with whole plasma maintained the same rapid flocculation times over a period of ten days. These observations are in harmony with many that have been made. A full treatment of the rise and fall in antibody titer can be found in The Production of Antibodies by Burnet and Fenner (101).

IV. Serological Analysis of Liver and Kidney Extracts

The most formidable obstacle to overcome in the analysis of the organ extracts for serum albumin or any antigenically related molecule was the contaminating plasma protein. Perfusion alone did not remove all of the blood proteins. Repeated washings of the mash were necessary before a constant level of reactive material was obtained. The significance of this finding will be referred to in the discussion. The reactive material was identified serologically as homologous to serum albumin.

Table 11 shows the results of an experiment performed with liver soluble proteins and antiserum CA1 course 1.

TABLE 11

Addition of Increasing Amounts of Undiluted Liver Soluble Protein to Constant Volumes of Antiserum*

CA1 course 1 0.5 ml. used

ml. Liver protein added	Total N pptd. ug.	Estimated serum albumin N pptd. ug.	Corrected to 1.0 ml. ug.	Tests on super.
0.1	9.7	0.8	8.0	B excess
0.1	11.2	0.9	9.0	B excess
0.3	28.5	2.2	7.3	B excess
0.3	28.0	2.1	7.0	B excess
0.5	51.9	4.2	8.4	B excess
0.5	45.8	3.5	7.0	B excess
0.7	67.2	6.1	8.7	B excess
0.7	67.2	6.1	8.7	B excess
1.0	85.6	9.6	9.6	B excess
1.0	80.5	8.5	8.5	B excess

AVERAGE = 8.2 ug. SaN/ml.

*The data are from single determinations.

The antiserum, normal serum and the extract were centrifuged for 45 minutes in a Sorvall angle centrifuge at 20,000 x g in the cold room (+3° C) before mixing. A small pellet was usually recovered from both sera and the soluble extract. Incubation time was the same as for the other precipitin tests. The controls were negative in this experiment. Where they were not, the values presented have been

corrected appropriately.

The total nitrogen in the soluble extract was 1,650 ug. per ml. On this basis the precipitating antigen comprised 0.5% of this total. Complete absorption of the serum was accomplished by the addition of the optimum concentration of soluble liver extract. Supernatants tested negative for antibody against the purified serum albumin.

If cross reactive antibodies were present in the serum, but in small amounts, the concentration of soluble nitrogen added could have been too far in antigen excess to permit precipitation. Under these circumstances soluble products would have been obtained, and the reaction overlooked. Accordingly, the experiment reported in Table 12 was performed.

TABLE 12

Addition of Increasing Amounts of Liver Soluble Protein to Constant Volumes of Antiserum

Serum CA1 course 1 2.0 ml. used. Total vol. 3.0 ml.

Incubated 6 days at 0° C to $+2^{\circ}$ C

Liver N added ug.	Total N pptd. ug.	Tests on super.
16.5	0.0	B excess
49.5	3.0	B excess
82.5	6.8	B excess

Although four times as much antibody was added as in the previous test, and the antigen concentration was one-tenth of that previously

employed, the precipitation that occurred could be entirely accounted for as an homologous reaction.

The duplicate supernatants were pooled from the precipitin tubes to which 82.5 ug. liver nitrogen had been added. One ml.quantities were precipitated in duplicate with known amounts of Sa. The experiment was designed to uncover any non-specific loss in antibody which might have resulted from the six day incubation with the liver proteins. Table 13 presents the data obtained.

TABLE 13

Addition of Increasing Amounts of Serum Albumin

to Constant Volumes of Supernatant

1.0 ml. super. used

Serum albumin N added ug.	Total N pptd. ug.	Antibody by diff. ug.	Ratio antibody N to serum albumin N in ppt.	Tests on super.
8.0	89.0	81.0	10.1	B excess
11.9	116.0	104.0	8.7	B excess

Total BN in 6.0 ml. of supernatant = 664 ug.

Calculated loss of BN due to precipitations with liver N = 12 ug.

Total BN remaining = 652 ug.

Total BN remaining per ml. = 109 ug.

