THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHEMICAL, PHYSICAL, AND BIOLOGICAL STUDIES OF A CHEMICALLY TREATED BOVINE PLASMA

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1956

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CHEMICAL, PHYSICAL, AND BIOLOGICAL STUDIES

OF A CHEMICALLY TREATED BOVINE PLASMA

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ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. M. R. Shetlar for his suggestions and support of the problem.

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CHEMICAL, PHYSICAL, AND BIOLOGICAL STUDIES OF A CHEMICALLY TREATED BOVINE PLASMA

CHAPTER I

INTRODUCTION

For many years the need for an ideal plasma substitute has been recognized, however, World War II gave a tremendous impetus to both study and research in blood substitutes. As yet, no substance has been found which will completely carry out the functions of unmodified whole blood. Strictly speaking, plasma and serum are not complete whole blood substitutes for they do not contain all the original properties of unmodified blood. However, the most important, most widely used and the most valuable whole blood substitute is human blood plasma. The search continues therefore for an ideal human plasma substitute. This ideal plasma substitute should be: 1) able to maintain colloidal osmotic pressure in the blood stream; 2) of suitable viscosity and molecular composition; 3) stable over long periods of time under a wide range of temperature changes; 4) non-allergenic and non-pyrogenic; 5) non-toxic; 6) inexpensive; and 7) available in large amounts.

Even containing all these qualities, a plasma substitute is not completely adequate if it cannot equal the nutritional value of whole plasma. It must be one that will not only maintain the blood

volume so that the patient can recover, but also one that will furnish amino acids with nutritional values. A need for a large stock of this ideal plasma substitute which has nutritional value is of importance from a civil defense standpoint, for it is known that in the case of a direct atomic attack on the United States the supply of human plasma which was so important during World War II will be completely inadequate.

At the present time many possible plasma substitutes are being considered. Some of these are: gum acacia, pectin, fish and animal gelatin, ascitic fluid, dextran, polyvinyl-pyrrolydone, and animal plasma. It would appear that the logical approach may be to modify animal plasma because of its close similarity to human plasma and because of the relatively large available sources.

History of Plasma Substitutes

In considering the numerous substances that may have value as substitutes for plasma, the first consideration has been the maintenance of the correct osmotic pressure; a substance which forms colloidal aqueous solutions is considered essential. Some such substances are: acacia, ascitic fluid, gelatins, pectin, dextran, polyvinyl-pyrrolydone and animal proteins.

Acacia. Acacia is a dried gummy polysaccharide of plant origin. It is insoluble in alcohol but almost completely soluble in cold water giving an acid solution. Both its molecular weight and osmotic pressure are comparable to that of normal plasma protein. Bayliss (1916) introduced the use of acacia intravenously. It was found to be highly antigenic, but Bayliss blamed this objection on its

method of preparation. Damashek (1944) concluded after experimental studies that an intravenous injection of acacia solution causes an extreme drop in platelet count with accompanying thrombus development.

Jackson and Frayser (1939) have described a depression of plasma protein production when acacia solution is injected. This, added to the fact that acacia is a polysaccharide, eliminates consideration of this produce for any lasting value. These workers also found an increase in bleeding time due to the mentioned depression in protein, which is probably due to depression of fibrinogen.

When acacia was injected in 6 per cent solution with potassium nitrate it was found by Goudsmit and Ringer (1940) to have a therapeutic value in the treatment of refractory nephrotic edema. This therapeutic value, however, was found to be only temporary with a subsequent recurrence of the edema. It would seem that acacia may have a place in medical usage only in the temporary treatment of nephrotic edema or as emergency treatment, but its harmful effects place it far from being a good substitute for plasma.

Ascitic Fluid. The first report in this country on the use of ascitic fluid was that of Davis and White (1938). The fluid was obtained in large amounts from human patients with cardiac decompensation or portal cirrhosis. Since this fluid contains proteins it must be checked with the blood of the recipient before use because of the danger of protein reactions. In the experiments of Davis and White, shock was produced in anesthetized dogs by graded hemorrhage, and then variable amounts of ascitic fluid were given intravenously. The blood pressure rose rapidly from 15 mm. to 30 mm. with the final survival of the

animal. Contrary results were shown by Choisser and Ramsey (1938) who found that initial restoration of blood pressure was followed by a rapid fall in blood pressure and eventual death of the animal. Other reports concerning ascitic fluid seem contradictory. Therefore, at this point it can be said ascitic fluid is not likely to become a substitute for human plasma because of scarcity, low protein content, and varied reactions.

Gelatins. Fish gelatin is prepared from the swimming bladders of some marine species such as the sea trout. This product was first used in transfusion by Taylor and Waters (1941). They found that a 7 per cent solution in saline adequately raises and maintains blood pressure and blood volume in dogs bled to the severe shock stage. They also stated that fish gelatin is superior to animal gelatin because there is no danger of contamination from anthrax or tetanus organisms. Later, in 1943, Taylor and co-workers reported the absence of pyrogenic and antigenic reactions to fish gelatin, and established that there was evidence of protein utilization.

Human experiments with fish gelatin were carried out by Pugsley and Farquharson (1943). In 61 patients only 8 cases showed febrile reaction and minor symptoms such as malaise, headache, and nausea. The main objection to fish gelatin is its low nutritional value, but it appears to have merit at the present time among the proposed plasma substitutes.

Animal Gelatin. Gelatin was first introduced by Hogan (1915) when he reported that gelatin restores blood pressure when given intravenously to patients with shock. At that time it was not met with much

interest, but during World War II, when great emphasis was placed on plasma substitutes, more studies on animal gelatin were undertaken.

Gelatin is a nutritionally incomplete protein derived from collagen of bones and skin and degraded by different chemical methods. Pauline (1942) prepared gelatin by using glyoxal and hydrogen peroxide to degrade the molecule to a smaller size. The resulting product "oxypolygelatin" had a molecular weight of about 30,000 and does not solidify at temperatures above 5° C. The osmotic pressure of this product is slightly above that of plasma protein. Pulaski (1952) has also recently prepared a fluid gelatin which solidifies at 0° C. after treatment with an aromatic amine.

During the years 1942 to 1945, Koop and his co-workers made extensive studies on gelatin. These workers found that bone gelatin prepared as 6 per cent solution was found to be both safe and effective when given intravenously for the treatment of shock. This gelatin prepared from bones is of a high molecular weight and is not excreted by the kidneys very fast. It remains in the blood about five days.

Very interesting results were reported by Heuper (1942) on dogs. He found that the production of plasma proteins was impaired by the gelatin and likewise production of hemoglobin was also impaired. In addition, he found arterial lesions of the sclerosis type. Gelatin was found by Koop (1945) to cause pseudoagglutination of blood. This is of great danger since it may cause immediate death by shock due to hemagglutination.

Other studies have been done on the nutritional value of gelatin. Brunschwig, Corbin and Johnston (1943) found that a rapid rise

in plasma protein can be obtained in protein depleted dogs by daily intravenous injection of gelatin. They also found that at least part of the gelatin was catabolyzed since increased urea nitrogen was noted during the intravenous injection period, but a large portion of the gelatin was excreted unchanged. The same workers found, in 1944, a 40 to 43 per cent retention of gelatin in adults with intravenous injections.

Another interesting value of gelatin was shown by Haimowici and Fine (1945). These workers found that gelatin exhibited a definite antithrombic action when given prophylactically in single injections immediately after thrombosis had been produced.

Felmus (1949) administered a 6 per cent macromolecular gelatin solution to 97 patients with varying degrees of shock. All the patients reacted well within 15 minutes of infusion. Felmus writes that gelatin solution is superior to pooled plasma because it eliminates the danger of serum jaundice.

Oxypolygelatin has been found by Barker and co-workers (1952) to be easily administered, non-toxic and non-antigenic, and to exhibit a good osmotic pressure suitable for combating shock.

The advantages of gelatin are many, as indicated in the above discussion, but several important findings about it have made it far from an ideal substitute for human plasma. For example, it is an incomplete protein, lacking many of the essential amino acids. Also, pseudoagglutination of erythrocytes has been reported in the administration of gelatin with reduction of protein generation encountered.

Pectin. Probably the first complete report of the use of pectin as a plasma substitute was made by Hartman and his group (1941).

Pectin is a colloidal carbohydrate of high molecular weight and rather complex composition. Upon hydrolysis it produces galacturonic acid, galactose, arabinose, xyloses, methanal, and acetic acid. The aforementioned workers found no traces of antigenicity or toxicity in guinea pigs, rabbits and dogs after intravenous injections. They did find that pectin was excreted unchanged in the urine. In experiments carried out to study the ability to restore blood pressure, all the results were satisfactory.

Adverse results were found by Ivy (1943). He reported that pectin solution caused pseudoagglutination and had lethal effects when used after massive hemorrhage. In a later report Hartman (1943), using 0.75 per cent pectin in traumatic shock, noted fairly good results in restoring blood pressure to normal condition. Popper and his co-workers (1945) found that pectin in large doses produced splenomegaly and a deposition of a fecular material in various organs. This material was found in phagocytic cells, capillaries, tissue spaces and infiltrating reticulum fibers in the spleen, kidney, liver, and lungs. It resembled deposits of amyloid and caused reactions of the reticulo-endothelial cells.

At the present time no definite conclusions can be drawn as to the value of pectin solutions as a plasma substitute. It raises blood pressure and volume, but as a carbohydrate, has no value as a source of protein and is deposited in vital organs. In final analysis, however, it may have value as a temporary substitute.

Polyvinylpyrrolidone (P.V.P.). The product, polyvinylpyrrolidone, was a polymer synthesized from ammonia, acetylene and water

which was used widely in Germany during World War II. It is non-antigenic, has a pH 7, and a satisfactory molecular weight of 40,000 to 50,000. It is readily soluble in water and can be stored indefinitely. Posner (1953) and McCarthy (1952), respectively, found that a 3 to 5 per cent solution in normal saline was equal to plasma in restoring reduced blood volumes following hemorrhage, in obstetric cases and in burns produced in rats.

The biggest question concerning the value of the product seems to be that the ultimate fate in the body is unknown. On this problem Ranin, Seligman and Fine (1952) carried out studies with radioactive iodine and revealed its retention in the reticulo-endothelial system for long periods of time. However, Reinhold, Van Frijtag, Newton and Thomas (1952) at the University of Pennsylvania found no apparent deleterious effect on the liver. Hartman and Behrmann (1953) also reported marked storage in the reticulo-endothelial cells, the liver, and the kidneys.

At this point it can be said that as long as the fate of P.V.P. is unknown and the reason for its retention not understood with relation to tissue damage, etc., P.V.P can not yet be accepted as a successful human blood plasma substitute.

Dextran. In 1943 Granwall and Ingelman, in Sweden, suggested the use of dextran as a plasma substitute. Dextran was first discovered in Germany as a mucoid material formed during the extraction of sugar from beets. Found only in those sugars infected with Leuconostoc mesentoroides, it is a polysaccharide formed from glucose molecules by this organism. For use as a plasma substitute dextran is hydrolysed so that its particles attain a range of molecular sizes close to that of

the plasma proteins, about 100,000.

Dextran has been used extensively in Sweden during the last few years with excellent results. Thorsen (1949) reported the use of dextran in 5,000 patients and stated that it was an effective plasma substitute, and that it was non-injurious to tissues locally or systemically. He stated that it was being used in the hospitals routinely in major operations. In this country dextran is readily available under various trade names and results obtained from this product are varied.

Lundy (1950) reports that 1500 cases in the Mayo Clinic have received dextran and the results appeared highly beneficial.

Pulaski (1953) found that the smaller molecules were rapidly excreted. He injected dextran tagged with C^{14} in the dog. Sixty-five to 70 per cent was found in the urine with 4 per cent to 6 per cent in the expired air (CO_2) and 3.5 per cent in the viscera at the end of 75 hours. About 25 per cent could not be found. Material marked with C^{14} was found in the liver, spleen, and lymph nodes. In relation to possible kidney damage, Michie and Ragni (1951) found no clear evidence of renal damage but marked depression of tubular function was noted.

Numerous pharmacological studies on dextran have been conducted by Granwall and Ingelman (1944). These workers found that in general, dextran solution was well tolerated and very effective in treating shock. Bull, Rickets, Squire and Maycok (1949) found dextran non-pyrogenic, non-toxic and non-antigenic. Similar results are reported by Bohmanson (1949) and Rousenquist (1947). Witham, Fleming, and Bloom (1951) showed dextran to increase blood volume consistently in proportion to the amount administered.

The value of dextran has been demonstrated by a report of Amspacher and Cuneri (1953) who gave the material to 60 men wounded in Korea. Most of the wounded soldiers were in state of serious shock and a few showed impending shock. No harmful reactions were noticed in any of the 60 patients, but there was a noticeable increase in blood volume.

At the present time dextran is probably the most widely used plasma expander, but it only serves the purpose of restoring circulating blood volume until human blood is available; it lacks the necessary protein composition to be an ideal blood substitute. Also, late reports indicate the possibility of latent antigenicity and temporary depression of renal function when large amounts are given (Metcalf, Rousselat, 1953).

Animal Plasma. The use of animal plasma as a substitute for human plasma goes back to Rosenau and Anderson (1906) who employed heat and chemical treatment on animal plasma in order to use it in humans, but no successful results were obtained. Later, in 1918, Saint-Girons successfully injected 500 ml. of horse serum with no visible bad results. He suggested the future use of animal plasma in transfusion. However, it was not until 1940 that the idea of a chemically treated animal plasma to be used as a plasma substitute began to be investigated more extensively. Wangensteen and his co-workers (1940) made use of bovine plasma in clinical cases. They concluded that bovine plasma could be administered intravenously to some patients in fairly large quantities. A patient was given 450 ml. of bovine plasma daily for five days and kept in positive nitrogen balance. These authors stated, however, that as long as antigenicity was present, it would be detrimental for general use. In a later report, Kremen, Hall, Koschmitzke, Stevens, and

Wangensteen (1942), at the University of Minnesota, found that positive nitrogen balance could be maintained with bovine plasma as the sole source of protein, again indicating possible utilization. The reactions were as follows: In 24 patients receiving whole bovine plasma, 58.3 per cent had immediate reactions and 43.7 per cent had delayed reactions. However, if the plasma were absorbed with human red cells, only 24.5 per cent had immediate reactions and 53.3 per cent delayed reactions. The remaining portion (22.2 per cent) showed no reaction. The authors, by these results, suggested the possibility that elimination of the globulin or chemical treatment may eliminate anaphylactogenic properties. Davis and Eaton (1942) noted no untoward reactions in injecting bovine albumins into human subjects; they found no albuminuria. Re (1940) in Argentina found that horse serum treated with formaldehyde and heat showed a considerable reduction of its antigenic properties. He was unable to cause uterine and intestinal contractions with the Schultz-Dale technique. Similarly, Gutfreund and Ogston (1945) found that in experiments with rabbits injected with bovine serum to which formaldehyde had been added and then heated, there occurred an appreciable loss in toxicity and antigenicity.

Massons (1946), the first investigator to use the formaldehyde, ammonia, and heat treatment on animal plasma, tabulated the following results: In 40 guinea pigs, 10 rabbits, and 2 dogs, no anaphylactic reactions were noticed. No uterine contraction was noted with the Schultz-Dale technique. Of 25 patients given as high as 1000 ml. of chemically treated bovine plasma, there was no toxicity or traces of allergic reactions. The same results were obtained by Edward in 1944.

He also found that destruction of agglutinins was associated with changes in the globulin fraction after heating above 72° C.

Probably the most extensive clinical study of a chemically treated bovine plasma was done by Larget and Culot (1952). These investigators found a definite increase in blood volume in 40 per cent of the cases given the product. A definite increase in blood proteins was found except in cases of advanced cancer. This protein increase is comparable to that obtained by human plasma. They also noted an elimination of polypeptides and albumoses in the urine which they considered as protein breakdown products.

Denatured calf plasma, prepared by Carlinfanti and Pontecorro (1948) in the same manner as by Massons, was found to possess strong anaphylactogenic properties on guinea pigs. They found that although the challenging power is completely absent, the sensitizing power is present. Massons ascribed this discrepancy to the technique used to test anaphylaxis. Goret, Jaubert, and Grosset (1954) obtained results to indicate that some antigenic properties are retained when normal bovine plasma is treated with formaldehyde and heat. Perez (1954) found that even though antigenicity remains on chemically treated bovine plasma it has a different specificity than normal bovine plasma protein.

Melka, Ranpant and Zapletal (1948) reported that:

1. Denatured calf plasma does not contain agglutinins for human blood corpuscles group A, B, AB.
2. No hemolysin was present.
3. In guinea pigs, rabbits, or human beings, denatured calf plasma does not elicit the formation

of precipitins against either denatured calf plasma or normal cow plasma.

