PART I

EFFECT OF HYDROGEN PEROXIDE OXIDATION ON THE ANTIGENICITY OF OVALBUMIN, BOVINE SERUM ALBUMIN, AND RABBIT GAMMA GLOBULIN

PART II

CORTICAL DISCONTINUITY AND PROPAGATION OF SPREADING DEPRESSION

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ABSTRACT

PART ONE

Three proteins were treated with solutions of hydrogen peroxide under a variety of conditions. The resulting products were soluble, heat stable, and showed some increase in heterogeneity of electrophoretic components. Osmotic pressure determinations indicated a marked reduction in number average molecular weights, and intrinsic viscosity studies showed increased frictional ratios for the treated proteins. UV absorption of the treated proteins indicated extensive oxidation of the tryptophane, tyrosine and phenylalanine residues in the protein. Chromatographic studies indicated that cystine and cysteine were oxidized to cysteic acid. Immunochemical investigation of the H_2O_2 treated proteins showed: (1) That treated ovalbumin had lost all its native specificity, and that treated bovine serum albumin and rabbit gamma globulin retained only traces; (2) Each protein apparently developed a new specificity as a result of treatment, but the antigenicity of such proteins was very low. Tests employed were the development of precipitins in rabbits and chickens, and Schultz-Dale and gross anaphylaxis in guinea pigs.

ABSTRACT

PART TWO

An investigation into the possible mechanism underlying the propagation of Leão's spreading depression (S.D.) was conducted in rabbits by cutting the cortex in chronic experiments and thus destroying neuronal continuity. The slow potential change (S.P.C.) concomitant with S.D. had been postulated as the agent of transmission in a manner similar to the nerve action potential associated with nerve conduction. It was found in this investigation that neither the S.D. or the S.P.C. crossed the cut even though in some cases the scar was only 0.1 mm thick and the cortical edges were well approximated. A small potential change was recorded as crossing the cut, but it was never instrumental in initiating a S.D. It was therefore concluded that either neuronal continuity or localized microfields smaller in radius than the scar are involved in the transmission of S.D. The possibility of a chemical agent being involved in the transmission of S.D. was not eliminated by these experiments.

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CORTICAL DISCONTINUITY AND PROPAGATION OF SPREADING DEPRESSION

by

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PART ONE

CHAPTER I

INTRODUCTION

Immunity reactions resulting from the injection of foreign proteins such as vaccines are extremely valuable with respect to protection against disease, but the same mechanism can also produce harmful allergic reactions which limit the use of foreign proteins for other purposes. If a method could be developed by which the antigenicity of proteins were destroyed (deantigenized) without seriously reducing their molecular size (e.g. not less than about 40,000), an abundant source of proteins would be available for use as plasma expanders. In addition, if a heterologous antiserum could be deantigenized without destroying its therapeutic value, passive immunization would then become more efficient and safer. Besides the practical applications resulting from the successful destruction of antigenicity, fundamental knowledge might also be obtained as to the properties essential to protein antigenicity.

The purpose of this thesis is to develop a method for the destruction or reduction of antigenicity, and to investigate the importance of aromatic amino acids, sulfhydryl groups, and molecular size to the antigenicity of proteins.

Proteins have in the past been treated by a variety of methods, including heat, acidification, alkalinization, oxida-

tion, and partial enzymatic digestion. In general the resulting products have shown some reduction in antigenicity. With the exception of oxidation, which has proven to be the most potent deantigenizing agent, the reduction in antigenicity has ranged from 0.1 to 0.001 of the original. Investigations by Henry (1) using photo-oxidation showed a reduction in the antigenicity of horse serum to 0.00005 of the original. For use as a plasma expander, complete destruction of a protein's antigenicity is necessary, and therefore the residual antigenic acitivity in photo-oxidized proteins eliminates them for this purpose. However, oxidation seemed to be the most promising lead in an attempt to remove antigenicity.