BN recovered as calculated from above data = 112 ug.

Two facts were established by these experiments. The first was that no cross reactions occurred; the second was that prolonged incubation of liver extract with antiserum did not inactivate or change the quantitative combining characteristics of the antibodies. The identical experiments, conducted with kidney soluble extracts, yielded similar results.

It may be argued that this level of approximately one-half of one per cent Sa in the liver soluble extracts was due solely to physical contamination with the plasma proteins. If so, repeated washing of the organ mash would be expected to reduce this value.

Perfused livers and kidneys were mashed and washed twice, three times, and five times before being homogenized. The soluble proteins recovered were examined for Sa. The data appear in Table 14.

TABLE 14

The Effect of Repeated Washing on the Concentration of Serum Albumin in the Soluble Extracts

Sera CA1 course 1 and CA5 used

	Liver		Kidney	
No. of washings	% Serum albumin N found	Highest conc. of sol. N used mg.	% Serum albumin N found	Highest conc. of sol. N used mg.
2	0.51	1.65	0.15	1.35
3	0.65	1.00	0.0	1.05
5	0.62	0.92	0.0	1.03

Although the concentration of Sa in the kidney extracts could be diminished to the point where it was no longer detectable by precipitin

analysis, the same was not true for the liver.

As a further check on the specificity of the antisera, analysis was made for Sa in whole chicken plasma by precipitin test. The results appear in Table 15.

TABLE 15

Addition of Increasing Amounts of Whole Chicken Plasma to Constant Volumes of Antisera*

0.5 ml. sera used

Anti-serum	Plasma N added ug.	Total N pptd. ug.	Tests on super.	Est. serum albumin N in ppt. ug.		% Serum albumin N in whole plasma
CA1 course 2	9.4	48.3	B excess	3.7		39.4
	9.4	48.6	B excess	3.8		40.4
	18.8	72.2	B excess?	7.8		41.4
	18.8	72.2	B excess?	7.8		41.4
					Av.	40.7
CA5	9.4	42.3	B excess	3.7		39.4
	9.4	40.7	B excess	3.5		37.2
	18.8	62.8	no B; no Sa	7.0		37.2
	18.8	62.3	no B; no Sa	6.8		36.2
					Av.	37.5

*The data are from single determinations.

Electrophoretic analysis of the plasma gave 43% Sa in the total protein, in good agreement with the precipitin tests. Chow (12) and

Sterling (102) observed that precipitin and electrophoretic analysis of whole plasma for albumin agreed within 10%.

Complement fixation studies were carried out with nuclei, mitochondria, microsomes and the supernatants from liver and kidney homogenates. No complement was fixed by any of the washed kidney cell preparations. The same cell fractions from livers did fix complement.

Antisera were absorbed with sheep r.b.c. and chicken r.b.c. before being used. No reactions were observed with sheep cells, but chicken cells removed trace amounts of antibody. Antigens were diluted with saline to the same T.C.A. precipitable "protein" concentration. This amount of 1.0 mg. per ml. was one-fifth to one-seventh of the lowest concentration which was anti-complementary. The results obtained with antiserum CA1 course 2, are presented in the following tables. The numbers indicate the degree of fixation: 4+ means complete fixation and no lysis; 3+, 2+, 1+, tr. and 0 refer to decreasing amounts of fixation based on increasing amounts of lysis. No exact values of fixation are implied by these numbers except for 4+.

TABLE 16

Complement Fixation Test

Serum CA1 course 2 Antigen Chicken Liver Nuclei 1.0 mg./ml.

Serum dilutions

Antigen dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640
undiluted	3+	2+	tr.	0	0	0	0
1:2	tr.	tr.	0	0	0	0	0
1:4	0	0	0	0	0	0	0
1:8	0	0	0	0	0	0	0
1:16	0	0	0	0	0	0	0
1:32	0	0	0	0	0	0	0

TABLE 17

Complement Fixation Test

Serum CA1 course 2

Antigen Chicken Liver Mitochondria 1.0 mg./ml.