4. Denatured calf plasma cannot sensitize guinea pigs to cause anaphylaxis.

In 1954 Cazal stated that in his opinion the enthusiasm over denatured calf plasma is not valid since he finds no increase in serum proteins, a fall in prothrombin level, and a definite pyrogenic action.

Very little has been done on the actual chemistry of denatured bovine plasma, but some electrophoretic studies have been completed by Regamey (1952) in France. He presented results showing that bovine plasma treated with heat and formaldehyde had no electrophoretic components with the mobility of globulin (pH 8.6 veronal buffer) but exhibited an entity with mobility similar to that of normal bovine albumin. Iachan, Tornasquis and Perrone (1955) found that crystallized serum albumin, when treated with heat and formaldehyde, increased in mobility at pH 7 and 11. Concerning the relation between chemical grouping and antigenicity Laiseleur and Catinot (1955) found that heating serum in the presence of formaldehyde at 95-100° decreased the number of basic functional groups to a minimum. The work of Landsteiner (1929) indicated that groups such as the amino groups -(NH_2) are very influential in antigen-antibody reactions. Similar results to those mentioned above were obtained by Sieber (1953) in his electrophoretic studies. He also suggested that the ability of this chemically treated bovine plasma to give a strong biuret reaction indicates the presence of many peptide linkages. This worker also indicated the possibilities of molecular weight changes in the original protein molecule. Preliminary

studies *in vitro* indicated possible utilization of the denatured protein in metabolism.

Koecher, Hansen and Burkett (1954) reported results comparable to those of Regamey (1952) and Sieber (1953) in that plasma proteins treated with formaldehyde and heat exhibit two components in electrophoresis. He reported that these two components in electrophoresis were not identical with either normal human albumin or globulin.

Concerning the molecular weight changes present in the altered protein, Gutfreund (1945) noted a decrease in osmotic pressure of the altered protein. From this he concluded that there was an aggregation of the proteins to form a polydisperse system of larger particles. However, Melka, et al (1947) found no changes in osmotic pressure with the treatment which indicated that no changes in molecular size had occurred.

In this work, information is presented concerning the chemical, physical, nutritional, and immunological properties of a bovine plasma which was altered in the hope of making it suitable for human use.

The product under study is a commercial preparation from the Lissa Laboratories in Havana, Cuba. Only an outline of the preparation is known.

CHAPTER II

MATERIAL AND METHODS

Normal bovine plasma was collected in sodium citrate after which formaldehyde was added. Ammonia was then added to neutralize the excess of formaldehyde and the solution was heated for one hour at 100° C. After heating, the solution was diluted with distilled water to approximately one-third the original concentration and invert sugar added.

Immunochemistry. Antigen-antibody cross reactions were studied by using the ring precipitation test which at the moment is the most sensitive *in vitro* antigen-antibody reaction known. The test consists generally in layering a known dilution of soluble antigen on top of a homologous antiserum or a heterologous antiserum to study cross reactions.

Immune sera were prepared against human serum, bovine serum, but could not be prepared from chemically treated bovine plasma. Rabbits were employed in developing the immune sera using the procedure as follows:

Two ml. of antigen were injected in the ear vein for three consecutive days. Five days after the third injection the animals were bled and their serum titer against their homologous serum determined. This step was followed by two ml. injections intraperitoneally and two consecutive injections in the ear vein. Again five days were allowed

to pass and the titer was rechecked. At this point positive precipitation was obtained and the animals were immediately bled from the heart. Fifty ml. of blood were obtained from which the immune serum was separated, and stored to be used in cross reaction studies and for the establishment of the presence of antigenicity.

Procedure for the ring precipitation test was as follows:

Tube No.	Antigen Dilution	Amount	Saline	Final Dil.
1	1/500	1 ml.	0 ml.	1/500
2	1/1000	3 ml.	0 ml.	1/1000
3	1/1000	1 ml.	1 ml.	1/2000
4	1/2000	1 ml.	1 ml.	1/4000
5	1/1000	0.5 ml.	2.5 ml.	1/6000
6	1/4000	1 ml.	1 ml.	1/8000
7	1/6000	1 ml.	1 ml.	1/12000
8	1/8000	1 ml.	1 ml.	1/16000

After the final dilutions for the antigens were made, and 0.5 ml. aliquots added into test tubes, 0.2 ml. of antibody was added very carefully in order to form a layer. The tubes were allowed to stand at room temperature for one to two hours and then investigated for a ring formation at the interface of the antigen-antibody.

The cross reaction studies were carried out with the above method using antiserum against normal bovine and human plasma matched against the antigens, treated plasma, normal bovine and human plasma.

Albumin Estimation by Immunochemical Methods. The quantitative determination of the albumin, if any, in treated plasma was carried out at room temperature using Chow's method (1947) as follows:

One ml. of a solution containing pure bovine albumin (Armour's) in a concentration of 0.05 mg./ml. through 0.50 mg./ml. was added to each of a series of Klett tubes containing 2.0 ml. of 0.85 per cent saline. Finally, 2 ml. of immune rabbit serum against the albumin was added to each tube. Also, 2 ml. of immune rabbit serum against the albumin was added to tubes containing 1.0 ml of diluted whole bovine serum and to tubes containing 1.0 ml. of treated plasma. After two hours the tubes were read in a Klett-Summerson electrophotometer with a blue filter of wave length 425 millimicrons. All tests were run in duplicate.

Turbidity readings at different concentrations of albumin were made to obtain a standard curve. The amount of albumin in an unknown sample may be estimated from this standard curve when the turbidity is known.

Electrophoretic Studies. Electrophoretic studies were carried out on an Aminco-Stern Model 5-8000 electrophoresis apparatus; before electrophoresis, samples were dialyzed for 24 hours against the buffer of the proper pH. Veronal buffers of pH 8.6 and phosphate buffers of pH 7.8 and 6.0 were used. The running time was two hours. These studies were carried out on formaldehyde and heat treated bovine plasma, normal bovine plasma, and mixtures of treated plasma and normal bovine at the stated pH values.

Salt Fractionation Studies. Salt fractionation studies were carried out with 33.33 per cent ammonium sulfate and 26 per cent sodium sulfate. The fraction precipitated with ammonium sulfate (Cohn, 1940) corresponds with electrophoretic γ-globulin fraction in normal human serum and the 26 per cent sodium sulfate (Howe, 1921) soluble fraction

corresponds to electrophoretic albumin.

The experimental procedures for these fractionations were as follows:

Precipitation with 1/3 saturation (Ammonium sulfate).

To 1 ml. of sample in a centrifuge tube, was added 0.5 ml. of saturated ammonium sulfate. The tube was allowed to stand at ice box temperature, (5° C.), overnight. This was followed by centrifugation and washing with one-third saturated ammonium sulfate. The samples were allowed to drain for one hour; after this the precipitate was dissolved in 10 ml. of saline. The protein content was determined by the biuret reaction using 5 ml. of the protein solution. Readings were taken on a Coleman spectrophotometer at 550 mmu. A blank consisted of 5 ml. of the protein solution plus 5 ml. of biuret reagent blank.

Precipitation with 26 per cent sodium sulfate.

To 0.5 ml. of sample was added 7.5 ml. of 27.7 per cent sodium sulfate. In order to have enough solution for biuret determination four tubes were run of each sample. After adding 3 ml. of ether to each tube, the tubes were shaken for 30 seconds and centrifuged. The insoluble globulin forms a layer at the interface above the albumin solution. In order to remove the albumin a 5 ml. volumetric pipette was inserted carefully into the solution; 3.2 ml. of this solution was used for the estimation of albumin by the biuret reaction.

Biuret determination for total proteins. The biuret reagent consists of a basic mixture of Rochelle salts (sodium potassium tartrate), copper sulfate and potassium iodide, and sodium hydroxide. The biuret blank consists of the same solution except that the copper sulfate is

omitted.

Samples were diluted to 5 ml. with saline and 5 ml. of biuret reagent was added to each. Blanks were set up in the same manner except that 5 ml. of the biuret blank was added instead of the biuret reagent. The samples were always run in duplicate. A protein standard of bovine albumin was made to contain approximately 4.5 g./100 ml. of protein and standardized by a Micro-Kjeldahl determination. The color was read on a Coleman Universal spectrophotometer at 550 millimicrons.

Micro-Kjeldahl Determination for Total Proteins. A two ml. sample was digested with 2 ml. of concentrated sulfuric acid. Digestion was continued in the presence of selenium granules until the digest became a clear yellow color and the neck of the flask was clear. After digestion was finished, the digest was diluted to 10 ml. in a volumetric flask. One ml. was then used for steam distillation into flasks containing 20 ml. of approximately 0.01 Normal solution of sulfuric acid. All the runs were made in triplicate. Back titration was made using 0.01006 N NaOH (sodium hydroxide) with methyl red as indicator.

The following formula was used to calculate the results:

(B-V) x 14.0 x N x 6.25 x 5 = mg. of protein/ml. of unknown.
B = ml. of .01006 N NaOH necessary to neutralize flasks in which distillate is recovered.
N = normality of NaOH.
V = ml. of .01006 N NaOH necessary to neutralize 20 ml. of .01 N acid

Hexosamine and Polysaccharide Determinations

Hexosamine Determinations. Procedure: 1 ml. of serum was added drop by drop to 18 ml. of absolute ethanol contained in a 15 x 150 mm.

pyrex test tube. After five minutes the tubes were centrifuged, the supernatant liquid was poured out and the tube inverted to drain off the excess alcohol from the precipitate. Two ml. of water were added to the precipitate and stirred with a glass rod, followed by 2 ml. of 8 N hydrochloric acid. The mixture was hydrolysed in a boiling water bath under reflux for 4-5 hours. It was filtered through Whatman #1 filter paper into 10 ml. volumetric flasks, made up to volume, and mixed. One to 2 ml. of the solution were pipetted into three 15 ml. calibrated centrifuge tubes, a drop of phenolphthalein indicator added to each and carefully neutralized with 2.5 N sodium hydroxide. The solution was made slightly acid by adding a drop of 2.5 N hydrochloric acid. Water was added to bring the total volume to 3.5 ml. Two ml. of acetyl-acetone solution was added to two of the tubes and 2 ml. of 0.5 M Na_2CO_3 to the third, which serves as a blank. The tubes were stoppered lightly and placed in a boiling water bath for 20 minutes.

After removal from the bath, and cooling below 37.5° C., the solutions were made to volume of 10 ml. with absolute ethanol, and exactly 2 ml. of Ehrlich's solution added. The tubes were stoppered lightly and incubated in a 37.5° C. oven for 45 minutes. The optical density was determined in a Coleman 11 Spectrophotometer at a wave length of 540 mμ. Each sample was read against its respective blank. A standard containing 100 μg. of glucosamine-HCl (83.4 μg. glucosamine) was subjected to the same procedure with each set of samples.

Calculations:

$$\frac{Kx \times 83.4 \times 10}{K_s \times V} = \mu g. \text{ of glucosamine per ml. of serum}$$

Kx = optical density of sample

K_S = optical density of standard

V = volume of aliquot of hydrolysate

Total Polysaccharide Determination. Polysaccharide determinations were carried out according to the tryptophan method of Shetlar, Foster and Everett (1948), as follows:

The serum was diluted 1/2 with distilled water. Two-tenths ml. of the diluted serum was pipetted drop by drop into 10 ml. of absolute ethanol in glass stoppered centrifuge tubes. The precipitate was centrifuged out and washed once with 10 ml. of absolute ethanol. After centrifuging as before, the ethanol was decanted and the ethanol drained out by inversion of the tubes. One ml. of distilled water and 7 ml. of 57 per cent (V/V) sulfuric acid were then added, the tubes were allowed to stand at room temperature for 10 minutes to dissolve the precipitate, and were then placed in an ice bath for 15 minutes. One ml. of cold 1 per cent aqueous tryptophan solution was then added to each tube without shaking. The contents of the tube were mixed and the tube placed immediately in a boiling water bath for a period of 20 minutes. The tube was shaken once after it had been heated 10 minutes. After removing from the boiling water the tubes were cooled for 5 minutes in an ice bath and then allowed to stand 25 minutes at room temperature. The color intensity was determined in a Coleman No. 11 spectrophotometer at a wave length of 500 mμ. by reading against a blank containing only water, tryptophan and acid, which was run through the above procedure.

Qualitative Determination of Amino Acids and Sugars

Paper chromatography was used to determine which amino acids and sugars were present in the chemically treated bovine plasma as compared to the normal bovine. Whatman #1 paper was used throughout the studies both in sugar and amino acid determinations.

Carbohydrates. Free sugar present in the sample was determined by first precipitating the protein with absolute ethanol. To 10 ml. of absolute ethanol was added 1 ml. of sample, the precipitate was then separated by centrifugation. The supernatant was then used for chromatography studies. One hundredth of a ml. was spotted on the paper and run for 18 hours on a normal butanol-pyridine-water solvent 6:4:3. The chromatograms were then dried, sprayed with phenylendiamine (Chernick, 1951) 0.2 per cent reagent, 2 per cent oxalic acid in 95 per cent alcohol and heated for 5 minutes at 105° C. Standard solutions (1 mg./ml.) of glucose, mannose, sucrose, fructose, glucosamine, and glucuronic acid were always run simultaneously.

The bound carbohydrate studies were carried out in the same manner except that the alcohol precipitate of protein was hydrolysed and then chromatographed.

Hydrolysis. The hydrolysis was carried out according to the method of Kunita and Sahasrabudhe (1955). The method consists in boiling with 85 per cent formic acid and N. hydrochloric acid for two hours. Amounts used were 5 ml. of serum, 1 ml. formic acid, and 10 ml. of 2 N. hydrochloric acid.

Desalting Procedure. The hydrolysates were desalted by the pyridine extraction method of Malpress (1949). The sugar solutions were dried on a steam bath and the residue was extracted with 5.0 ml.

of pyridine at 100° C. for 10 minutes. The resulting mixture was cooled and filtered and the pyridine was removed by distillation under reduced pressure at a temperature not exceeding 40° C. The residue was dissolved in 60 per cent isopropanol and aliquots used for chromatography.

Amino Acid Chromatography

Qualitative studies of amino acids were made by paper chromatography following hydrolysis of the sample. This hydrolysis consists of refluxing 5 ml. of sample with 6 N. hydrochloric acid for 12 hours. The excess hydrochloric acid is removed by evaporation to dryness in vacuo at 35° C. The hydrolysate was then dissolved in warm water. The solvents used in the chromatographic studies of the amino acids were butanol-pyridine-water (6:4:3) V/V, phenol-water (100:40) V/V, butanol-acetic acid-water (40:10:50) V/V.

Quantitative Determination of Amino Acids by Microbiological Assay

The quantitative determination of bound and free amino acids was carried out by microbiological assay. The organism used was Leuconostoc mesenteroides P. 60 and the essential amino acids assayed were L-lysine, L-leucine, and L-arginine.

The innoculum for the assay was prepared by subculturing from a stock culture to 10 ml. of Bacto-Micro Innoculum broth. After 16-24 hours incubation at 30-37° C., the cells were centrifuged under aseptic conditions and the supernatant liquid decanted. The cells were re-suspended in 10 ml. sterile isotonic solution of sodium chloride. The cell suspension was diluted 5 to 100 and one drop of this latter suspension was used to inoculate each of the assay tubes. Assay tubes were

incubated for 18 hours at 33° C. Readings were taken on the Klett-Summerson electrophotometer at 425 millimicrons.

A standard curve for lysine was obtained at levels of 0.0, 30, 60, 90, 120, 150, 180, 240, 300 micrograms per assay tube containing 10 ml. of solution. This concentration was prepared by dissolving 6.0 gm. of l-lysine assay medium in 1000 ml. distilled water. This stock solution was diluted 1 to 99 parts distilled water and the following amounts were used in each tube: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 ml.

For l-leucine the standard curve was obtained at levels of 0.0, 10.0, 20, 30, 40, 50, 60, 80, and 100 micrograms per assay tube containing 10 ml. of solution. The stock solution was prepared by dissolving 2.0 g. of l-leucine in 1000 ml. of distilled water. This stock solution was further diluted 1 to 99 parts of distilled water and the following amounts were used in each tube: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, ml. per tube.

The levels obtained for the l-arginine assay were 0.0, 20, 40, 60, 80, 100, 120, 160, and 200 micrograms per assay tube (10 ml.). In order to prepare these concentrations 4.0 g. of l-arginine assay medium was dissolved in 1000 ml. of distilled water. After diluting this stock solution 1 to 99 parts of distilled water the following amounts were used in each tube: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 ml. per tube.