Various methods of oxidizing proteins have been tried previously and the physical, chemical and immunochemical properties of some of the derived proteins studied. A review of this earlier work is now presented.

Literature Review

Immunological properties of oxidized proteins.--The first and only work on changes in immunological properties of proteins oxidized by potassium permanganate was reported by Obermayer and Pick (2) in 1906. The product of their oxidation was termed "oxyprot-sulfonic acid." The oxyprot-sulfonic acid was reported to have lost its precipitability with an anti-serum against the original protein, but had acquired its own characteristic antigenic specificity.

A second method of oxidizing proteins and perhaps the most extensively investigated is that of photo-oxidation. Photo-oxidation is accomplished either by irradiation with visible light plus a sensitizer (e.g. eosin or hematoporphyrin), or by irradiation with ultra violet. The apparent function of the sensitizer in visible light is to supply structures (e.g. porphin ring) capable of resonating at the longer wave lengths and of transferring the energy required for the rupture to the peptide chain. The earliest work reported was that of Fleischmann (3) in 1905, who irradiated a mixture of proteins (antigens or anti-serum) and eosin with glass filtered sunlight, and found a destruction in the precipitating properties of the system. This method was termed "photo-dynamic" by Fleischmann. The same results have been accomplished by irradiation with ultra violet (4X5) in the neighborhood of 2537 Å without a sensitizer.

Antigenicity studies made with photo-oxidized proteins revolve mainly around three investigators, Smetana and Shemin, and Henry, which I would like to discuss separately and in some detail.

Smetana and Shemin (6) worked with egg albumin and used hematoporphyrin as a photo-sensitizing agent. These workers showed that as oxidation progressed the antigen lost its ability to precipitate with the corresponding rabbit anti-serum. The susceptibility of anti-serum to photo-oxidation was greater by a factor of

ten than the antigen as judged by its precipitating ability. The study of cross reactivity of the treated and untreated material was extended to an investigation of passive immunization of guinea pigs, and of gross anaphylaxis. As a result of these studies, it was found that guinea pigs either passively or actively sensitized to ovalbumin would not go into anaphylactic shock when challenged with treated (photo-oxidized) ovalbumin. Attempts to sensitize guinea pigs with 45 mg of the oxidized material failed, as did the attempts to produce precipitins in rabbits which received 80 mg of oxidized protein in 16 injections. The conclusion was drawn that as a result of irradiation, the native antigenicity was destroyed without the production of a new one.

The work of Henry (1) was primarily concerned with quantitating the antigenic response of photo-oxidized horse serum. The primary purpose underlying this work was the possibility of employing de-antigenized serum proteins as plasma expanders. The degree to which the antigenicity should be lowered was based on the observation (7) that 0.01 cc of horse serum would sensitize an individual for years, as determined by skin testing. Since in practical use in humans the average quantity of a plasma expander is about 50 grams, it was necessary to reduce the antigenicity so that this quantity was equivalent to less than that of 0.01 cc of horse serum. Thus the antigenicity of the photo-oxidized serum would have to be 0.00001 of the original serum. Following the lead of

Smetana and Shemin (6), Henry irradiated horse serum with both ultra violet and visible light plus a hematoporphyrin sensitizer. The course of oxidation was followed by the loss of precipitability of the oxidized protein with an anti-serum to the original protein.