Serum dilutions

Antigen dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Undiluted	4+	4+	2+	0	0	0	0
1:2	4+	3+	2+	tr.	tr.	0	0
1:4	3+	2+	1+	tr.	0	0	0
1:8	tr.	tr.	tr.	tr.	tr.	0	0
1:16	0	0	0	0	0	0	0
1:32	0	0	0	0	0	0	0

TABLE 18

Complement Fixation Test

Serum CA1 course 2

Antigen Chicken Liver Microsomes 1.0 mg./ml.

Serum dilutions

Antigen dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Undiluted	4+	4+	3+	tr.	tr.	0	0
1:2	4+	4+	3+	1+	tr.	tr.	0
1:4	4+	4+	3+	2+	tr.	tr.	tr.
1:8	3+	2+	1+	tr.	tr.	tr.	0
1:16	tr.	tr.	tr.	0	0	0	0
1:32	0	0	0	0	0	0	0

TABLE 19

Complement Fixation Test

Serum CA1 course 2

Antigen Chicken Liver Soluble Protein 1.0 mg./ml.

Serum dilutions

Antigen dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Undiluted	4+	3+	0	0	0	0	0
1:2	4+	4+	2+	0	0	0	0
1:4	4+	4+	3+	tr.	0	0	0
1:8	3+	3+	2+	tr.	0	0	0
1:16	tr.	tr.	tr.	0	0	0	0
1:32	0	0	0	0	0	0	0

The results of complement fixation tests using antisera CA5 and CA6 were similar. The only difference was a shift in complete fixation of one or two tubes in the direction of more concentrated antigen and antiserum in these sera as compared with CA1 course 2. Considering the differences in the amount of antibody in these antisera, as determined by previous precipitin tests, the data are consistent with the above findings. All necessary antiserum, complement and antigen controls were run with each test. The control reactions were either 4+ or 0 appropriately.

Calculations of the antibody/antigen ratios in Table 19, based on the precipitin data, indicated that fixation occurred in those tubes which contained antibody in excess and fell off abruptly where the antigen concentration exceeded the optimum. From these data it was estimated that the microsomes had approximately the same amount of complement fixing antigen per mg. of T.C.A. precipitable "protein," as the soluble protein had. Mitochondria and nuclei had less of the antigen associated with them. The supernatant in the above experiment came from a different homogenate than the cell particles. For this reason, the results are not strictly comparable. In another experiment in which all the cell fractions were from the same homogenate, the supernatant possessed the most complement-fixing antigen. Microsomes were next, followed by mitochondria and nuclei.

Absorption analyses were carried out in an attempt to distinguish between an homologous or heterologous reaction. Antiserum absorbed with homologous serum albumin gave doubtful complement-fixing reactions with the different cell components at their highest concentrations. The absorbed antiserum still precipitated with Sa, suggesting that a

trace of antibody remained. This apparently incomplete absorption might well explain the doubtful complement fixation reaction. Absorptions with the other cell constituents produced highly anti-complementary sera, with the result that the data were inconclusive. Diluting the antisera to the point where they were no longer anti-complementary was not feasible, since at these dilutions even unabsorbed antisera gave doubtful reactions. Numerous different procedures were tried in an attempt to obtain non-anti-complementary sera. Absorptions were made with fresh and frozen preparations, at room temperature and at 0° C. Antisera were heated at 56° C for 20 minutes after absorption or used without heating. In all cases anti-complementary activity remained. Dialysis in the cold room against M/15 phosphate buffer overnight also failed to remove the anti-complementary material. The significance of the complement-fixing tests after absorption, therefore, remains dubious. The appearance of anti-complementary activity raises some interesting questions, for in other control experiments where the different cell particles were incubated with normal serum, no anti-complementary activity was observed. This phenomenon will be referred to again in the discussion.

Despite this difficulty, the tests suggested that absorption with one cell fraction reduced the complement fixing titer for all fractions simultaneously. The amount of reactive antigen in the particle preparations must be very small. Precipitin tests made with the supernatant following one ten minute absorption with approximately 19 mg. of mitochondria showed a drop in titer of only five to ten per cent.