Preparation of unknowns for the microbiological assays of bound amino acids were prepared as follows: hydrolysis was carried out as mentioned earlier in the chromatographic studies, but the hydrolysates were diluted to 10 ml. with water. Out of the latter dilution 2 ml. were

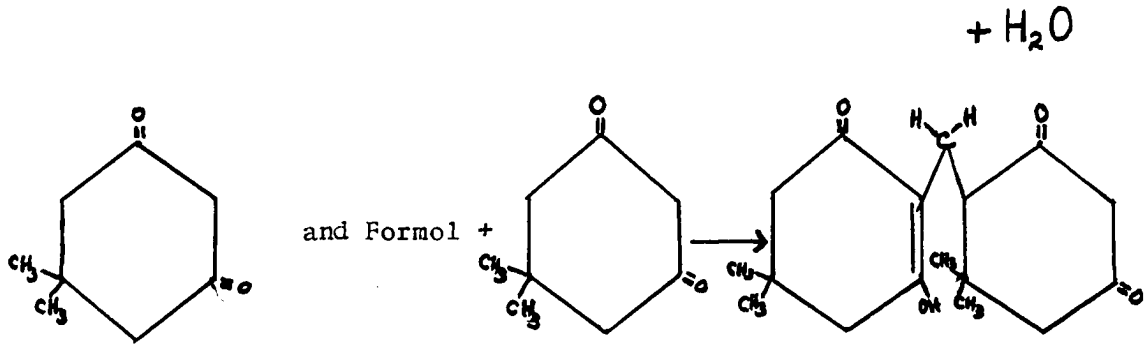
placed in a 50 ml. volumetric flask. To test tubes containing the basal medium were added 0.5, 1.0, 1.5 ml. of the last dilution. All determinations were done in triplicate.

For the assays of the free amino acids 5 ml. of whole serum plasma was used as a sample after proteins had been precipitated with absolute ethanol.

Formaldehyde Determination

The method used for formaldehyde determination (Pangborn, 1936) is essentially that recommended by Velluz and depends on the fact that the precipitation of formaldehyde by dimethyldihydroresorcinol (dimedon) can be made quantitative by establishing the appropriate condition.

Reaction:



Dimedon Dimedon Formol Dimedon

Free formaldehyde was determined by mixing 1 ml. of sample with 20 ml. of dimedon (4.5 g./1.). After the mixture was left standing at room temperature for 4 hours the precipitate was collected on a Whatman #50 filter paper previously weighed after being kept in a desiccator. The precipitate was then washed with water, dried for 2 hours at 110° C., and weighed on an analytical balance.

Bound formaldehyde was determined by the same procedure except

that the mixture of sample with dimedon was allowed to stand for 72 hours. By this method 1 mg. of formol-dimedon is equal to 0.1027 mg. formaldehyde.

Molecular Weight Determination

For the determination of molecular weights, advantage was taken of the fact that colloidal solutions such as proteins scatter part of the incident light.

The intensity of the scattered light increases with the number and size of the colloidal particles according to the formula:

(A) $T = MH\,c$

T = excess turbidity

M = molecular weight

c = concentration in grams/cm.^{3}

The proportionality constant H can be found if we know the refractive indices of the solution and solvent from Debye's equation (Debye, 1949):

(B) $$H = \frac{32\pi^3}{3} \cdot \frac{M_o^2}{N\lambda^4} \cdot \left(\frac{M - M_o}{c}\right)^2$$

Where: N = Avogadro's number

M = Refractive index of solution

M_o = Refractive index of solvent

λ = Wave length of light used

C = Concentration in g./cm.^{3}

Light scattering determination was made according to the method outlined by Holmes, Nutting and Brice (1951), with some alteration, such as using the biuret reaction as a method of protein concentration rather than the dry weights method.

Method

The instruments used were an Aminco Light Scattering Microphotometer No. 4-6001 and an Abbey Refractometer for refractive index measurement.

Proteins after being precipitated with 1/3 saturated, $\frac{1}{2}$ saturated and saturated ammonium sulfate, were washed three times with distilled water and put in solution with 0.9 per cent sodium chloride. Careful washing is very important since the refractive index of ammonium sulfate solution will be different from that of sodium chloride solution. After the proteins were suspended in saline, the protein concentration was determined by the biuret reaction and the refractive indices of both the protein solutions and the 0.90 per cent sodium chloride solution were determined in an Abbey refractometer using sodium light of wave length 589 millimicrons. The error due to using the D line of sodium instead of the blue light of mercury (436 millimicrons) was approximately 10 per cent.

A square cross-section cell (2.40 cm. in length) was used for scattering readings. Distilled water was placed in the cell and for a given wave length the sensitivity of the instrument at 0° was adjusted to give a large meter deflection (I_{o}). The water was then replaced with a solution of "Ludox" of approximately 3 per cent concentration. "Ludox" is a stable silica suspension whose optical density at a 3 per cent level is inversely proportional to the fourth power of the wave length. Thus, keeping the sensitivity constant and using blue light, of wave length 436 mu., a deflection caused by an unknown can be expressed in turbidity. Formula C was used to calculate turbidity.

$$(C) \qquad t = \frac{2.303}{d} \cdot \log \frac{I_o}{I} = .958 (D - D_o)$$

Where: d = path length of cell

I_o = transmission in water

I = transmission in Ludox

Animal Experiments

Urea Production in Normal Dogs and Dogs Injected with Altered Bovine Plasma. A 19 pound dog was kept on a protein free diet and urea excreted in the urine determined for three consecutive days. The method used was that of Folin and Youngburg (1919). After the normal value for this dog was determined treated plasma was injected in 30 ml. volumes for 6 consecutive days and urea determination in the urine carried out on the third and sixth day of injection.

Procedure. Five ml. of urine was diluted to 100 ml. in a volumetric flask and mixed well. One ml. of the diluted urine was transferred to a series of test tubes to which were later added 1 ml. of an alcoholic urease solution and a drop of buffer solution. The tubes were digested in a beaker of warm water (40° to 55° C.) for five minutes or at room temperature for 15 minutes, at the end of which time the contents of the test tubes were transferred with rinsings to 100 ml. volumetric flasks, diluting to a volume of about 80 ml. A standard was prepared in another flask by adding sufficient standard nitrogen solution to contain 0.5 mg. of nitrogen, 1 ml. of urease solution, and water to a volume of

about 80 ml. To each flask was added 10 ml. of Nessler's reagent, then diluted immediately to 100 ml., mixed, and allowed to stand for five minutes. Readings were made in a photometer in the usual way. For photometric measurement the photometer was set to zero density with a blank prepared similarly to the standard except that the standard nitrogen solution was omitted. Ammonia determinations were made in the same manner except that urease was omitted.

For photometric measurement:

$$\frac{\text{Density of Unknown} \times \text{mg. of N. in Standard Solution} \times \text{Dilution}}{\text{Density of Standard}} = \text{mg. of urea plus ammonia nitrogen in 1 ml. of undiluted urine.}$$

Protein Plasma Levels in Rabbits Given Altered Bovine Plasma after Protein Value had been Reduced by Consecutive Bleeding. Procedure:

The plasma protein levels of rabbits A and B were determined by the biuret method. For seven consecutive days both rabbits were bled at the rate of 30 ml. a day by cardiac puncture. Only at the end of the sixth day, blood protein values were determined. For the next six consecutive days rabbit A was injected with 120 ml. of altered bovine plasma intravenously using the ear-veins. Both rabbits were fed a protein free diet. Rabbit B served as a control and was not treated. At the end of the 12th day, plasma protein levels were again determined in both rabbits A and B. Rabbit A was given again 100 ml. of altered plasma in the next five consecutive days while both rabbits were kept on the

protein free diet. After this, rabbit B was given 20 ml. of altered plasma each day for six consecutive days while rabbit A was allowed to continue this time on a protein free diet. At the end of this last period the plasma protein levels were determined for both rabbits A and B.

Studies with Radioactive I^{131} labeled Treated Plasma, Rat Plasma, Normal Bovine Plasma and RISA. Treated plasma, rat plasma, and normal bovine plasma were labeled with Abbott I^{131} sodium iodide. The procedure used was that outlined by Crispell, Porter and Nieset (1950) which consists of the following steps. To five mg. potassium iodide were added 10 ml. of the original I^{131} which contained 250 microcurie. The mixture was acidified with 1 ml. of 0.08 N solution of sulfuric acid and 60 ml. of 4 per cent hydrogen peroxide was added. This solution was allowed to stand for one hour at room temperature. One hundred ml. of the sample to be labeled was added to this mixture together with 50 ml. of 7 per cent sodium bicarbonate. This was allowed to stand for 12 hours with occasional agitation.

This iodo-protein mixture was dialysed against distilled ice cold water. Dialysis was run for approximately 48 hours in order to remove all possible iodide which may have remained free within the dialysis bag. During the dialysis 2 ml. aliquots of the ice water bath (dialysate) were counted from time to time for radioactivity. When no radioactivity remained in the bath the dialysis was stopped.

After the dialysed protein sample was labeled, 3 ml. aliquots were injected into rats. Three sets of 5 rats each were run with each labeled product. One set of rats was sacrificed at the end of 24 hours,

another at the end of 48 hours, and the final batch after 72 hours.

The counting of the radioactive material was done with a Tracerlab SC-18A Super scaler using a P20W Scintillation well counter equipped with a sodium iodide crystal activated with thallium.

Lungs, kidneys, liver, and spleen were removed from each sacrificed animal. Plasma and urine were collected for the time the animals were under study. All the mentioned samples were counted for radioactivity with the proper corrections for decay. Each organ was properly weighed.

CHAPTER III

RESULTS

Some data were obtained showing the lack of antigenicity of bovine plasma after being treated with formaldehyde and heat. Ring precipitation studies indicated as shown by Table I that both human serum and normal bovine serum are capable of inciting the production of antibodies but that chemically treated bovine plasma does not incite the production of a homologous antiserum. It is clearly seen that human plasma when mixed with its homologous antiserum causes precipitation in titer as high as 1/4000; normal bovine plasma reacts similarly, but chemically treated bovine plasma when mixed with its homologous antiserum results in no precipitation. This indicates the absence of antigenicity in the treated plasma.

Further cross reaction experiments indicate (Table II) that immune serum against human serum will cross react with normal bovine plasma antigen in titer as high as 1/1000 and with human serum antigen in 1/4000 titer but that it will not react at all with chemically treated bovine plasma. Results in Table III show that immune serum against bovine plasma will cross react with human serum antigen in titer as high as 1/2000 and with its homologous antigen in titer as high as 1/4000 but in no case precipitation was obtained with chemically treated

TABLE I

ANTIGEN-ANTIBODY REACTIONS BETWEEN TREATED PLASMA, NORMAL BOVINE PLASMA AND HUMAN PLASMA, EACH AGAINST ITS HOMOLOGOUS ANTISERUM

Antigen (1/10 dilution)	Dilution of serum								
	Saline control	1/500	1/1000	1/2000	1/4000	1/6000	1/8000	1/12000	1/16000
Treated plasma	-	-	-	-	-	-	-	-	-
Normal bovine plasma	-	+	+	±	-	-	-	-	-
Human plasma	-	+	+	+	±	-	-	-	-

Experimental animal: 3 rabbits

+ = strong precipitation

± = mild precipitation

- = negative

TABLE II

ANTIGEN-ANTIBODY REACTIONS BETWEEN TREATED PLASMA, NORMAL BOVINE PLASMA AND HUMAN PLASMA AGAINST IMMUNE SERUM DEVELOPED BY THE INJECTION OF HUMAN PLASMA

	Dilution of immune serum against human plasma								
Antigen 1/10 dilution	Saline control	1/500	1/1000	1/2000	1/4000	1/6000	1/8000	1/12000	1/16000
Treated plasma	-	-	-	-	-	-	-	-	-
Normal bovine plasma	-	±	±	-	-	-	-	-	-
Human plasma	-	+	+	±	±	-	-	-	-

+ = strong precipitation

± = mild precipitation

- = negative

TABLE III

ANTIGEN-ANTIBODY REACTION BETWEEN TREATED PLASMA, NORMAL BOVINE PLASMA AND HUMAN PLASMA AGAINST IMMUNE SERUM DEVELOPED BY THE INJECTION OF NORMAL BOVINE PLASMA

Antigen 1/10 dilution	Dilution of immune serum against human plasma								
	Saline control	1/500	1/1000	1/2000	1/4000	1/6000	1/8000	1/12000	1/16000
Treated plasma	-	-	-	-	-	-	-	-	-
Normal bovine plasma	-	+	+	+	±	-	-	-	-
Human plasma	-	+	+	±	-	-	-	-	-

+ = strong precipitation

± = mild precipitation

- = negative

bovine plasma. These results correlate closely with those of Massons (1946) and Larget and Culot (1952) who found that chemically treated bovine plasma lost the capacity to produce antibodies and hence no anaphylactic reaction occurred in guinea pigs. Re (1940) found no uterine contraction when chemically treated bovine plasma was used as the challenging dose. Contradictory results on the antigenicity of chemically treated bovine plasma were reported by Carlinfanti and Pontecorro (1948), who found strong anaphylactogenic reactions on guinea pigs sensitized with chemically treated bovine plasma.

Highly interesting results were obtained with cross matching experiments of human blood groups O^+, A^+, B^+, AB^+ against both normal bovine plasma and chemically treated bovine plasma. In Table IV it is apparent that chemically treated bovine plasma does not agglutinate or hemolyse any of the human blood types within 30 or 60 minutes, while complete hemolysis is found with normal bovine plasma. This seems to indicate that chemical treatment causes the removal of non-specific agglutinins and hemolysins present in normal bovine plasma. The elimination of such non-specific hemolysis represents an advantage over the other protein plasma substitute gelatin, since it has been found by Koop (1945) that gelatin causes hemoagglutination of human erythrocytes.

Protein Studies on Chemically Treated Bovine Plasma

Results. Electrophoretic patterns, pH 8.6 in veronal buffer, showed the disappearance of the globulin fractions in chemically treated bovine plasma (Fig. 1a, b). Similar results were obtained by Regamey (1952) and Sieber (1953). Studies of a mixture of treated plasma and

TABLE IV

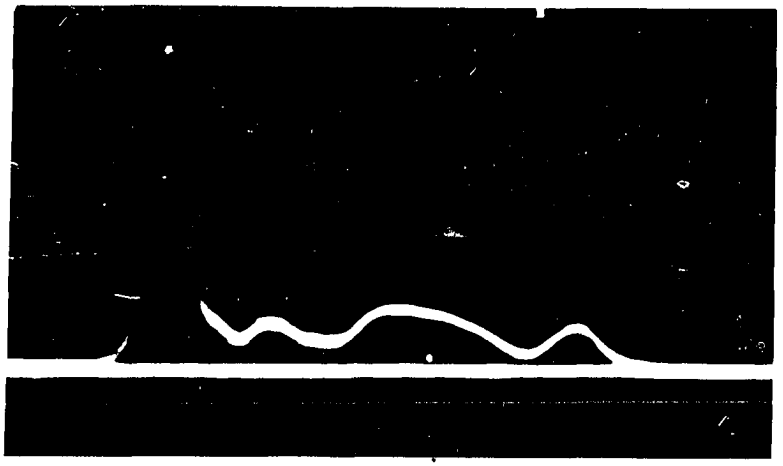
IMMUNOLOGICAL STUDIES OF NORMAL AND CHEMICALLY

TREATED BOVINE PLASMA AGAINST HUMAN ERYTHROCYTES

Cells	Normal bovine plasma	Chemically treated bovine plasma
O+	complete hemolysis *	negative hemolysis **
A+	complete hemolysis	negative hemolysis
B+	complete hemolysis	negative hemolysis
AB+	complete hemolysis	negative hemolysis

* complete hemolysis within 30 minutes

** no hemolysis after 60 minutes



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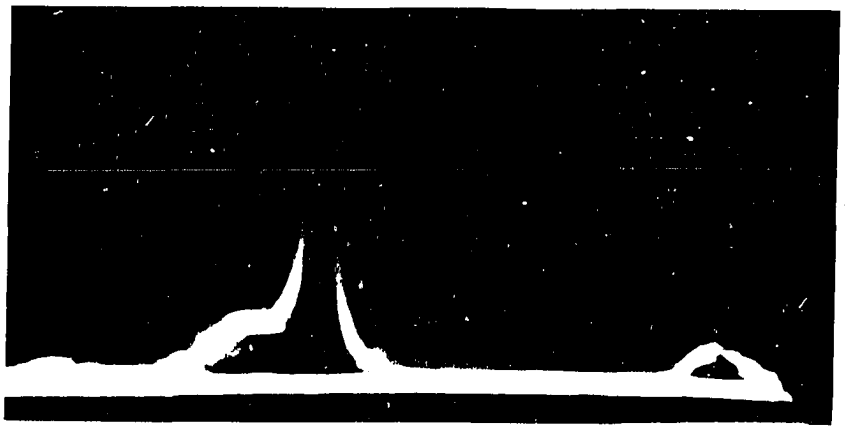