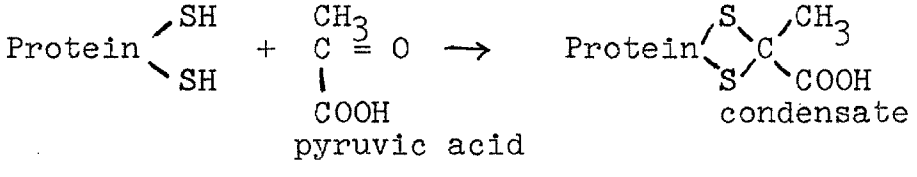
The antigenicity was followed in guinea pigs which were injected intra-peritoneally with one mg of antigen. The Schultz-Dale technique and gross anaphylaxis were employed as means of determining active immunization. Henry found that by gross anaphylaxis he was able to induce shock in guinea pigs which had previously received one mg of horse serum proteins by the injection of 0.01 cc of horse serum. If 2 cc of the treated horse serum failed to shock a normal horse serum sensitized animal, he concluded that 2 cc of treated material had less antigenic potency toward untreated material than was contained in 0.01 cc of unoxidized horse serum. Therefore, the photo-oxidized horse serum had 0.005 the residual potency of the original material. These quantitative interpretations are only a very rough approximation since biological variations in susceptibility to sensitization as well as testing animals precludes any exacting measurement of antigenicity.

Henry concluded from his results that the treated horse serum (THS) contained sufficient amount of the original antigenicity to sensitize but insufficient amount to shock an animal sensitized to NHS. In addition, he found that a new antigeni-

city had developed with a higher potency than the residual native antigenicity. Henry's best results in deantigenation were a reduction in the native antigenicity to 0.00001 of the original, and for the new specificity an antigenicity equivalent to 0.00005 of the original protein.

With the Schultz-Dale technique, he showed that the original and oxidized protein do not cross react or mutually inhibit.

The investigations of Williams (8) were concerned with the role played by sulfhydryl and disulfide group in the antigenicity of bovine serum albumin. Methods employed by Williams to alter the degree of antigenicity in bovine serum albumin were (1) oxidizing the sulfhydryl and disulfhydryl groups with either iodine or hydrogen peroxide, (2) blocking the sulfhydryl groups by condensation with pyruvic acid as shown in the following equation,



or (3) oxidizing the condensate obtained by procedure (2) with hydrogen peroxide. The condensate resulting from the reaction of pyruvic acid and bovine serum albumin was found to be stable at neutral pHs. It was felt by Williams that oxidation of the condensate with hydrogen peroxide would lead to the formation of a stable sulfone, RSO_2. The antigenicity

of the various preparations were tested by gross anaphylaxis in guinea pigs. The guinea pigs were given sensitizing injections intra-peritoneally of 5 to 10 mg, and challenged 20 to 60 days later with 20 mg intravenously. From his results Williams concluded that a positive correlation existed between the antigenicity of bovine serum albumin and the "reactive sulfhydryl" groups in the protein. To preclude the possibility that the reduction in antigenicity might be explained on the basis of iodine substitution on the tyrosine, colorimetric tests were made with the Folin-Ciocalteau reagent which gives different color reactions with tyrosine than with diiodotyrosine. It was found that 80 to 90% of the tyrosine which remained after iodine oxidation was unsubstituted.

Chemical and physical properties of oxidized proteins.-- Ultra violet absorption has been commonly used to follow changes in the content of aromatic amino acids and cystine of oxidized proteins. Since this method is common to several of the investigations to be reported, the rationale behind its use is here presented.

Proteins are essentially transparent between the wave lengths 2500 Å to 3000 Å unless tyrosine, tryptophane, phenylalanine, or cystine are present. It has been reported by Beaver (9) that mixtures of cystine and the aromatic amino acids representative of a specific protein give essentially the identical absorption curve in the ultra violet as that protein. The degree of identity between the absorption spec-

trum of a representative mixture of amino acids and the original protein is so close that a quantitative method for the estimation of the aromatic amino acids based on ultraviolet absorption has been proposed (10). From these considerations it is therefore concluded that any change in the ultra violet absorption spectrum of a protein may be taken as indicative of changes in the aromatic amino acids and cystine.