V. Precipitin Analysis in Liver Slice Experiments

Peters and Anfinsen (61) reported that incubated chicken liver slices gave off into the medium a protein which appeared to be serum albumin. No pre-incubation levels for serum albumin in the slices were obtainable, since their assays were performed on proteins made radioactive by C^{14} uptake.

In the present study, serum albumin was determined serologically in (a) the slices before and after incubation and, (b) the slices and the media after incubation with either KCl metabolite, normal serum or anti-serum albumin.

Although the number of experiments was small, certain consistencies were evident. These were (a) the total amount of serum albumin detected in the KCl controls after incubation was more than that found before incubation and, (b) the slices incubated with antiserum showed a distribution of serum albumin which was different from that observed with the normal serum-treated slices.

Preliminary experiments established certain important facts, namely (a) minced livers in contrast to liver slices did not release serum albumin into the medium; nor did chicken red blood cells and, (b) no consistent results were obtainable unless the livers were perfused and the slices washed before incubation. The data summarized in Tables 20-22 are from experiments conducted in accordance with the preliminary observations.

TABLE 22

Experiment No. 4 10 slices/beaker

Specific Activities

	mg. SaP in Slice / g. Sol. Slice Protein	mg.SaP in Med. / g. Total Protein	mg. SaP Total* / g. Total Protein
Pre-incubation	4.6	0.0	1.5
Incubated with KCl	4.8	0.53	2.0
0.4 ml. Normal serum	5.4	0.46	2.1
0.4 ml. CAl course 2	6.2	0.05	1.9

SaP = SaN x 6.25

Medium Protein = Total medium protein - serum protein added

Total Protein = Total slice protein + medium protein

*Total SaP = Medium SaP + corrected SaP** of slice

**The amount of SaP in the slice was corrected in the following way. The per cent soluble protein extracted from the slices was calculated. In general, the percentages varied between 30% and 40% of the total slice protein. The highest per cent found in each experiment was given the value

of 1. Its per cent was divided by the other percentages found, which yielded factors greater than 1. The serum albumin detected in the soluble slice prctein was multiplied by its appropriate factor. This procedure was necessary in order to compare the quantities of SaP found in the soluble slice protein with the total protein. The correction was not applied in calculating the ratio of SaP in the slice to the grams of soluble slice protein, since it was not necessary.

TABLE 23

Experiment No. 5 15 slices/beaker

Specific Activities

	mg. SaP in Slice / g. Sol. Slice Protein	mg. SaP in Med. / g. Total Protein	mg. SaP Total / g. Total Protein
Pre-incubation	3.7	0.0	1.5
Incubated with KCl	3.5	1.6	2.9
0.4 ml. Normal serum	3.7	1.4	2.6
0.4 ml. CAl course 2	4.6	0.95	2.7

TABLE 24

Experiment No. 6 10 slices/beaker

Specific Activities

	mg. SaP in Slice / g. Sol. Slice Protein	mg. SaP in Med. / g. Total Protein	mg. SaP Total / g. Total Protein
Pre-incubation	4.6	0.0	2.0
Incubated with KCl	4.2	1.3	3.0
0.2 ml. CA5	4.2	1.0	2.6
0.4 ml. CA5	3.6	0.82	2.3
0.8 ml. CA5	6.1	0.01	2.6
0.8 ml. Normal Serum	4.9	1.5	3.5

The data show that the total serum albumin detected after incubation was greater than before. Also, much less serum albumin was found in the media from slices incubated with antisera than in normal sera or KCl metabolite.

To evaluate the results it was necessary to assess how much serum albumin in the media came from such non-specific sources as plasma contamination and autolysis. This was done for the pre-incubation, KCl and normal serum controls. The antiserum beakers were treated separately. In analyzing the data, it was assumed that in each experiment the slices were distributed at random and that they came from a homogeneous population. Two further assumptions were tested.

Assumption 1. The serum albumin in the media was due to contaminating plasma protein.