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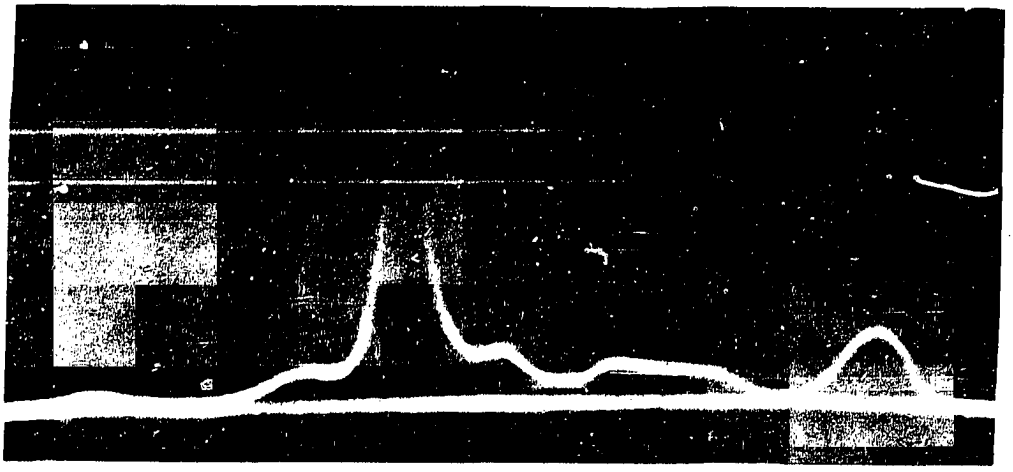

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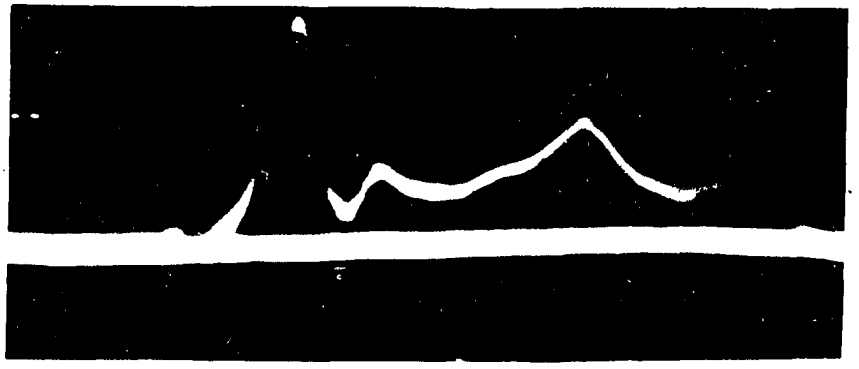
Fig. 1. Electrophoretic patterns, ascending boundaries, 0.1 N barbiturate buffer, pH 8.6. a. Normal bovine serum, b. bovine plasma treated with formaldehyde and heat, c. mixture of a and b.

normal bovine plasma at pH 8.6 (Fig. 1c) seemed to indicate, as stated by Regamey (1952), that the globulins, because of treatment, increased in mobility, joining the albumin as a single fraction. However, at pH 7.8 a resolution of a mixture of chemically treated bovine plasma and normal bovine plasma occurred (Fig. 2c), with the chemically treated bovine plasma protein moving ahead of the normal plasma albumin. This indicated that chemically treated bovine plasma contained proteins of a completely altered nature as compared to the original bovine plasma.

Fractionation studies indicate that 85 per cent of the chemically treated bovine plasma is precipitated in 33 per cent saturated ammonium sulfate. This fraction corresponds in its solubility properties to γ-globulin in normal human serum (Cohn, 1940). Similarly, the fraction not precipitated with 26 per cent sodium sulfate, which is a measure of albumin in human sera, was found to be only 12 per cent of the total protein. In normal bovine plasma 50-60 per cent is not precipitated in 26 per cent sodium sulfate.

Massons (1946) believes that by chemical treatment of bovine plasma with formaldehyde and heat, the albumin-globulin ratio remains normal. In this study no evidence for this postulation can be cited except that it was found that the hexosamine values in total protein did not change by treatment (Table V). As the hexosamine content of albumin is very low, as compared to that of globulin, this would indicate that the treatment has not changed the original proportions of albumin and globulin.

The total protein content of the chemically treated bovine plasma as determined by the biuret reaction was less than the normal



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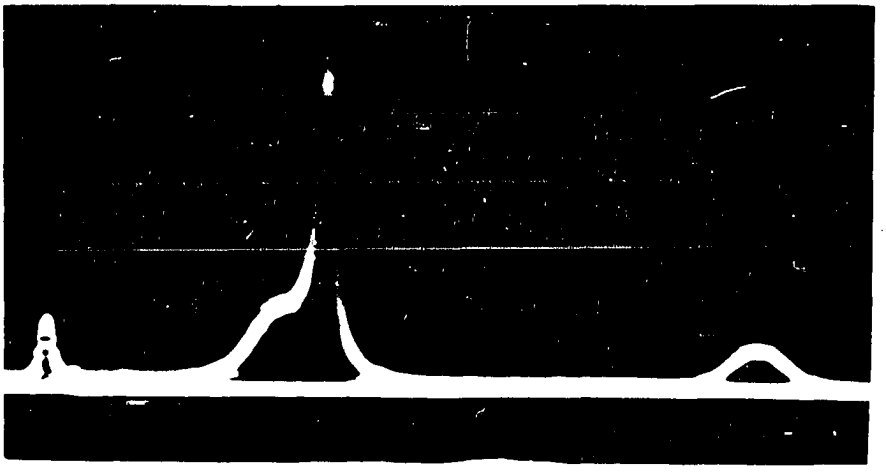

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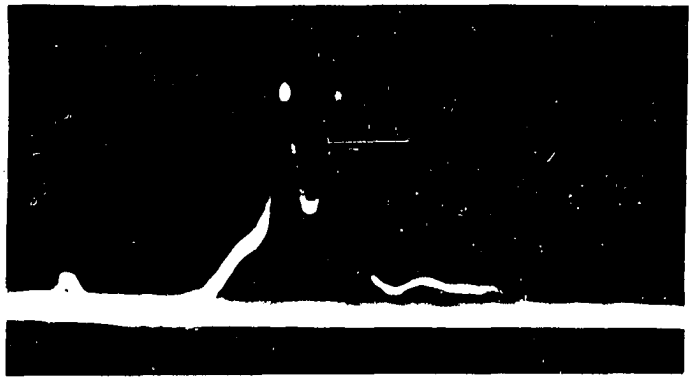

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Fig. 2 Electrophoretic patterns, ascending boundaries, 0.16 N phosphate buffer, pH 7.8. a. Normal bovine serum, b. bovine plasma treated with formaldehyde, c. Mixture of a and b.

TABLE V

COMPARATIVE PROTEIN STUDIES OF CHEMICALLY TREATED AND UNTREATED BOVINE PLASMA*

Determination	Treated	Untreated
Total protein** biuret	2.26 (2.08-2.63)	6.58 (6.28-6.89)
Micro-Kjeldahl (N x 6.26)	2.55 (2.45-2.67)	6.54 (6.27-6.96)
Ppt. with 33 1/3 NH_4SO_4***	85%	20%
Soluble in 26% Na_2SO_4***	12.4%	60%
Immunochemical albumin***	0	61%
Electrophoretic albumin****	90%	57.0%
Electrophoretic globulins	0	43.0%
Bound hexosamine***	1.19 (1.14-1.26)	1.12 (1.10-1.13)
Specific gravity	1.0347 (1.034-1.036)	1.026
pH	6.8-7.2	7.4

* Based on analysis of 4 treated and 3 untreated samples

** Grams per 100 ml. of plasma

*** Percentage of the total plasma protein

**** At pH 8.6 veronal buffer, 0.1 M.

bovine plasma (Table V). This was probably due to the dilution of the product during the treatment process. Also, it was found that the Kjeldahl values for total protein of the chemically treated bovine plasma were slightly higher than the values found by the biuret reaction. Similar results were found by Ardry and Stark (1954).

As it was mentioned earlier, the chemical treatment seems to have eliminated non-specific agglutinins or hemolysins from the bovine plasma. It is very possible, therefore, that the elimination of the conventional globulin fractions, as shown by electrophoretic determinations, is related to the disappearance of the mentioned non-specific antibodies. Edward (1942, 1943) noted that by elimination of the globulin fraction from normal bovine plasma he eliminated both non-specific agglutination and hemolysis.

At this point it may be mentioned that as shown by Chow's method (Figure 3) the fraction which moves together with normal albumin at pH 8.6 is immunochemically different from normal albumin. Normal bovine plasma readily reacted with antibody formed against bovine albumin (Armour), while no reaction occurred with the treated plasma fraction mentioned.

Electrophoretic studies agree with molecular weight determination by light scattering. Table VI indicates the particle weights of fractions precipitated with 33 per cent ammonium sulfate from four different batches of treated bovine plasma. Fractions from three batches showed particle weights of 61,000, 70,000, and 51,000. The fraction from one batch, however, showed a particle weight of 200,000. One reason for this great difference could be an alteration in the

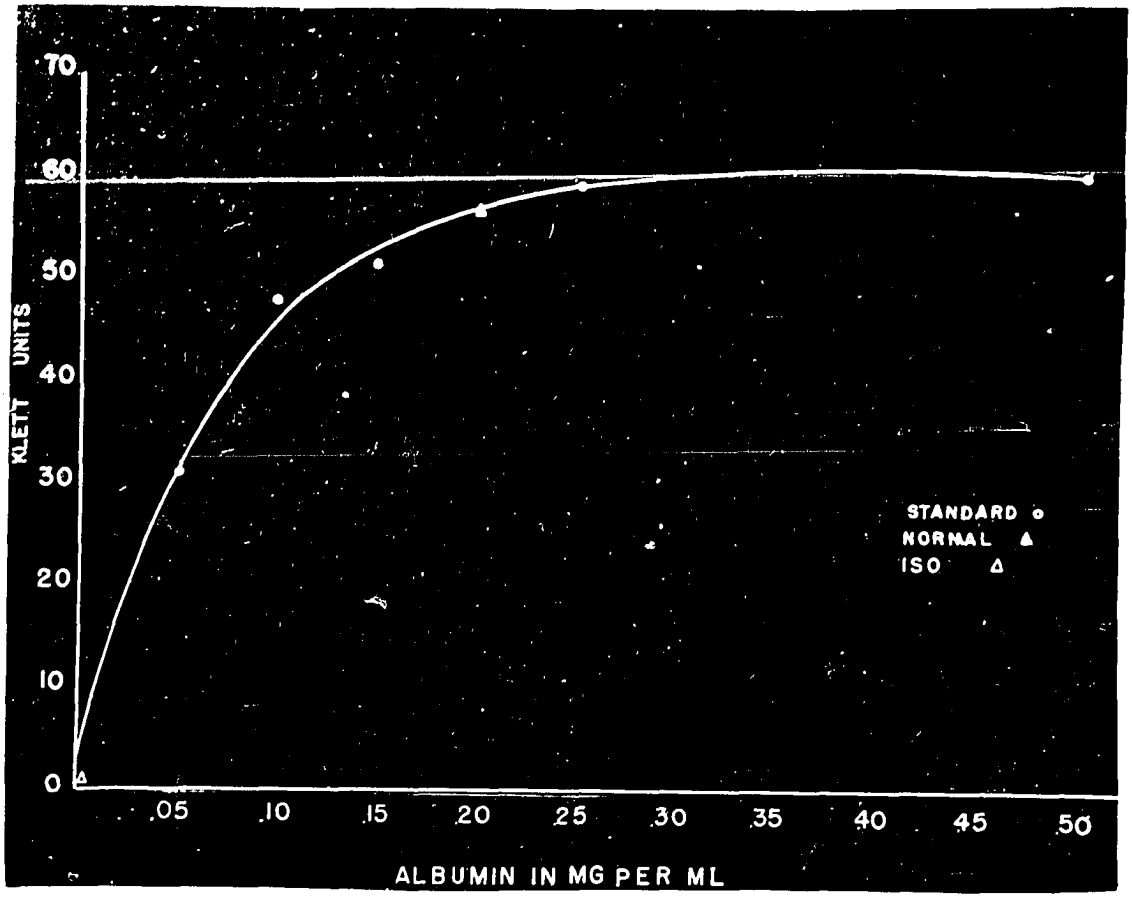


Figure 3. Standard antigen-antibody precipitation curve for albumin in antibody excess. Unknowns, normal bovine plasma, and chemically treated bovine plasma are analyzed for the presence of albumin.

TABLE VI

PARTICLE WEIGHTS DETERMINED BY LIGHT SCATTERING

Protein chemically treated bovine plasma	Concentration*	Turbidities	Refractive index of solution-refractive index of solvent	Mean weight
1	0.42	0.152	0.0060	61,000
2	0.42	0.172	0.0060	70,000
3	0.42	0.138	0.0065	51,000
4	0.80	0.256	0.0060	200,000
Crystalline bovine albumin	2.69	0.0344	0.0070	70,000
Normal bovine plasma				
A	1.90	0.0572	0.0050	160,000
B	1.61	0.0375	0.0042	140,000
C	1.96	0.0322	0.0055	95,000
(1)	0.61	0.171	0.0065	100,000
(2)	0.62	0.132	0.0060	79,000

Chemically treated bovine plasma (fraction precipitated with 1/3 saturated ammonium sulfate represents 85% of total protein)

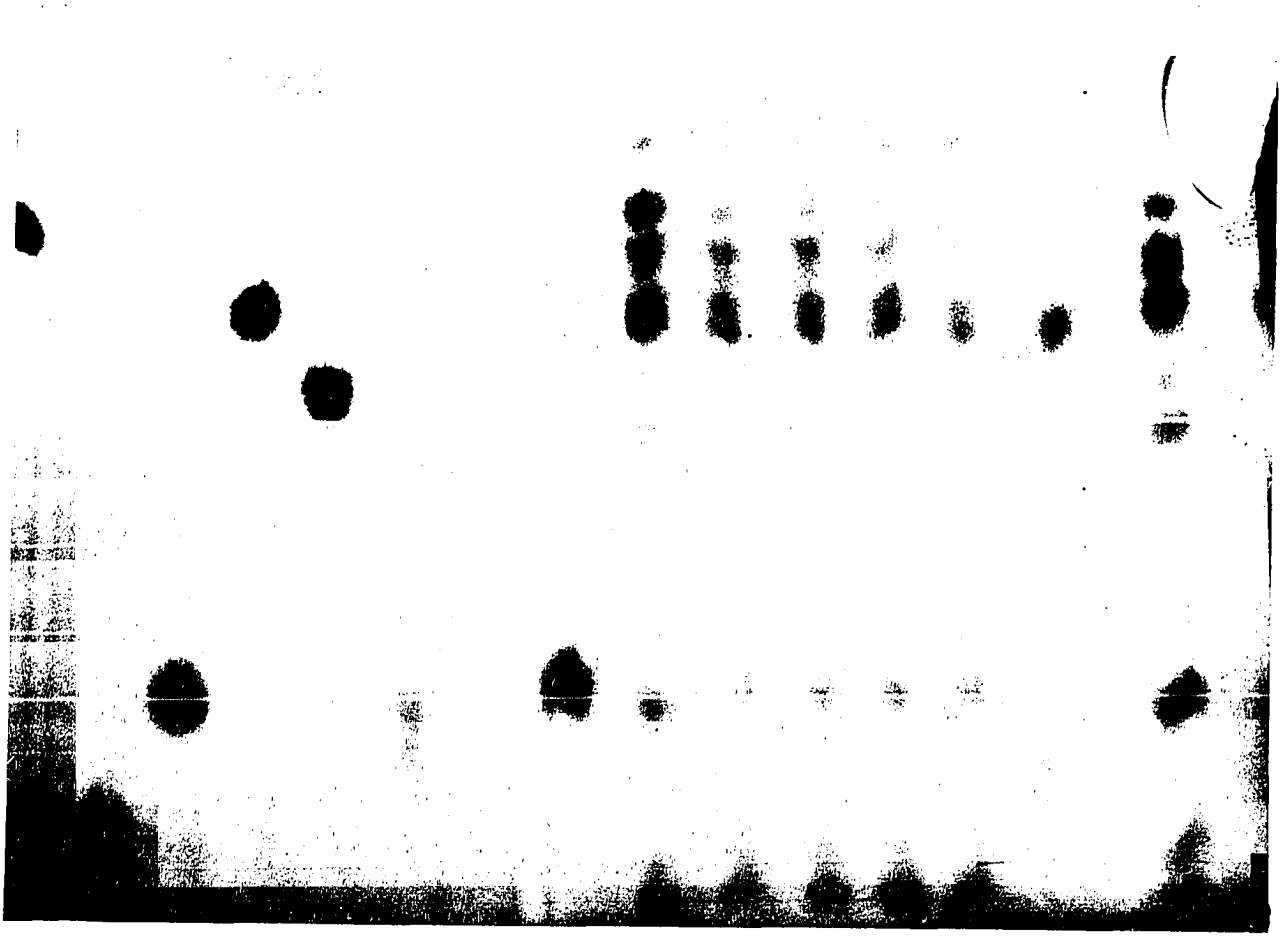
Normal bovine plasma A - Fraction precipitated with 1/3 $(NH_4)_2SO_4$ (ammonium sulfate)
B - Fraction precipitated with 1/2 $(NH_4)_2SO_4$ (ammonium sulfate)
C - Fraction precipitated with sat.$(NH_4)_2SO_4$ (ammonium sulfate)
(1) and (2) Chemically treated bovine plasma precipitated with saturated $(NH_4)_2SO_4$ (ammonium sulfate)

* Concentration in g./100 ml. of sample

preparation.