The investigation of the chemical alterations of proteins by $KMnO_4$ oxidation was made by Haas et al. (11). The changes in proteins and individual amino acids were followed by means of ultra violet absorption. Of the amino acids studied, phenylalanine was reported to be the most resistant to $KMnO_4$ oxidation, but nevertheless under "extreme treatment" the resulting spectrum showed only continuous absorption, which was interpreted as meaning a complete destruction of the ring structures. The overall effect of the oxidation of tyrosine and tryptophane was the reduction in absorption at the previous maximum (2800 Å), with a simultaneous increase in absorption at the original minimum (2500 Å). With a gradation in the degree of oxidation, the results showed that the product resulting from tyrosine oxidation first went through an increased absorption at 2800 Å. This increase in absorption was interpreted to be the result of an intermediate product in the oxidation of tyrosine, and was postulated to be dopa (3,4 di-hydroxyl-phenylalanine), which is characterized

by having a higher extinction coefficient than tyrosine. With respect to tryptophane, $KMnO_4$ oxidation produced only an "unselected reduction of total absorption" in contrast to an initial rise in absorption, characteristic of oxidation of the other two aromatic amino acids. Haas et al. (11), also studied the effect of oxidation on insulin and pepsin. Oxidation of proteins produced about the same change in light absorption as did oxidation of their component amino acids. The proteins gave an initial increase in absorption at 2800 Å, but a decrease with prolonged oxidation. From these observations it was concluded that as a result of potassium permanganate oxidation of proteins, incorporated aromatic amino acids are changed in a manner identical to the oxidation of free amino acids.

Chemical changes resulting from either ultra violet or visible light irradiation have been studied by several investigators. McLean and Giese (12) found by irradiating egg albumin with ultra violet that the spectrum of the treated protein lost its characteristic aromatic maximum at 2800 Å and showed a general increase in its extinction coefficient between the wave length of 2350 Å and 3000 Å. When individual aromatic amino acids were similarily irradiated, the resulting changes in spectrum paralleled those obtained with the protein. These results were found to be similar to those obtained by the action of tyrosinase on proteins. Since the extinction coefficient of the benzene ring is a direct function of the number of hydroxyl

groups present on the ring (12), and since their results show an increase in the extinction coefficient with oxidation, it was concluded by McLean and Giese (12) that the oxidation of phenylalanine and tyrosine proceeds as follows:

$$\text{Phenylalanine} \xrightarrow{\text{U.V.}} \text{Tyrosine} \xrightarrow{\text{U.V.}} \text{Dopa.}$$

They further postulated, as a result of comparing irradiated quinones with dihydroxyl substituted phenol compounds, that photo-oxidized phenylalanine and tyrosine must have a quinone as an intermediate. Speculation as to the ultimate end product of this treatment was not made. Doty and Geiduschek (13) also have reported that phenylalanine and tyrosine form dopa as an intermediate of photo-oxidation, as well as mentioning the converting of cystine to cysteine, destruction of histidine, and the breaking of hydrogen bonding.

Colorimetric determinations on irradiated proteins have been run for the purpose of assaying changes in the aromatic amino acids, histidine, and cystine. The results reported by Smetana and Shemin (6) and Henry (1) are in general agreement. The percentage of tyrosine or cystine in the molecule does not show an appreciable change as a result of photo-oxidation. Tryptophane and histidine show a significant drop as does the number of sulfhydryl groups. The paradox that arises from the results obtained with ultra violet absorption and the colorimetric determinations may in part be resolved from the

fact that tryptophane is characterized by an extinction coefficient five times greater than tyrosine. Thus pronounced changes in spectra may occur which involve only tryptophane.

As a result of studies to determine the effects of ultra violet irradiation on proteins, Rideal and Mitchell (14) have postulated a rupture in the polypeptide chain immediately adjacent to the aromatic amino acids. Their theory is based on the irradiation of compounds such as stearic anilide, which were found to split at the keto imino link with ultra violet, and on the irradiation of monolayer proteins. Carpenter (15) repeated their work and extended the distance between the benzene ring and the keto imino link by irradiating compounds such as beta phenylethylstearylamine. It was concluded from these investigations that the benzene ring acts as a chromophore, absorbing energy which can be transferred along the chain to split the keto imino link. Further support was obtained when it was noted that the upper wave length limit of this photo-chemical process coincides with the upper limits of absorption of the aromatic nucleus. When irradiations of longer wave lengths are employed in the absence of any sensitizer, the benzene ring will not resonate, and photolysis will not occur. The importance of the aromatic nucleus in the above scheme was further emphasized when it was noted by Harris (16) that O_2 uptake as the result of ultra violet irradiation was only characteristic of those proteins containing aromatic amino acids. Proteins such as gelatin

which have no tryptophane and only a very small amount of tyrosine and phenylalanine take up very little O_2 when irradiated.