Since the total amount of contaminant was constant, loss of part to the media would have diminished the amount remaining with the slices. Consequently, the specific activities, mg. SaP in Slice/g. Sol. Slice Protein, after incubation should have been less than before incubation. This was not observed. In Experiment 3, which is not reported, the chicken was bled maximally by cardiac puncture before it was sacrificed. The ratio mg. SaP in Slice/g. Sol. Slice Protein before incubation was 12.0. After incubation, the ratio obtained for the KCl control was 5.6.

Assumption 2. The serum albumin in the media came from autolysis of a fraction of the cells in the slices.

It was assumed that the concentration of serum albumin in the slices was directly proportional to the soluble slice protein. On this basis, the amounts of serum albumin observed in the media were used

to calculate the amounts of soluble protein expected there. No agreement between the expected and the observed amounts was found. The differences between the observed and expected values were as much as nine fold.

The above analysis was presented, not as evidence against contamination and lysis, but rather to suggest that the contribution of serum albumin to the media due to these causes must have been small (10%?) and may be neglected in interpreting the data.

The data obtained from the slices incubated in antisera consistently showed less serum albumin in the media, and more in the slices than in the controls after incubation.

How did the presence of antibodies in the media affect the levels of serum albumin observed? Were the low values in the media due solely to precipitation by antisera? How can the high levels associated with the slices be explained? Could precipitation alone account for the observed data, or would some other assumption be necessary? As a first approach, the data were analyzed without attributing to the antibody any special property other than its ability to precipitate serum albumin. Other assumptions will be treated in the discussion.

Barring precipitation, how much serum albumin should have appeared in the medium? Estimates based on the amounts of serum albumin observed in the normal serum controls gave two expected values. One value was estimated directly by multiplying the total protein in the antiserum beakers by the ratio mg. SaP Med./g. Total Protein of the normal serum controls. The other value was obtained by calculating the total mg. of SaP expected and subtracting from this the amounts of SaP actually found in the slices. These subtracted values were consistently

lower than those of the direct estimate. The data are shown in Table 25.

TABLE 25

Estimated Amounts of Serum Albumin In The Media From Slices Incubated With Antiserum

	Direct Estimate mg.	Subtracted Estimate mg.
Exp. 4	0.040	0.023
Exp. 5	0.198	0.122
Exp. 6	0.085	0.066

In Experiment 6, only the 0.8 ml. antiserum beakers were considered, since no antiserum effect was noted with the lower antiserum concentrations.

The differences between the two expected values suggested that the release of serum albumin from the slices was partly inhibited. The extent of this inhibition is considered an order of magnitude rather than a precise amount.

Whether the inhibition was real or not depended upon the estimate of the SaP in the slices. If this figure was correct, an antiserum effect was suggested. If not, no such effect should be postulated.

The amounts of SaP found in the slices could have been overestimated if antibody/antigen complexes were carried along with the slices. This could have happened had precipitation occurred in the interstitial spaces of the slices or if the precipitates stuck to the slices and were not removed by washing. In either case, the precipitates would have encountered excess antigen after the slices were homogenized with

the result that new complexes would have formed. Some of these complexes would have been soluble, others not. The insoluble precipitates would have been discarded, but the soluble products and free antigen would have remained in the supernatant after centrifugation. All of this specific nitrogen would have precipitated after the addition of excess antibody, with the result that the Sa estimated would have been too high.

If precipitation had occurred only in the medium, and no precipitates had adhered to the slices, the values for Sa in the slices would have been valid. In this case, no specific nitrogen other than SaN would have been precipitated from the soluble slice protein. Since the whole question of inhibition depends upon where precipitation occurred, an independent experiment is needed to decide the point. Such an experiment will be suggested in the discussion.

DISCUSSION

Of the six rabbits immunized against purified chicken serum albumin, three produced antisera which could be used in this study. The other antisera were not employed for the reasons given in the experimental section. Rabbit CA1 was successfully reimmunized once, but failed to produce a specific antiserum after the second reimmunization. The four antisera which were studied showed similar quantitative combining characteristics. They obeyed the Heidelberger and Kendall equation for precipitation throughout the region of antibody excess. The observed amounts of antibody precipitated agreed well with the calculated amounts based on this equation.