The particle weights being approximately equal to that of normal albumin (Table VI), agree with the electrophoretic patterns described, since it is known that the size of the particle is an important factor in mobility. Also, these data indicate that some complexing may have occurred in the batch whose particle weight was found to be 200,000. These results correlate with those of Gutfreund and Ogston (1945) who found, as mentioned before, that when serum is heated with formaldehyde there is an aggregation of protein to form a polydisperse system of larger particles with a decrease in osmotic pressure. But the studies of Melka (1947) with the osmotic pressure led to the conclusion that no change in molecular size had occurred.

Qualitative Determination of Amino Acids and Carbohydrates. - Results. The results obtained from paper chromatography of amino acids indicate that chemical treatment of bovine plasma does not change the qualitative content of the amino acids as compared to normal bovine plasma (Figures 4A and B, 5A and B, 6A and B). Of the amino acids found by Rose (1937) to be essential for rats, valine, leucine, threonine, histidine, and arginine were found in the acid hydrolysates of chemically treated bovine plasma. Methionine, tryptophan and phenylalanine were not found. There was no direct proof, according to chromatography, for the presence of lysine because its Rf value was very close to those of glycine and aspartic acid; however, its presence was indicated by the growth of Leuconostoc mesenteroides in a lysine free medium to which the hydrolysate of the treated plasma had been added. Finding phenylalanine is always



mixture
glucosamine
batch 4 treated plasma
batch 3 treated plasma
batch 2 treated plasma
batch 1 treated plasma
normal bovine plasma
valine
tyrosine
tryptophan
threonine
serine
methionine
phenylalanine
lysine

Figure 4A. Paper chromatography of amino acids-butanol-acetic acid-water solvent.



Figure 4B. Paper chromatography of amino acids in butanol:acetic acid: water solvent.

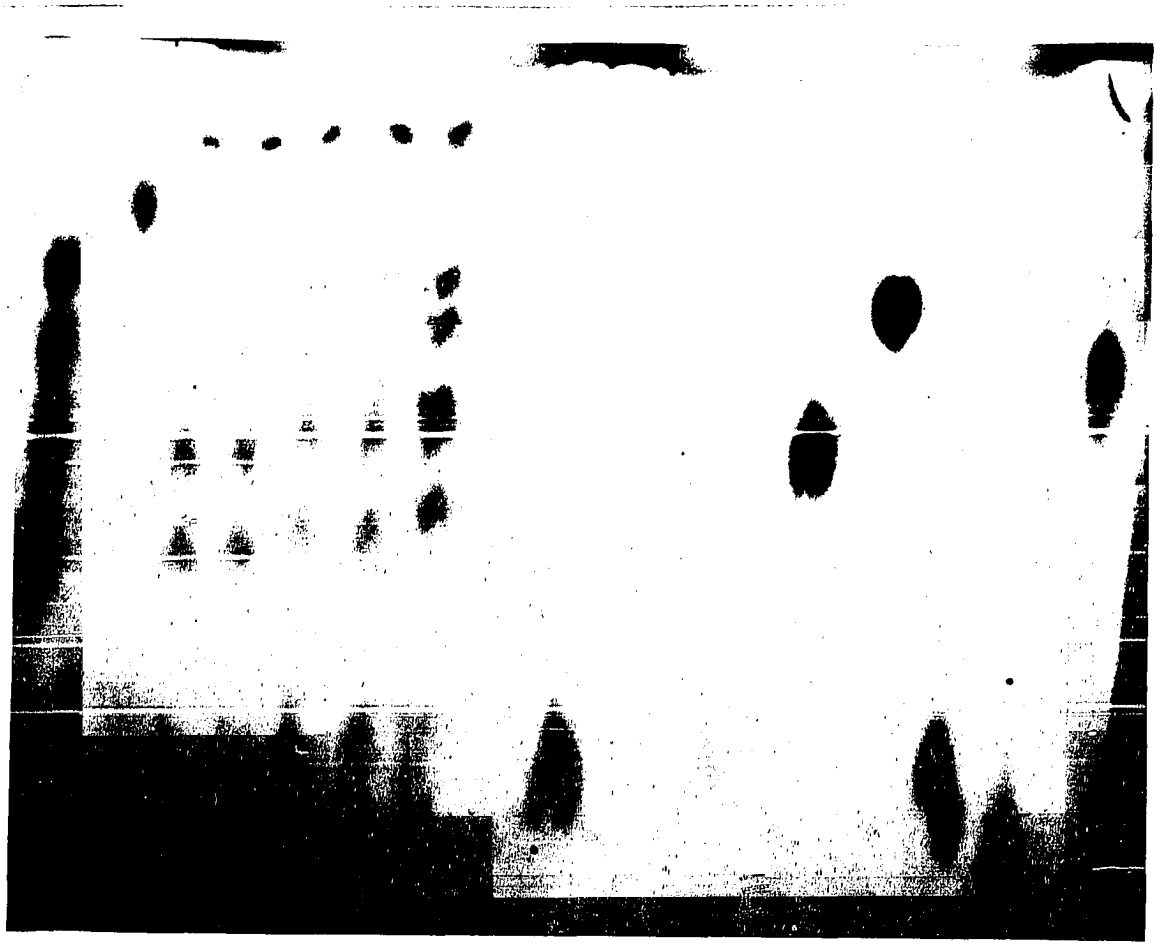


Figure 5A. Paper chromatography of amino acids in phenol:water solvent.

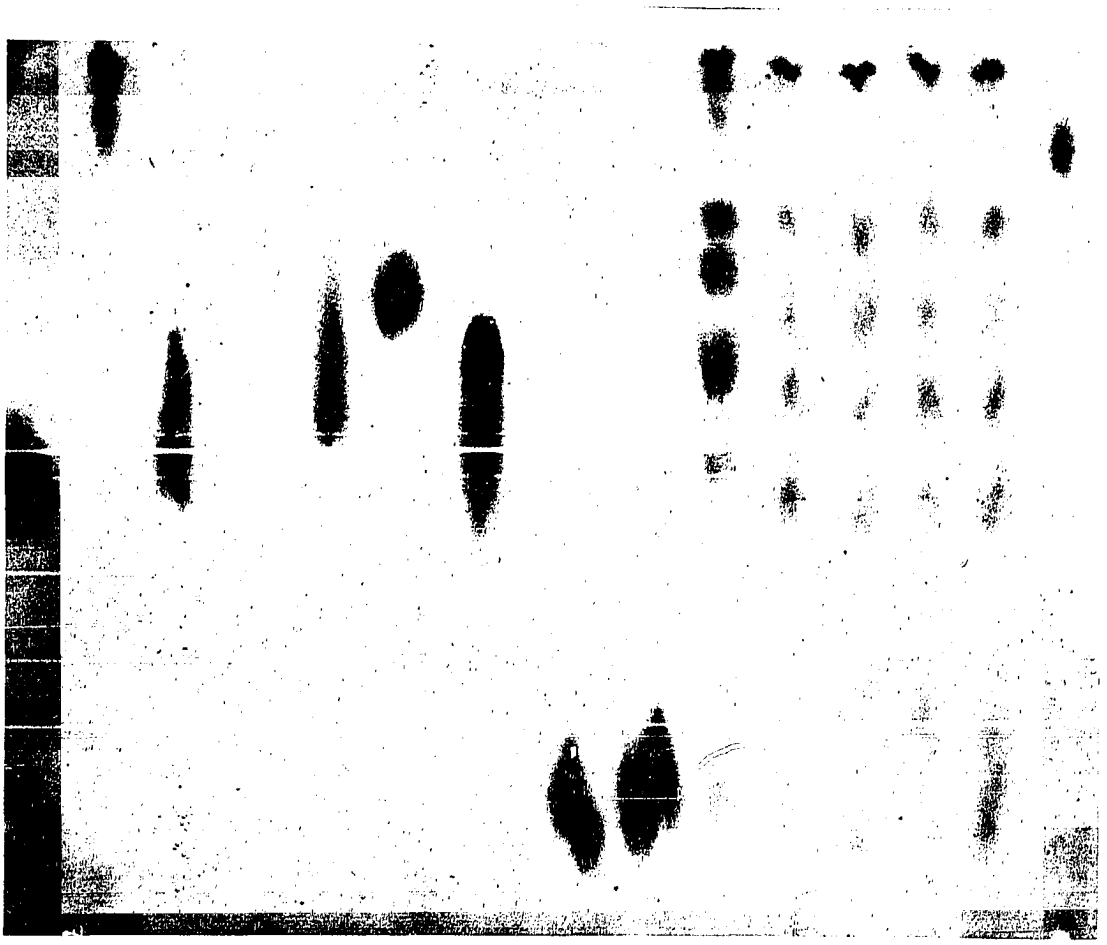
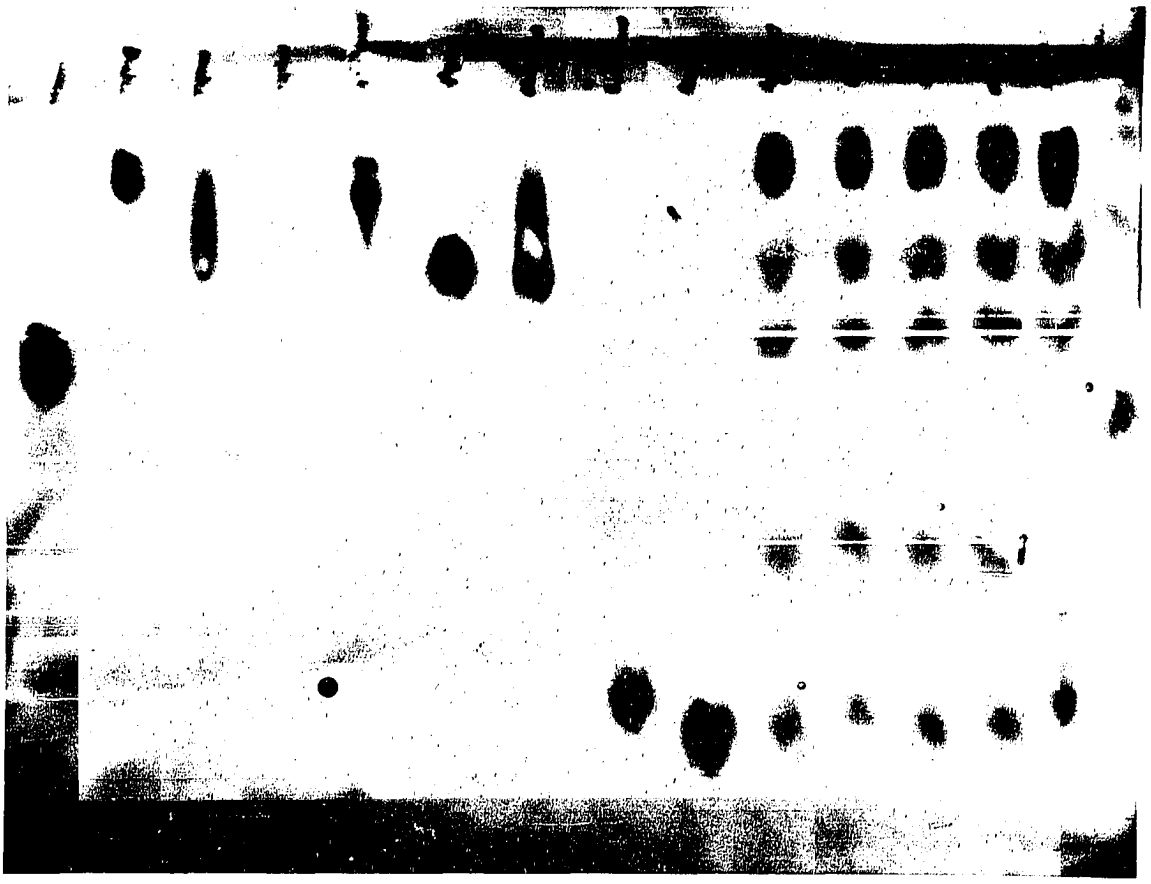


Figure 5B. Paper chromatography of amino acids in phenol:water solvent.



glucosamine
normal bovine plasma
batch 4 treated plasma
batch 3 treated plasma
batch 2 treated plasma
batch 1 treated plasma
leucine
isoleucine
histidine
glycine
glutamic acid
cystine
arginine
aspartic acid
alanine

Figure 6A. Paper chromatography of amino acids in butanol:pyridine: water solvent.

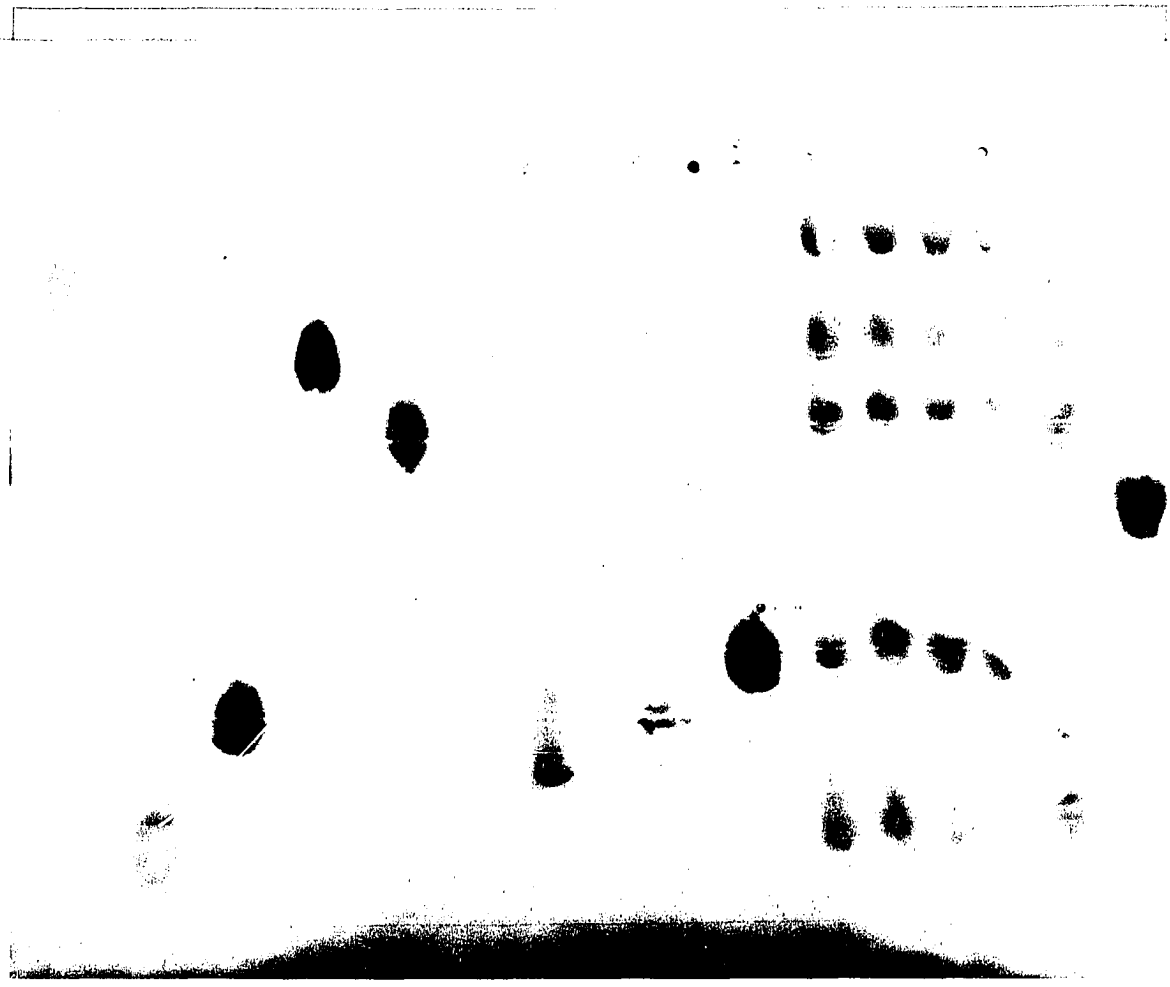


Figure 6B. Paper chromatography of amino acids in butanol:pyridine: water solvent.

troublesome by this technique since it travels closely to the leucine, making its identification by paper chromatography difficult. Tryptophan is lost in acid hydrolysis. The Rf values obtained in the three different solvents are in close correlation with those published (Block, 1955).