Due to the importance of molecular size in any interpretation of antigenic properties of proteins, the molecular size of the various proteins irradiated for antigenic study has been investigated by Harris (16), Rideal and Mitchell (14), and Carpenter (15), whose results suggested that the molecular weight of photo-oxidized proteins was reduced. With hemocyanin a reduction in size was found to occur when irradiated with ultra violet. However, ultra violet irradiation of hemoglobin indicated a heterogeneous mixture of both larger and smaller size particles than the original (17). Sanigar and Kerjei (18) working with human serum albumin concluded from sedimentation studies that the molecular size increased as much as 2 or 3 times the original with oxidation. The mechanism underlying the increase in molecular size was apparently aggregation (18). Davis et al. (19) were led to the same conclusion while determining the osmotic pressures of irradiated horse serum, and human serum albumin and gamma globulin by the Hepp method. They observed that the osmotic pressure decreased as a function of the duration of the determination and interpreted it as meaning aggregation. Further evidence for aggregation was obtained in the studies (19, 20a) of electrophoretic patterns from photo-oxidized proteins. An increase in the homogeneity of serums was reported as a result of irradiation. This increase in homogeneity was demonstrated

by the appearance of a new component, D, moving with a mobility between alpha and beta globulin. The concentration of the new component was a function of the quantity of irradiation, and its mobility was a function of the quantity and kind of components irradiated. When the experiment was repeated with electrophoretically pure protein, e.g. serum albumin, the resulting pattern of the photo-oxidized protein was a single component with approximately the same mobility as the original proteins. Apparently as a result of photo-oxidation, there occurred a splitting of the molecule followed by a fusion of various fragments to give component D.

Davis et al. (19) have reported a linear increase in the relative viscosity of the treated material, horse serum, as a function of the duration of exposure. Since the resulting viscosity of only the photo-oxidized material was shown to be a function of external pressure, it was concluded that the increase in viscosity was due to an increase in the asymmetry of the molecule, resulting from the unfolding of the peptide chain.

In summary, the following scheme was proposed by Davis et al. (19).

$$\text{Protein} \xrightarrow{\text{U.V.}} \text{Unfolding} \xrightarrow{\text{U.V.}} \text{Splitting} \longrightarrow \text{Aggregation. (Molecular size distribution.)}$$

Williams (8) found that 58% of the tyrosine in BSA was left after oxidation with hydrogen peroxide, and that oxidation

of pyruvic acid-protein condensate resulted in complete oxidation of sulfhydryl and disulfide groups. A very limited investigation was made into the physical chemical properties of the derived proteins. The sedimentation patterns showed that fragmentation as well as aggregation had occurred from the oxidative methods employed. Osmotic pressure determinations gave a p/c value for normal bovine serum albumin of 4.00, and of 5.57 for the treated material. Since experimental details were not given it is hazardous to use his values for the calculation of the molecular weight of the oxidized protein. If, nevertheless, we assume that sufficient salt was present, and that the untreated material had a molecular weight of 69,000, and that both materials were measured under the same conditions, we find the oxidized product had a molecular weight of 50,000.