The changes observed in the plasma proteins of New Hampshire Red chickens with maturity confirm the work of Brandt (74). In the present study, the total protein increased with the age of the birds from 3.0% to 5.8% per 100 ml. of plasma, while the albumin/globulin ratios, as determined from electrophoretic patterns, fell from 0.75 to 0.25.

A fast moving component, first observed by Deutsch and Goodloe (73) in the electrophoretic patterns of White Leghorns, was also found in the New Hampshire Reds. However, the different mobilities that the authors reported for serum albumin in the two sexes of White Leghorns were not apparent in New Hampshire Reds, although in both experiments the same buffer was used (68). It would be interesting from a genetic standpoint to examine the plasma of hybrids from these two breeds.

The results of the electrophoretic analysis of liver and kidney soluble proteins show that more than 95% of the proteins migrate with mobilities different from that of serum albumin. This is in accord with other studies mentioned on rabbit and rat liver proteins. No

more than 5% can be considered "albumin" on this basis. In contrast to the electrophoretic data, the serological tests suggested that approximately one-tenth of this amount was actually serum albumin. Quantitative precipitation analyses performed on different liver soluble protein preparations gave values for serum albumin which ranged from 0.35% to 0.65% of the soluble cell protein.

The reactive organ antigen was proven homologous to serum albumin by the absorption experiments. Addition of the calculated amount of liver soluble extract to the antiserum completely removed the precipitating antibody for native serum albumin.

The results of the wash experiments (Table 14) showed that small concentrations of serum albumin always remained with the liver. This was supported by the data from the slice experiments, where the levels of serum albumin found in thoroughly washed slices from perfused livers were essentially the same before and after incubation. The wash experiments further demonstrated that perfusion alone did not remove all of the blood proteins. Repeated washings were necessary in order to reduce the concentration of the blood contaminants below the level of detection. This was the case with the serum albumin associated with the kidneys. Three washes of perfused kidneys were sufficient to reduce the concentration of serum albumin associated with the organ to the point where it was no longer measurable by precipitin analysis. Also, none of the washed kidney cell fractions was reactive in the more sensitive complement fixation tests. For these reasons the serum albumin associated with the kidneys was considered a plasma contaminant. The different behavior of the liver in this respect is compatible with the opinion that small quantities of serum albumin are present in the

liver cells, and do not exist there as simple contaminants.

These experiments demonstrated that the quantitative precipitin technique was capable of estimating small concentrations of a specific protein (serum albumin) in a mixture of proteins with an accuracy unobtainable with conventional physical and chemical methods. The method was also shown to be applicable in estimating larger amounts of serum albumin as are found in whole plasma. The results of such an experiment appear in Table 15. On a serological basis approximately 39% of the plasma proteins were serum albumin, as contrasted with 43% calculated from the electrophoretic patterns. The data are in reasonable agreement, and compare well with the observations of other workers (12, 102).

The data reported in Tables 11 and 12 suggest that no true cross reaction occurred between the antiserum and the soluble organ extracts, although an extensive range of antibody and antigen combinations was investigated. Either the concentrations of cross reactive antigens were small, or few cross reactive antibodies were present in the serum. Furthermore, no obvious non-specific inactivation of antibody occurred even after six days of incubation with the soluble organ proteins.

Although the precipitin reactions obtained with the soluble extracts were considered homologous, they do not imply that the physicochemical properties of the organ antigens are necessarily identical to the circulating serum albumin. It is conceivable that a difference does exist between the two proteins, but none other than an antigenic one would have been detected in this analysis. No antigenic difference was found.

Experiments such as Singer performed (103) to determine the valence of antibody might be used to decide the issue. In brief, the

precipitates obtained from the reaction between soluble liver extracts and anti-serum albumin could be dissolved in excess serum albumin to yield soluble products. The solutions could then be examined in the electrophoresis apparatus and the ultracentrifuge. The appropriate control would be a serum albumin anti-serum albumin precipitate dissolved in excess serum albumin. A component different from those found in the controls would suggest that the organ antigen differed physically from serum albumin.