From Tables VII, VIII, and IX, the following amino acids were identified in the treated plasma: alanine, cystine, arginine, isoleucine, leucine, threonine, valine, aspartic acid, serine, histidine, glutamic acid, and glycine. These results agree with those of Giri (1952) who found the following amino acids in serum: leucine, methionine, valine, alanine, lysine, histidine, glutamic acid, glycine, arginine, cystine, and serine. Likewise, Block has noted the presence of the following amino acids in serum: arginine, histidine, lysine, tyrosine, tryptophan, cystine, threonine, leucine, isoleucine, glutamic, aspartic, glycine, alanine, proline. It is worth mentioning that phenylalanine was not found by either workers and methionine only by Giri.

The studies of free and bound carbohydrates with paper chromatography indicated an increase of free sugars in chemically treated bovine plasma as compared to the normal. Figure 7 indicates the presence of free glucosamine, fructose and glucose in all the chemically treated bovine plasma while only glucose was detected in protein free normal bovine. The presence of fructose can be explained as due to the invert sugar added to the product in the manufacturing, but the existence of an amino sugar indicated that either some amination of the sugar had occurred during the treatment or that some of the bound hexosamine had been freed from its protein complex. The former process is feasible since the plasma was heated for one hour at 100° C. during the process

TABLE VII

AMINO ACIDS-Rf VALUES IN BUTANOL-ACETIC ACID-WATER SOLVENT

	Rf values	Normal bovine	Chemically treated bovine plasma			
			1	2	3	4
Alanine	0.300	0.081	0.081	0.080	0.079	0.079
Aspartic acid	0.136	0.132	0.133	0.130	0.130	0.130
Arginine	0.180	0.165	0.163	0.160	0.165	0.160
Cystine	0.080	0.180	0.181	0.180	0.179	0.179
Glutamic acid	0.230	0.228	0.228	0.233	0.220	0.220
Glycine	0.220	0.270	0.273	0.271	0.270	0.270
Histidine	0.101	0.304	0.308	0.305	0.303	0.300
Isoleucine	0.630	0.360	0.370	0.370	0.370	0.370
Leucine	0.651	0.490	0.470	0.480	0.486	0.490
Proline	0.373	0.625	0.627	0.627	0.627	0.620
Lysine	0.132	0.649	0.648	0.649	0.647	0.638
Phenylalenine	0.573					
Methionine	0.485					
Serine	0.200					
Threonine	0.270					
Tryptophan	0.522					
Tyrosine	0.407					
Valine	0.486					

TABLE VIII

AMINO ACIDS-Rf VALUES IN BUTANOL-PYRIDINE-WATER SOLVENT

	Known Rf values	Normal bovine	Chemically treated bovine plasma 1	2	3	4
Alanine	0.21	0.062	0.052	0.052	0.053	0.052
Aspartic acid	0.052	0.140	0.120	0.130	0.120	0.130
Arginine	0.120	0.194	0.191	0.186	0.186	0.181
Cystine	0.110	0.351	0.351	0.350	0.361	0.352
Glutamic acid	0.088	0.510	0.500		0.508	0.508
Glycine	0.130					
Histidine	0.140					
Isoleucine	0.490					
Leucine	0.510					
Lysine	0.074					
Phenylalanine	0.51					
Methionine	0.38					
Serine	0.13					
Threonine	0.192					
Tryptophan	0.430					
Tyrosine	0.380					
Valine	0.360					

TABLE IX

AMINO ACIDS-Rf VALUES IN PHENOL-WATER SOLVENT

	Rf values	Normal bovine	Chemically treated bovine plasma 1	2	3	4
Alanine	0.554	0.181	0.185	0.188	0.182	0.186
Aspartic acid	0.181	0.324	0.329	0.324	0.328	0.315
Arginine	0.537	0.400	0.409	0.405	0.400	0.404
Cystine	-	0.469	0.476	0.478	0.475	0.486
Glutamic acid	0.490	0.560	0.565	0.577	0.565	0.574
Glycine	0.398	0.828	0.822	0.820	0.820	0.828
Histidine	0.571					
Isoleucine	0.852					
Leucine	0.819					
Lysine	0.406					
Phenylalanine	0.846					
Methionine	0.803					
Serine	0.314					
Threonine	0.478					
Tryptophan	0.768					
Tyrosine	0.584					
Valine	0.772					
Proline	yellow color					

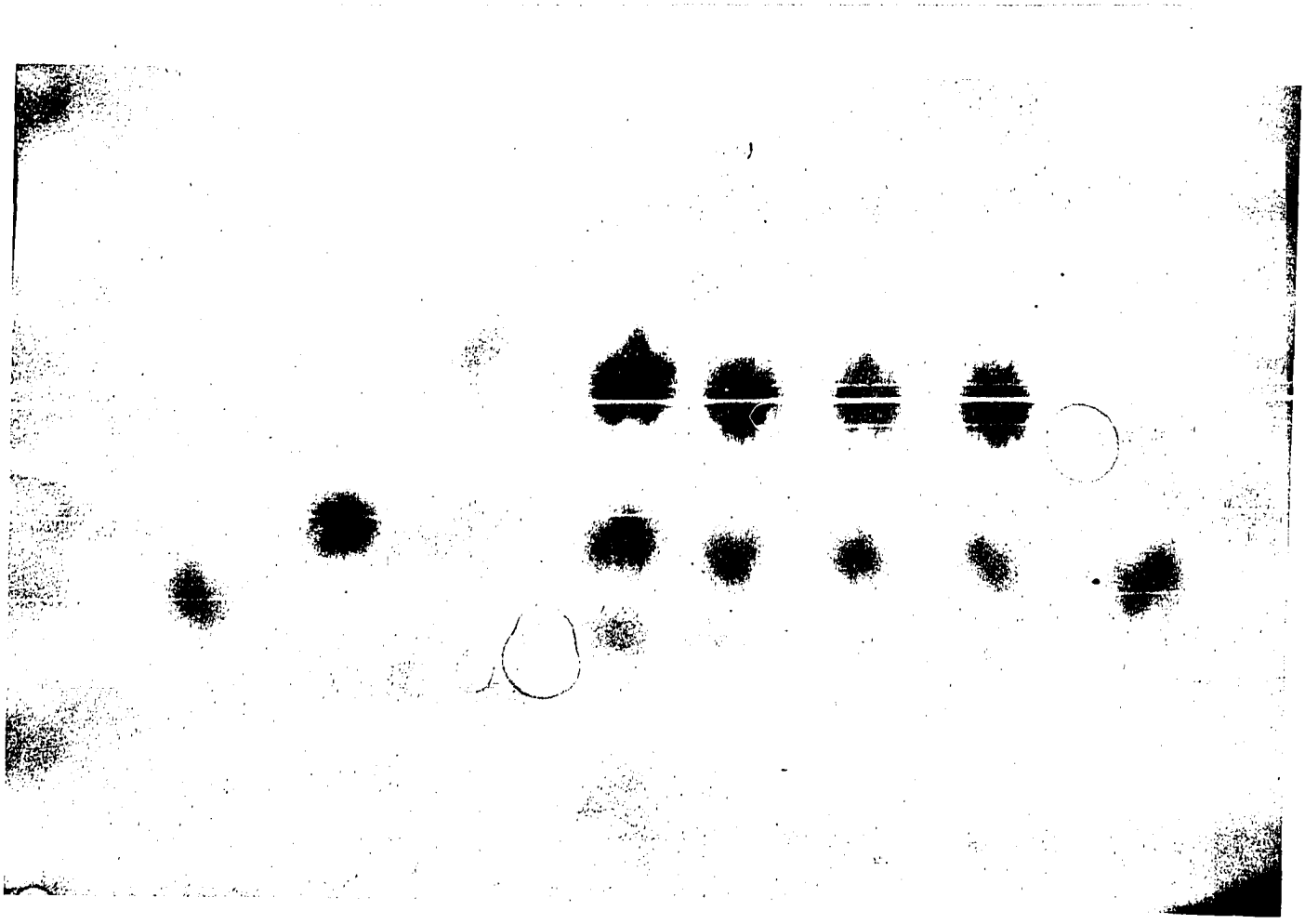


Figure 7. Paper chromatography of free carbohydrates in butanol: pyridine: water solvent.

after ammonia was added. Furthermore, no quantitative change was noted in the protein bound hexosamine (Table V).

It is well established that the polysaccharide moieties associated with serum glycoproteins of human blood contain galactose, mannose, and glucosamine (Waldron, 1952). It was found by paper chromatographic studies (Figure 8) that galactose was present in protein with glucosamine but mannose was not detected. Glucosamine was detected by the ninhydrin reaction. From the chromatograms no qualitative difference between the normal and chemically treated bovine plasma was detected in relation to bound carbohydrates.

Quantitative Determination of Three Essential Amino Acids. Comparison Between Normal and Chemically Treated Bovine Plasma. Results. From the standard curves shown in Figures 9, 10, and 11, and the turbidity readings shown in Table X, the values for leucine, lysine, and arginine were obtained both in the free and protein bound forms. In Table X are given the readings obtained in the Klett-Summerson electrophotometer for chemically treated bovine plasma and normal bovine plasma for both free and bound amino acids. From these readings the values for amino acid content were calculated as follows: a horizontal line was drawn from the point on the ordinate corresponding to the turbidity until it crossed the standard curve. From this point a perpendicular was drawn which crossed the abscissa representing the concentration in micrograms per milliliter.

The normal values obtained for free amino acids, as shown by Table XI agree closely with those obtained by Harper, Hutchin and Kimmell (1952) for free amino acids in human blood. They found leucine to range

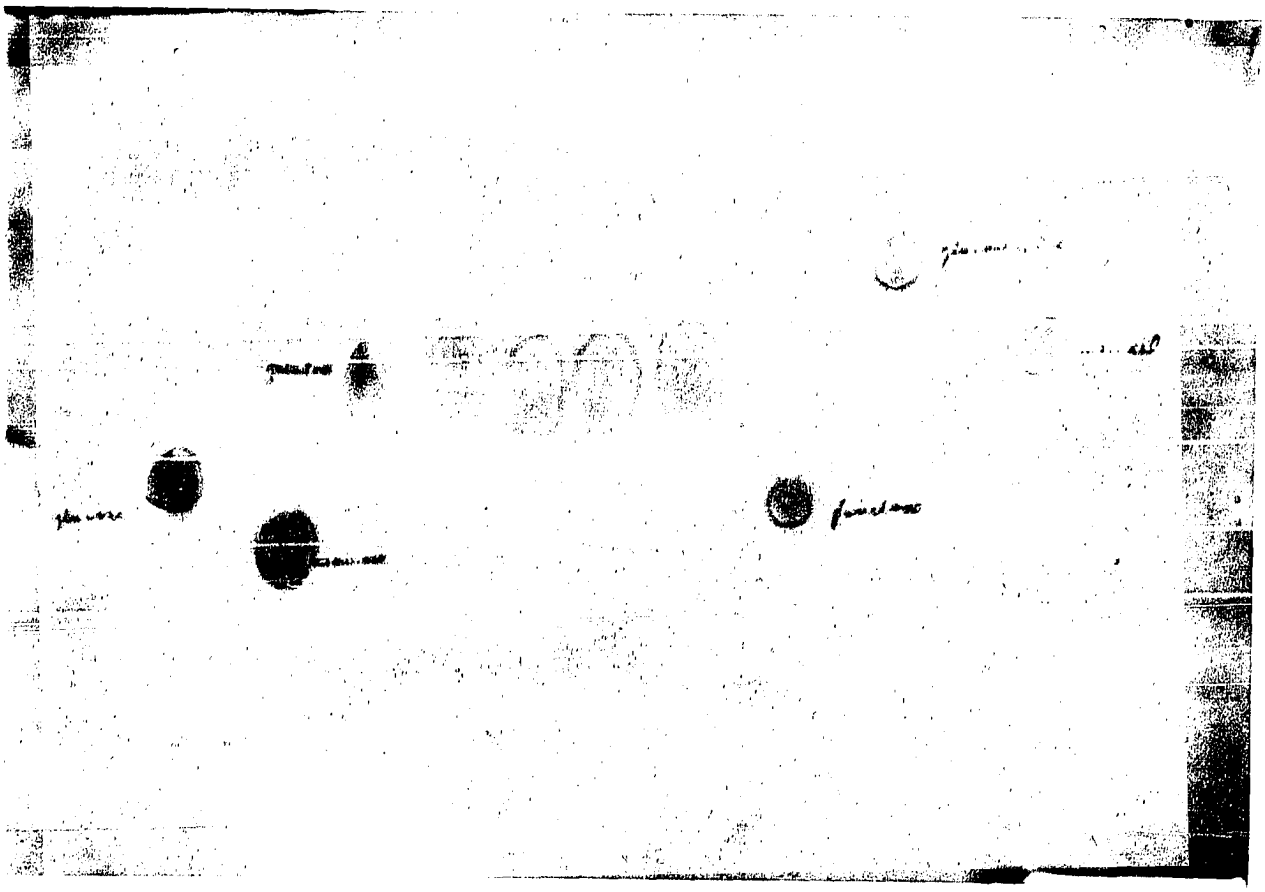


Figure 8. Paper chromatography of bound carbohydrates in butanol: pyridine: water solvent.

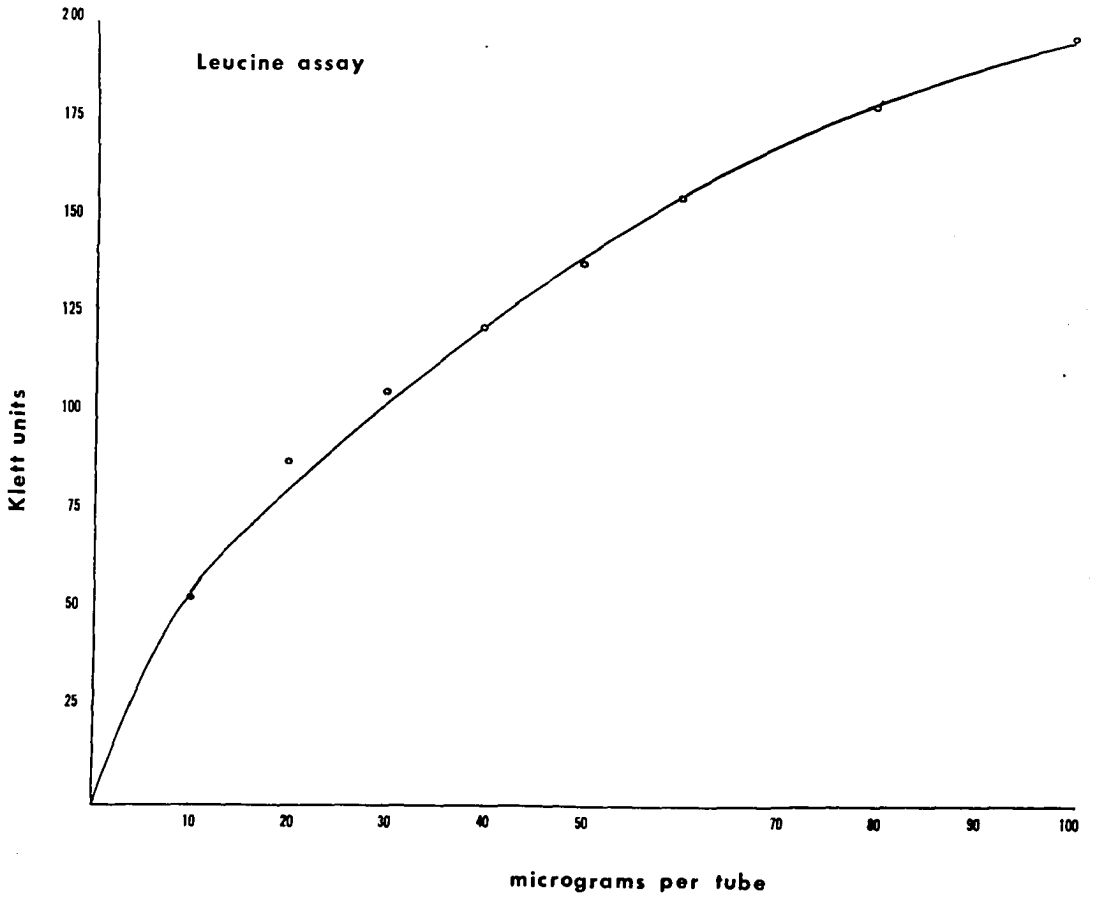


Figure 9. Standard microbiological assay curve for leucine.