Experimental Approach

Of the many methods by which proteins might be modified, oxidation appeared the most promising approach for the deantigenation of proteins. The results obtained by the various methods of oxidation are in general agreement. Oxidation of proteins has resulted in either the partial or full replacement of the native specificity with a new specificity. The antigenic potency of the new specificity has appeared to be, in part, a function of the oxidative method employed. Both photo-oxidation and hydrogen peroxide oxida-

tion have resulted in reductions in native antigenicity of the treated proteins. For use as a plasma expander, complete deantigenation of a protein is necessary, and therefore the procedures previously employed are automatically eliminated on the basis of their residual and newly acquired antigenicity.

Since, in the procedures described, there appeared to be a survival of intact tyrosine and perhaps phenylalanine, the assumption can be made that these elements are at least in part responsible for the continued antigenicity. The theory that aromatic amino acids are responsible for the antigenic nature of proteins dates back to Obermayer and Pick (2) who first proposed the theory based on the observation that gelatin is virtually lacking in aromatic amino acids and is non-antigenic. Williams' contention that sulfhydryls are largely responsible for the antigenicity of bovine serum albumin may find wider application since gelatin contains no cysteine or cystine. It is known that hydrogen peroxide oxidation destroys the aromatic amino acids (21) and converts cysteine and cystine to cysteic acid (2); it was decided to determine if under the proper conditions oxidation of proteins by hydrogen peroxide might not completely destroy the antigenicity. The purpose of the experiment was therefore to oxidize various proteins, and to determine the immunological and physical chemical properties of the protein thus derived.

CHAPTER II

MATERIALS AND METHODS

Preparations of Proteins

Crystalline ovalbumin.--Crystalline ovalbumin was prepared by the method of Kekwick and Cannan (23). To insure the proper salt concentration, a saturated solution of sodium sulfate was made and kept at 37°C. The egg white from two dozen fresh eggs was collected and the membranes were broken up by stirring. Equal volumes of egg white and saturated sodium sulfate solution at 37°C were mixed and allowed to stand for two hours at room temperature. The precipitated material was then removed by filtration and discarded, and the filtrate adjusted to pH 4.7 by the addition of 6N H_2SO_4. A slight precipitation occurred during the pH adjustment, and it was dissolved by the addition of a small amount of distilled water. With the filtrate at pH 4.7, saturated sodium sulfate at 37°C was added with constant stirring until an opalescence developed. The mixture was allowed to stand at room temperature (23°C) for an hour before addition of a final 25 ml of saturated sodium sulfate. After 24-48 hours the mixture was examined microscopically for needle like crystalline albumin. The crystals were then centrifuged out at room temperature and redissolved in distilled water in one-half the original

volume of egg white. This procedure of crystallization was repeated three or four times, with the final crystals being dissolved in and dialyzed against distilled water. The crystalline ovalbumin thus obtained was lyophilized and stored.

Crystalline bovine serum albumin.--The crystalline bovine serum albumin used in this study was obtained in the dry form from the Armour Laboratories.

Rabbit gamma globulin.--The procedure employed for the separation of gamma globulin from rabbit serum is that described by Campbell and Bulman (24). A saturated solution of ammonium sulfate was slowly added at room temperature to the serum to give a final one third saturated salt solution. The pH was adjusted to 7.8 and the mixture allowed to stand under refrigeration for two hours. The precipitate was removed by centrifugation, and then dissolved in 1% sodium chloride solution to one half the original serum volume. The gamma globulin was redissolved and reprecipitated three times. The final solution was dialyzed against 1% saline until free of NH_4^+. The final product had a small percentage of alpha and beta globulin as shown by electrophoretic analysis.

Alum precipitation.--Several of the proteins investigated were injected as alum precipitated proteins. The alum precipitated proteins were prepared by the addition of 5 ml of 1% alum to 45 ml of 1% protein solution. The solution was then adjusted cautiously with 1N HCl to achieve the maximum precipitation.

Oxidation of Proteins

Williams (8) employed hydrogen peroxide oxidation of proteins using concentrations of peroxide up to 7.5%. His investigation into the antigenicity of the oxidized protein showed some reduction dependent on the oxidative procedure as determined by gross anaphylaxis in guinea pigs. Since complete destruction of antigenicity was required for use as a plasma expander, it was decided first to try higher concentrations (20% and greater) of hydrogen peroxide and if antigenicity persisted, it would then be possible to eliminate the method at the start. If, however, successful destruction of antigenicity were attained, the minimum requirements could then be established.