The results of the complement fixation tests showed that no antigen related to serum albumin was associated with washed kidney cell fractions. This confirmed the previous negative finding in the precipitin analysis of the soluble kidney protein. It also extended the negative finding to include all of the kidney cell particles.

Positive complement fixation tests were obtained with each of the liver cell fractions. Since serum albumin was known from the precipitin analysis to be present in the soluble liver protein, the particles may have been contaminated with it. Mitochondria and nuclear fractions were washed three times; the microsome fraction was washed twice. One would imagine that since the amount of the serum albumin in the soluble protein was small, two to three washings would have reduced the concentration of any antigen present as a free contaminant below the limits of detection. This was not found. The data in Tables 16, 17, 18 and 19 show complement fixation reactions with microsomes and mitochondria which are comparable to those observed with the soluble protein. The simplest explanation for these positive results would be a non-specific adsorption of serum albumin from the soluble protein on the surface of the particles. This may not be correct.

The one suggestion that some antigen other than serum albumin was involved, was the anti-complementary effect observed after absorption of antisera with any of the cell fractions. Absorption with purified serum albumin did not produce anti-complementary sera. One possible basis for this observation may be that some non-specific anti-complementary substance associated with the fractions was liberated into the antiserum during the absorption. However, control absorptions made with normal rabbit serum and liver particles failed to yield an anti-complementary serum. This suggested that a specific action of the antiserum was necessary in order to produce the anti-complementary substance, but the basis of this postulated action remains a mystery.

During the reaction with antibody, some substance in the fractions may have been released or changed which was capable of inactivating guinea pig complement. This antigen-antibody reaction responsible for the production of the anti-complementary sera must have been small, and possibly cross reactive in nature, since particle absorptions removed only small amounts of precipitins. The anti-complementary activity observed may not have been due to a serological reaction as such, but rather to some product of a specific reaction.

Is there a substance antigenically related to serum albumin associated with the liver cell particles as well as with the soluble protein? Studies on the localization of antigen (31) gave the surprising result that much antigen became localized in the nuclei. The results of Haurowitz and Crampton's experiments (104, 105) with intravenously injected I^{131}-iodoovalbumin showed that the protein was first localized predominantly in the microsomes of the liver and spleen of the rabbit and then appeared associated largely with the mitochondria. The

supernatant and nuclei were less active in this respect. Considering that the cytoplasmic granules are the seat of intense biochemical activity, it is not surprising that they may be involved in antibody synthesis as proposed by these authors. But if they are involved in γ-globulin synthesis, do they take part as well in albumin synthesis? Possibly, but it is not known. The findings of the present study suggest that they may well do so, but the status of this important point must be regarded as still hypothetical.

It would be interesting to subject the particles to sonic vibration in an attempt to recover the antigen suspected of being involved in the production of the anti-complementary substance in a soluble form. It could be concentrated and perhaps purified in order that a more complete chemical and immunochemical study be performed.

The tissue slice experiments showed more serum albumin detectable after incubation than before. The data in Tables 22, 23, and 24 suggest increases of 0.3 to 0.9 times the base level observed in the KCl controls. One would like to know whether this implied a net synthesis of albumin. Unfortunately, no conclusive statement is possible at present. Although the differences were observed consistently, their magnitudes were not great. For this reason, some factor other than synthesis might explain the results. Particularly, the assay should be suspected, for in one respect it was not adequately controlled. Serum albumin was determined in only the soluble protein of the control slices, whereas it was estimated in two places after incubation; namely, the soluble slice protein and the medium. If the errors in the tests were all on the high side, this could have made the difference. The proper control should have been another set of duplicate beakers in which

the slices were not separated from the media, but homogenized with it. The values of serum albumin obtained from these controls would have to be equal to the sum of the medium and slice albumin before any conclusions could be drawn. This control was not included, since it did not appear necessary at the time the experiments were performed. It did appear so only after the data were analyzed.

At any rate, more experiments need to be done along these lines. Additional information could be obtained if the serological analyses were combined with a tracer study.