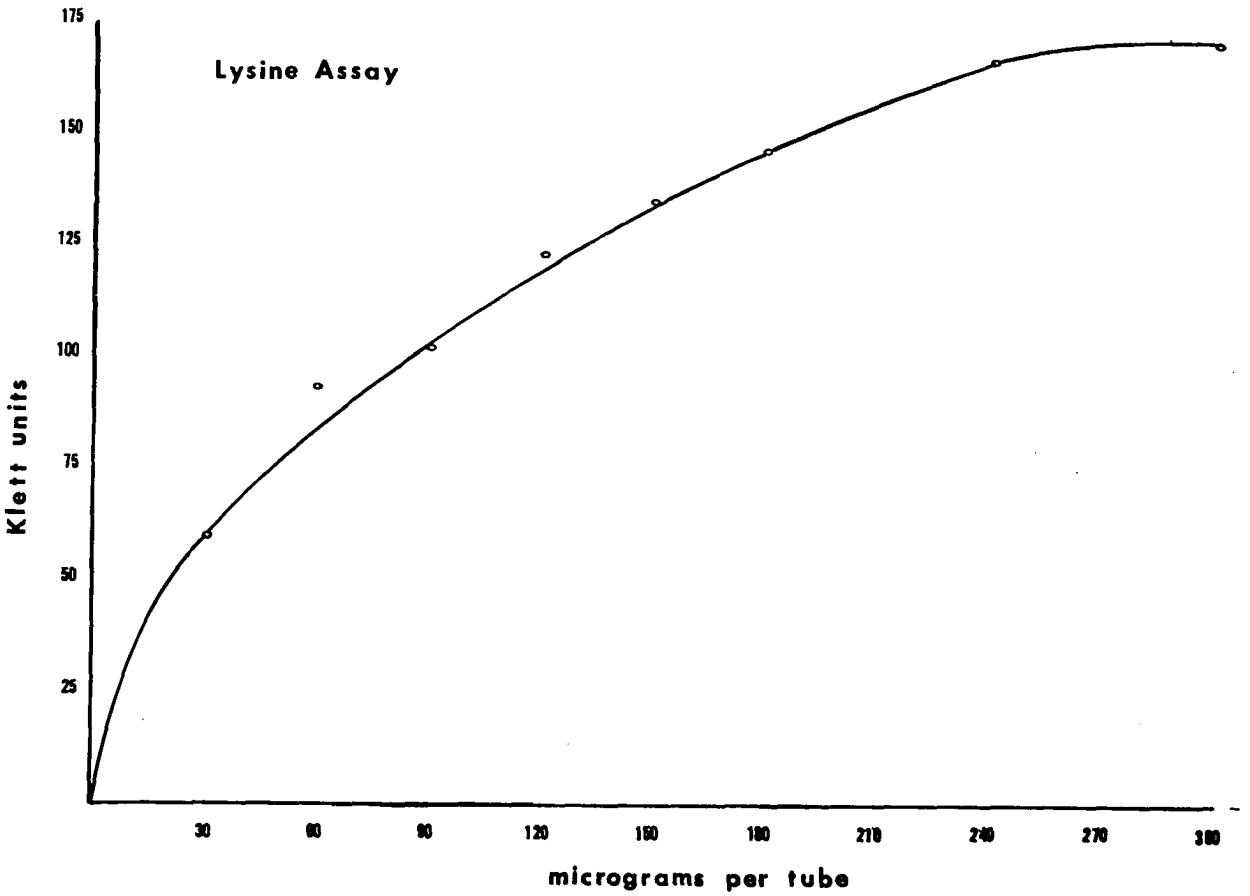


Figure 10. Standard microbiological curve for lysine.

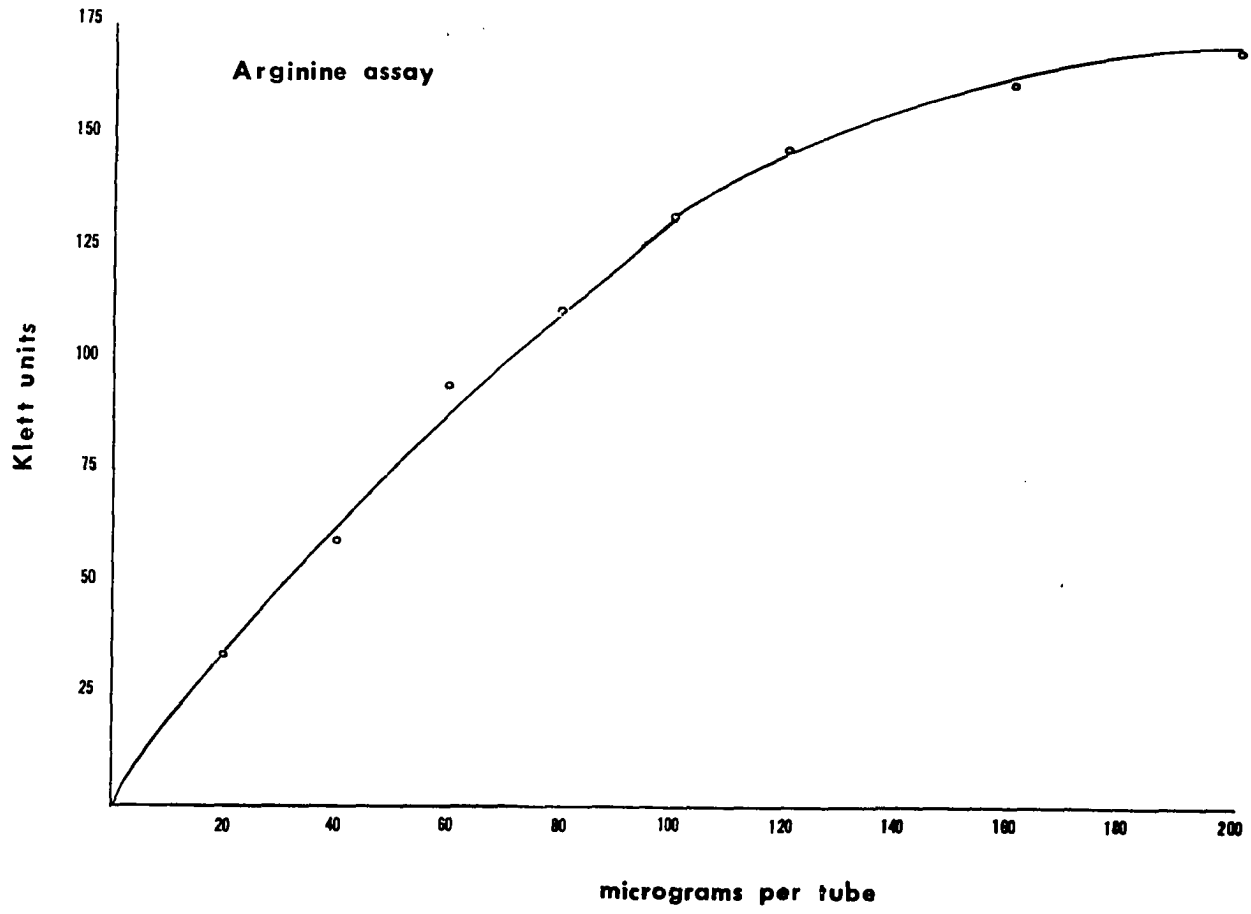


Figure 11. Standard microbiological assay curve for arginine.

TABLE X

TURBIDITY READING DETERMINED FOR BOTH FREE AND BOUND AMINO ACIDS FROM NORMAL AND CHEMICALLY TREATED BOVINE PLASMA

Substrate	Leucine		Lysine		Arginine	
Chemically treated bovine plasma	Bound	Free	Bound	Free	Bound	Free
1	116*	116	106	89	65	84
2	109	108	102	81	61	88
3	109	109	103	86	60	86
4	121	106	109	96	72	85
Normal bovine plasma	165	177	134	126	116	134

* All numerical values represent Klett units.

TABLE XI

CONCENTRATION OF FREE AND BOUND AMINO ACIDS AS DETERMINED BY MICROBIOLOGICAL ASSAY

Substrate	Free amino acid*			Bound amino acid*			Bound amino acid as percentage of total protein		
Chemically treated bovine plasma	Lysine	Leucine	Arginine	Lysine	Leucine	Arginine	Lysine	Leucine	Arginine
1	1.44*	0.720	1.44	395	360	215	17.6	16.1	9.6
2	1.20	0.680	1.49	360	325	180	17.3	15.6	8.7
3	1.22	0.68	1.50	365	321	176	17.5	15.4	8.5
4	1.48	0.66	1.55	435	390	230	16.5	14.8	8.5
Normal bovine	2.65	1.56	2.16	750	675	425	11.5	10.4	6.5

* Expressed in mg./100 ml. of sample.

from 1.0-5.2 mg./100 ml. Experimental results indicate an average value for leucine of 1.56 mg./100 ml. for normal bovine serum. Lysine ranges, according to these same workers, from 2.3 to 5.8 mg./100 ml. Experimental results indicate an average value for lysine in normal bovine plasma around 2.65 mg./100 ml. The arginine value was, according to these workers, 1.2 to 3.0 mg./100 ml. Experimental results indicated an average value for arginine of 2.16 mg./100 ml. in normal bovine plasma. Hier and Bergeim (1946) obtained similar results with the following values reported: leucine 2.03 mg./100 ml., lysine 2.97 mg./100 ml., and arginine 2.32 mg./100 ml.

The values obtained for the chemically treated bovine plasma indicated that there had been a dilution of the amino acids found free in the product due to the addition of distilled water and sodium citrate as anticoagulant. These results indicated likewise that bovine plasma does not differ strikingly from human plasma in free amino acid content.

Values obtained for arginine, leucine, and lysine in serum protein, Table XI, correlate closely with those reported by Block (1951) for human and bovine plasma proteins. He reported: For arginine, leucine, and lysine in human plasma protein 350-413 mg./100 ml., 1260 mg./100 ml., 469-672 mg./100 ml.; for bovine plasma protein the values were arginine 399 mg./100 ml., leucine 707 mg./100 ml., and lysine 546 mg./100 ml. It is worthwhile to indicate at this point that although the concentration of material is higher in normal bovine or human plasma, the milligrams of amino acids per gram of protein present was found to be larger in the chemically treated bovine plasma when total protein was determined by the biuret reaction or Kjeldahl method. It is important to mention that no

difference was noted on the amino acid studies both quantitatively and qualitatively between batches of chemically treated bovine plasma prepared at different occasions.

Since formaldehyde was used in the treatment of bovine plasma, and since this compound is toxic and an irritant in certain concentration, it was considered correct to determine quantitatively the amount of formaldehyde in the chemically treated bovine plasma. Both free formaldehyde and bound formaldehyde were determined.

The results as outlined in Table XII show concentration of 0.375 per cent to 0.318 per cent of free formaldehyde and concentrations of .059 per cent to 0.082 per cent of bound formaldehyde. These concentrations are below those used in the preparation of common vaccine, as typhoid, in which 0.5 per cent free formaldehyde may occur. Also, they are below the values indicated as safe in the Manual of Pharmacology (Sollmann). According to Boesen, Larsen and Nilsen (1948) the portion of formaldehyde which is protein free is actually in union with ammonia so that no toxicity exists. Even if the concentration of free formaldehyde is not in the range of toxicity, large amounts of protein bound formaldehyde could release excessive amounts of free formaldehyde which could be toxic unless the formaldehyde has formed methenamine, $N_4(CH_2)_6$, by the reaction $4\ NH_3 + 6\ H\text{-}C(=O)\text{-}H \longrightarrow N_4(CH_2)_6 + 6\ H_2O$. Methenamine is devoid of toxic or irritant action and it is stable at neutral pH

Animal Experimentation

Results. One of the main objectives in the animal studies of a chemically treated bovine plasma is to determine what type of action

TABLE XII

BOUND AND FREE FORMALDEHYDE

Chemically treated bovine plasma	After 4 hours Formol-Dimedon	After 96 hours Formol-Dimedon	Formaldehyde Free	Formaldehyde Bound
1	3.62*	4.23	0.371	0.063
2	3.11	3.67	0.319	0.058
3	3.13	3.91	0.321	0.079
4	3.68	4.32	0.377	0.066

* All numbers represent weight/volume percentage of formaldehyde.

such a product could have upon the experimental animal.

Toxicity. One dog was bled 250 ml. and given 100 ml. doses of the altered plasma intravenously for three consecutive days. The animal died during the night after the third injection. Autopsy was carried out and specimens of liver, kidney, spleen, heart, and lung were taken for histological study. Necrosis of the liver and damaged kidneys was noted by the pathologist upon examining these tissues. However, as the time of death was not definitely known, it was not possible to critically evaluate these observations. The death of the dog could be attributed to the toxic action of the product due to excess of free formaldehyde. This could be very possible since it has been shown that methenamine will release ammonia as the pH becomes acid.

Another experimental dog, weighing 25 lbs., was bled 250 ml. but was given immediately 270 ml. of altered plasma intravenously. The dog recovered and two weeks later the animal was given 100 ml. doses of altered plasma, intravenously, daily for seven days with no traces of bad effects either anaphylactic or toxic being noted.

A third dog, weighing 19 lbs., was given smaller doses in order to find possible anaphylaxis. For six consecutive days 30 ml. of altered plasma were given intravenously, two weeks after the animal was challenged with 80 ml. of altered plasma intravenously. In no instance were either immediate or delayed untoward reactions observed.

Intravenous Feeding of Treated Plasma after Excessive Bleeding. Three animal experiments have been done in order to study the action of the chemically treated bovine plasma. The first set of experiments indicates, as shown in Table XIII, that chemically treated bovine plasma

TABLE XIII

PLASMA PROTEIN LEVEL IN RABBITS WHICH WERE BLED AND GIVEN ALTERED BOVINE PLASMA INTRAVENOUSLY

Days	Amount bled ***	Treated plasma given rabbit No. 1 **	Total protein rabbit No. 1 *	Treated plasma given rabbit No. 2 **	Total protein rabbit No. 2 *
0	-	-	6.40	-	6.24
1	30	-	-	-	-
2	30	-	-	-	-
3	30	-	-	-	-
4	30	-	-	-	-
5	30	-	-	-	-
6	30	-	-	-	-
7	30	-	-	-	-
8	-	20	3.08	-	4.40
9	-	20	-	-	-
10	-	20	-	-	-
11	-	20	-	-	-
12	-	20	-	-	-
13	-	20	-	-	-

TABLE XIII - cont.