It was observed that liver slices incubated in antiserum had more serum albumin associated with the soluble slice protein than did the slices incubated in normal serum. One analysis of the data suggested that the release of the serum protein from the cells was inhibited when antibody was present. The case in favor of this inhibition, however, is tenuous. Much depends on the answer to the following question: How much of the antibody added to the media remained with the slices either as free antibody or in combination with antigen, after the slices were separated from the media? An answer to this question can be obtained.

If any precipitation occurred in the media, the specific precipitates along with small pellets of debris would be recovered after centrifugation. Appropriate amounts of antiserum or serum albumin may be added to these washed duplicate pellets. Uptake of antigen by these pellets would suggest that antibody was present in them. Uptake of antibody would suggest the presence of antigen. With these data it would be possible to estimate how much antibody was recovered in the media, and consequently how much remained with the slices.

Some of these data were obtained in an experiment designed to answer a related problem. It was assumed that all of the antibody was precipitated in the medium, and that the conditions for precipitation were such that the ratio of antibody to antigen in the precipitate would be at its maximum. It was expected in this case that no more antibody would be taken up by this precipitate if it were resuspended in excess antibody. The complete experiment as suggested above was not performed because the precipitate was not considered to have remained with the slices. Known amounts of antibody were added to the pellets obtained from media to which antiserum was added. The results suggested that a small amount of antibody was removed. Control pellets from KCl incubated slices did not remove any of the antibody. Since antigen was not added to a duplicate pellet, the experiment is inconclusive as it stands. However, it suggests that some antigen was present, and further that some antibody may have been present also.

It is likely that precipitation occurred in the media and in the interstitial spaces of the slices. For this reason, the inhibition may not be as pronounced as suggested in Table 25. Nevertheless it may be a real inhibition. If so, then certain other observations can be explained. First, the inhibition appeared to be roughly proportional to the ratio of antibody/total protein, but there was a difference in the capacity of the two antisera, CA1 course 2 and CA5, to elicit this response. Antiserum CA5 was not as effective in this regard as was CA1 course 2. The difference in the capacity of the two antisera to inhibit the release of serum albumin may depend on the amount of cross reactive antibodies they contain. CA1 course 2 may have a higher concentration of these cross reactive antibodies than CA5. Since

CA1 course 2 was the result of a reimmunization, this is not unlikely.

A possible mechanism for the inhibition of the release of serum albumin from the cells may be suggested. On the surface of the hepatic cell there may be antigens which are serologically related to serum albumin. These antigenic sites may be associated with or border on portions of the cell membrane whose functions are related to protein permeability. Cross reactive antibodies in low concentration in the antisera may combine with these sites and inhibit the release of serum albumin from the cell. Not all of the sites may be covered and some serum albumin might emerge. The combination of antibody and cell site may not be a firm one, and dissociation could occur. The affinity of the antibody may be greater for the antigenic structure of the serum albumin, and if dissociation occurred, antibody could combine with the serum albumin. This effectively would remove the inhibiting antibody. At the end of a two hour incubation, most or all of the inhibitor could be removed in this way leaving a back log of serum albumin in the cell. This amount of serum albumin which did not appear in the medium, would be recovered in the soluble slice protein. Higher concentrations of the inhibiting antibody would produce a greater percentage inhibition. Conceivably, inhibition could be complete under the appropriate conditions.

Large protein molecules are known to pass through cell membranes, but the mechanism is not understood. If the cell membrane were permeable to serum albumin at specific sites at or near where antibodies were attached, the inhibition could be envisioned as a steric hindrance. Alternatively, the protein permeability of the cell membrane could be a general phenomenon of the whole cell surface with no fixed sites necessarily involved. In this case, the combination of antibody with

the cell membrane might alter the permeability of the membrane as a whole, thereby inhibiting serum albumin release. It is realized that this is speculation, but it need not appear unreasonable in the light of the data at hand.

One other question is worthy of mention. Are the "serum albumin molecules" found in the liver cell the fragments of circulating protein postulated by Madden and Whipple? Possibly they are, but a physico-chemical study would be necessary before the issue could be decided. This point may be an important one, since it may be the clue to the rapid mobilization of new plasma protein after loss of some from the circulation.

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