Days	Amount bled***	Treated plasma given rabbit No. 1 **	Total protein rabbit No. 1 *	Treated plasma given rabbit No. 2 **	Total protein rabbit No. 2 *
14	-	20	4.20	-	4.60
15	-	20	-	-	-
16	-	20	-	-	-
17	-	20	-	-	-
18	-	20	-	-	-
19	-	-	5.56	20	4.36
20	-	-	-	20	-
21	-	-	-	20	-
22	-	-	-	20	-
23	-	-	-	20	-
24	-	-	-	20	-
25	-	-	5.60	-	5.69

* grams per 100 ml. of sample
** ml. of treated plasma given
*** ml. bled

will raise the total protein level in an animal which has been partially depleted of its plasma protein by continuous bleeding and kept on a protein free diet. The protein concentration was reduced in rabbit No. A from 6.40 g./100 ml. to 3.08 g./100 ml. After feeding intravenously 120 ml. of chemically treated bovine plasma the protein concentration was raised at the end of the sixth day to 4.20 g./100 ml. The feeding was continued for the next five days and the plasma protein concentration increased to 5.56 g./100 ml. The animal was allowed to go seven days on a protein free diet and at the end of that time the protein level was 5.60 g./100 ml. If chemically treated bovine plasma would have been excreted instead of retained as shown by this experiment, the results should have been the same as shown by rabbit No. B which was bled in the same manner as rabbit No. A except that no intravenous feeding was undertaken. The total protein in rabbit No. B was lowered to 4.40 g./ 100 ml. but remained at that level on a protein free diet. Immediately after rabbit No. B was fed chemically treated bovine plasma intravenously its total protein rose to 5.69 g./100 ml. within six days. Larget and Culot (1952), showing similar results as those outlined, found that chemically treated bovine plasma raised plasma protein levels in humans to values as high as those found by treatment with human plasma transfusions.

Production of Urea. In 1842 Dumas and Cahours suggested that urea was an oxidation product of nitrogenous metabolism. Becham (1856) sought to confirm this experimentally and claimed to have obtained urea by oxidation of native proteins by means of potassium permanganate. A

very long controversy followed until the unequivocal results of Fosse (1912) proved the correctness of Becham's early conclusion. Fearon (1906) found that urea formed when aerated solutions of amino acid in blood or saline are perfused through surviving mammalian liver. Conclusive evidence that the liver is the site of urea formation when amino acids or proteins are perfused through it, was afforded by the work of Ballman, Mann and Magath (1924).

In a current experiment, a dog whose urinary excretion was followed for three consecutive days had a normal urine urea excretion of 1773 mg. per day. The published amount of excretion for a dog is 1640 mg./100 ml. of urine (Marshall and Davis, 1914). After the dog was fed intravenously with 180 ml. of chemically treated bovine plasma (6 daily doses of 30 ml.) the urea excretion increased to 2596 mg. per day. It is indicated from these results that there is a breakdown of protein which caused the increase in urea excretion. This breakdown however, does not occur totally as a result of the injected protein, since approximately 2.40 g. of protein are catabolized to produce the mentioned daily increase in urea excretion, whereas only 0.65 g. of protein were given daily to the animal.

Radioisotope Studies. Studies with radioactive I^{131} labeled protein indicated that there were some differences in the amounts of I^{131} incorporated in different organs.

At the end of 24 hours, groups of five rats demonstrated as shown in Table XIV, that an average of 68 per cent of the I^{131} injected as labeled normal bovine plasma was recovered in the urine and serum. Approximately 74 per cent average of the amount given was demonstrated

TABLE XIV

PERCENTAGE OF RADIOACTIVITY IN DIFFERENT TISSUES 24, 48, AND 72 HOURS AFTER INTRAVENOUS INJECTION OF I^{131} LABELED PROTEIN*

	24 HOURS				48 HOURS				72 HOURS			
	1 **	2	3	4	1	2	3	4	1	2	3	4
urine	21.42	17.79	44.93	14.94	27.90	24.42	57.76	19.47	29.38	31.81	58.29	24.85
serum	9.56	10.84	23.77	60.67	11.68	8.63	10.95	23.64	1.08	9.46	7.72	12.75
spleen	0.49	0.32	0.06	0.13	0.22	0.21	0.03	0.06	0.13	0.13	0.02	0.04
kidney	0.81	0.94	0.35	0.76	0.50	0.46	0.19	0.42	0.50	0.54	0.13	0.25
lungs	0.23	0.54	0.32	0.92	0.085	0.12	0.12	0.52	0.32	0.17	0.07	0.21
liver	8.62	9.98	0.75	2.55	5.71	6.82	0.48	1.24	4.41	2.32	0.36	0.96

* 3 cc. of protein was given by cardiac puncture

** All numbers represent percentage of total protein I^{131} given. These values are averages from 5 rats.

1 - Altered plasma labeled with I^{131}

2 - Rat serum labeled with I^{131}

3 - Normal bovine serum labeled with I^{131}

4 - RISA

in the urine and serum of five rats given RISA (Radioactive iodine human serum albumin). Only 30 per cent and 28 per cent could be demonstrated in the urine of rats (5 rat groups) given I^{131} labeled treated bovine plasma and I^{131} rat labeled serum. In the latter two groups of rats, it was found that in the livers there were 8.62 per cent and 9.98 per cent of the original amount of I^{131} given, as compared to 0.75 per cent and 2.55 per cent in the rats given normal bovine plasma and RISA respectively. Likewise, the spleens showed some differences. As much as 0.49 per cent and 0.32 per cent were found in the spleen after giving the I^{131} treated bovine plasma and I^{131} rat serum respectively, and only 0.06 per cent and 0.13 per cent were found in the I^{131} labeled normal bovine plasma and RISA. Concerning the kidney, it seems that both the I^{131} rat serum and the I^{131} labeled treated plasma were more concentrated in this organ as compared to labeled normal bovine plasma. No unusual trend was found in the radioactive material found in the lungs. The same pattern as outlined for the 24 hour period continued through the 48 hours and the 72 hour experimental rats (Table XIV), with the exception that there was a slight decrease in the total radioactivity found in these four organs.

If the radioactivity found in each organ is calculated as percentage of total radioactivity found in all the four organs (Table XV), the amount retained by the liver is very striking. At the end of 24 hours, 84.9 per cent of the total radioactivity found was in the liver of the rats which had been given labeled treated bovine plasma and 83.5 per cent in the liver of rats given labeled rat plasma. In the case of rats given I^{131} labeled normal bovine plasma and RISA the activity in

TABLE XV

PERCENTAGE OF RADIOACTIVITY IN SPLEEN, KIDNEY, LUNGS, AND LIVER FROM TOTAL ACTIVITY FOUND IN ALL FOUR ORGANS. COUNTS PER GRAM OF EACH ORGAN AND WEIGHT OF EACH ORGAN. DETERMINATIONS ARE MADE AT THE END OF 24, 48, AND 72 HOURS

		Altered plasma I^{131}			Rat serum I^{131}			Normal bovine serum I^{131}			RISA	
		%	cts/g	wt.*	%	cts/g	wt.	%	cts/g	wt.	%	cts/g	wt.
24	Spleen	4.2	1706	0.73	3.8	1161	0.81	4.3	489	.74	3.1	1043	0.8
	Kidney	8.5	1357	1.9	8.0	1361	1.9	24.3	904	2.4	17.4	1943	2.4
	Lung	2.4	446	1.4	4.3	562	1.7	21.6	1049	1.8	20.9	2931	1.9
	Liver	84.9	3215	8.1	83.5	3400	8.5	49.81	402	10.1	57.3	1301	11.1
48	Spleen	4.2	534	1.5	2.1	767	0.8	4.42	300	0.7	3.1	431	1.00
	Kidney	7.8	459	3.2	7.6	685	2.0	23.2	507	2.3	18.7	747	2.7
	Lungs	1.6	108	2.0	1.3	219	1.7	14.9	500	1.6	22.5	1266	2.2
	Liver	86.4	998	15.0	89.0	2662	9.5	57.5	277	9.8	55.6	580	12.5
72	Spleen	3.5	1060	0.95	4.4	249	1.02	3.3	170	0.6	2.9	323	0.8
	Kidney	10.8	1159	2.0	17.0	358	2.7	22.6	335	2.3	16.9	634	2.4
	Lungs	6.1	863	1.6	6.0	168	1.9	12.9	281	1.5	14.4	724	1.8
	Liver	79.6	2298	8.6	72.9	350	12.3	61.2	203	10.0	64.2	495	12.0

* Weight in grams

** Each numerical value represents average of 5 rats

the liver was 49.8 per cent and 57.3 per cent.

One may postulate that an animal will concentrate large amounts of homologous protein in the liver for metabolism and synthesis of new proteins. This shows that I^{131} labeled rat plasma was concentrated by the liver more than either RISA or normal bovine plasma. But bovine plasma which had been chemically treated showed a similar recovery of I^{131}, as in the case of labeled rat plasma. This may indicate that chemical treatment of foreign plasma protein had perhaps made it acceptable on the same basis as homologous protein. Another fact in favor of this conclusion is that the animals given RISA concentrated more I^{131} in the liver than the animal given normal bovine plasma, although both were foreign proteins. According to Edwards (1942-44), the elimination of the globulin fraction of a protein decreases its antigenicity and its non-specific agglutination capacity. Therefore, the explanation for the difference between RISA and normal bovine I^{131} may be that RISA is an albumin preparation and contains no globulins. These findings agreed with those of Addis, Lee, Lew and Poo (1936, 1940) who showed that when the dietary protein of a rat was increased the greatest increase in tissue protein occurred in the liver and kidneys. These workers also found that a seven day fast caused the loss of 40 per cent of the liver protein. It was indicated that liver proteins are most rapidly broken down for amino acids and, in the same manner, proteins formed very rapidly.

The specific activity shown in Table XV also demonstrated a larger activity per gram of liver for the treated bovine plasma and rat plasma as compared to the normal bovine plasma and RISA.

CHAPTER IV

DISCUSSION

Chemical treatment of bovine plasma with formaldehyde and heat seem to be accompanied in many instances by a loss of antigenicity and non-specific agglutinins. It has been demonstrated experimentally that the chemically treated plasma lacked the capacity to incite antibody production in rabbits and did not cause either non-specific hemagglutination, or hemolysis of human erythrocytes. These results are in general agreement with previous reports. Massons (1944) noted the absence of anaphylaxis in 40 guinea pigs, 10 rabbits, and 2 dogs. He reported the absence of toxic effects in patients given as high a dose as 1000 ml. of the treated plasma. Melka (1947) found that chemically treated bovine plasma did not contain agglutinins for human red blood cells and did not produce precipitins against chemically treated bovine plasma nor against the normal bovine plasma.

Although many investigators have confirmed the above mentioned results, the point as to whether antigenicity is totally lost, is not completely answered since Carlinfanti (1948) and Regamey (1952) found the presence of antigenicity and hemagglutination in their preparations of chemically treated bovine plasma.

It is very possible that the few exceptions in which anaphylaxis

and toxicity were still found to be present, is due to differences in the treatment of the original bovine plasma. The level of free formaldehyde, if not carefully controlled, could be high enough to cause toxic symptoms. However, if most of the formaldehyde is bound, both toxicity and antigenicity are greatly reduced. It would appear that a determination of free and bound formaldehyde should be a required control procedure in the manufacture of this product.

Results of electrophoretic studies and salt fractionations seem to indicate that the normal proteins have been physically altered in their electrophoretic patterns and solubility properties. The conventional globulin fractions had disappeared, and electrophoretically appeared to move as albumin at pH 8.6 (veronal buffer); however, immunochemical studies and electrophoresis at pH 7.8 (phosphate buffer) indicate the actual absence of conventional albumin. The disappearance of the conventional globulin fraction may explain the absence of serum sickness or non-specific, in vitro, agglutination and hemolysis, since it has been shown by Edward (1944) and Regamey (1952) that the globulin fractions are chiefly responsible for the non-specific agglutination and the acute anaphylactic properties.

One of the important points in a good plasma substitute is the average molecular size because it is not desirable to have a large molecule which may cause obstruction of essential organs such as the kidneys. On the other hand, small molecules result in the osmotic pressure being dangerously high.

In the four different batches studied the mean particle weight ranged from 51,000 to 70,000 with the other sample being 200,000.

The 200,000 value on one of the batches may indicate the possibility of particle aggregation into large size particles as reported by Van der Sheer, Wyckoff and Clarke (1941). Again, it is worth mentioning the importance in the preparation of the product since large size particles could possess more antigenicity as shown by Kleczkowski (1941) or could be damaging to proper kidney function.

Although no previous studies have been made on the alteration of the sugar and amino acid components of chemically treated bovine plasma, studies here reported indicate the possible formation of free amino sugars which were absent in normal bovine plasma. This may be due to the action of the ammonia added.

Concerning both the bound and free amino acids, no qualitative difference was found between treated and normal bovine plasma by chromatographic studies, but quantitative results obtained by microbiological assays indicated that the concentration of bound amino acids per gram of protein was greater in the chemically treated bovine plasma than in the normal bovine plasma when the total protein was determined by the biuret reaction. When the Kjeldahl values for proteins were used, the concentration of amino acids per gram of treated plasma protein were closer to that of normal bovine plasma. The discrepancy between biuret and Kjeldahl values was noted by Ardry and Stark (1953).

Concerning the utilization of the plasma expanders, only gelatin of the commonly used substitutes had been shown to furnish amino acids as a source of nutrition. Brusching (1945) noted an increase in urea nitrogen when daily intravenous injections were given to dogs. The mentioned evidence is only an indication of breakdown and not of

utilization.

Basic findings by Salaskin (1898) and Bollman, et al (1924) gave conclusive evidence which demonstrated that the liver was the site of protein metabolism (anabolism and catabolism) and urea formation. However, urea excretion only indicates metabolism by the liver, of proteins and amino acids and gives no indication of utilization for building new proteins.

The results obtained demonstrated an increase in urea excretion in amounts which could not be ascribed to the protein administered. The increase could be due to either toxic effects or mechanical damage to the kidneys which may hinder reabsorption of glucose. As in the case of phlorizin (Houssay, 1944) poisoning, this may result in a state of gluconeogenesis which will increase the urea excretion. However, part of the excreted urea may have resulted from the specific dynamic actions due to the administered protein.

From radioisotope studies it was demonstrated that the liver incorporates larger amounts of I^{131} when the radioactive material was introduced as labeled treated bovine plasma or labeled homologous rat plasma when compared with labeled normal bovine plasma and RISA. Similar results were found concerning the kidneys. It is indicated that chemical treatment may have changed the bovine plasma in such a way that it can not be distinguished by the liver and other tissues from normal rat plasma and hence incorporated in equal amounts to be metabolized.

Larget and Culot (1952) found that when a chemically treated plasma was given to 24 patients an increase was found in blood volumes and plasma protein levels in the same proportions as when human plasma

was given. Similar results were obtained in this work when rabbits were administered chemically treated bovine plasma after excessive bleeding. This could mean that the raised protein is due to the administered protein which remained in circulation but at no instance has incorporation of the injected protein into tissue proteins been demonstrated. However, the equivalent of 38 per cent of the administered protein is retained while approximately 62 per cent is lost by excretion or deposition. It is important to mention that if such a large deposition occurred in the kidney serious damage may result with a consequent alteration in the metabolism of the animal.

Chromatographic patterns indicated that the chemically treated bovine plasma contained fructose in addition to those carbohydrates normally present. This is to be expected since invert sugar (fructose and glucose) had been added. The addition of invert sugar may be of importance in the light of the findings of Pollack (1954), in that truly effective protein therapy depends upon the ability of the body to supply enough calories in the form of carbohydrates and fats so that the administered protein will not be used as a source of energy.

CHAPTER V

SUMMARY

1. Bovine plasma chemically treated with formaldehyde and heat has lost the capacity to incite the production of antibodies in rabbits. Similar results had been obtained by Regamey (1952) and Massons (1948).

2. The ability to cause either non-specific hemagglutination or hemolysis of human erythrocytes, as in the case of normal bovine plasma, has been completely eliminated.

3. Electrophoretic and immunochemical studies demonstrate the disappearance of the conventional globulin and albumin fractions. This also had been shown by Sieber (1953) and Koecher, et al (1954).

4. Average particle weights were similar to those of normal plasma proteins but increased particle weight in one preparation indicated that aggregation may occur in the process under certain conditions.

5. No qualitative differences in the amino acid chromatographic patterns of chemically treated bovine plasma and normal bovine plasma were noted. In quantitative determinations an increase in the concentrations of lysine, leucine, and arginine was obtained.

6. Free amino sugars, fructose, and glucose were found in chemically treated bovine plasma as compared to only glucose in normal bovine plasma. No differences were noted in the bound sugars found in

chemically treated bovine plasma and normal bovine plasma (galactose and glucosamine were found in both cases).

7. In no instance were differences in amino acids, carbohydrates and formaldehyde content noted among the various batches of chemically treated bovine plasma.

8. Increased urea excretion was found to be in values beyond those which can be ascribed to the injected proteins. This indicated a process of catabolism induced by the injected protein.

9. Chemically treated bovine plasma increased the plasma protein levels after the animals had been subjected to excessive bleeding. Approximately only one-third of the injected proteins can be accounted as present in the plasma.

10. Larger amounts of I^{131} were found in rats injected with I^{131} labeled rat plasma and I^{131} labeled chemically treated bovine plasma as compared to those given I^{131} human serum albumin (RISA) and I^{131} labeled normal bovine plasma.

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