In any experiment designed to test the antigenicity of a substance, special care must be taken to prevent bacterial contamination of the preparation. This factor is critically important when considered with respect to the susceptibility of guinea pigs to sensitization in the Schultz-Dale technique. Using this method Henry (1) reported positive sensitization with 0.00001 ml of horse serum which would contain about six micrograms of protein. Therefore, to insure against contamination, all preparations were either filtered through a Seitz pad or a fine sintered glass filter and preserved with merthiolate prior to storage. Sterile technique after the peroxide treatment was rigorously observed. The method of oxidation was essentially the same in all cases, and the final concentrations

used, hydrogen peroxide and protein, are summarized in Table 1.

In all cases except in preparation 7-III, the protein was dissolved in 1% NaCl and 30% H_2O_2 was added. In the preparations listed in Table 1, the concentration and volume of the protein solution before the addition of H_2O_2, and the relative volume of 30% H_2O_2 used were varied. The final concentration of both H_2O_2 and protein assuming that no volume changes occurred are given in Table 1. From these data the relative quantities of reagents used may be calculated.

In preparing 3-I, 1.0 gram serum albumin was dissolved in 20 ml of 1% NaCl. Eighty ml of thirty percent hydrogen peroxide were added gradually to the solution which was allowed to stand 18 hours at room temperature. No attempt was made to control pH which fell to about 4.2. The solution gradually turned slightly amber on standing, and eventually the protein precipitated as a gel. After 18 hours, the solution was cooled to 4.0° C and the pH adjusted to 7.8 with 0.1N sodium hydroxide. As a result of the pH adjustment, the hydrogen peroxide decomposed and the precipitated protein re-dissolved. To catalyze the hydrogen peroxide break down, trace amounts of manganese dioxide were added. The treated protein was dialyzed against several changes of 1% sodium chloride to eliminate any residual peroxide. The resulting products were then preserved by the addition of merthiolate (1 to 10,000), filtered, and stored.

Preparation of Anti-serum

Rabbit.--The standard procedure in the preparation of

TABLE 1

SUMMARY OF VARIOUS CONCENTRATIONS OF PROTEINS AND HYDROGEN PEROXIDE USED IN THE VARIOUS PREPARATIONS

Material	Experiment	Final Protein Conc.	Final H_2O_2 Conc.
BSA	3-I	1.0%	24%
	3-II	4.0%	24%
	3-III	2.0%	27%
	3-IV	2.0%	27%
	3-VI	2.5%	28%
	3-VII	2.5%	28%
	3-VIII	1.0%	25%
Ovalbumin	7-I	4.0%	20%
	7-II	5.0%	27%
	7-III	5.5%	30%
Rabbit ɣ globulin	6-IV	1.5%	25%
Gelatin	13-I	2.0%	18%

rabbit anti-serum was as follows. Sensitizing shots of 1.0 cc of 1.0% antigen were given through the marginal vein of the rabbits ear every other day. This sensitizing course was continued for a six week period, after which the animals were rested for an additional week prior to bleeding. The animals were bled by cardiac puncture, the blood allowed to clot and the serum removed 24 hours later.

Chicken.--Chickens were given three injections intramuscularly on successive days. The concentration of the antigen used was 1.0 ml of a 1.0% solution, but the animals in this case were bled four days after the last injection.

Storage of anti-serum.--Anti-serums had merthiolate (1/10,000) added and were then frozen and stored. It was found by Sutherland and Terres (25) who investigated the effect of merthiolate, filtration by Seitz pad, and several slow freezing and thawings, that the titer was identical in all combinations with that of the serum sample having been just merthiolated.

Quantitative Precipitin Determination

Preparation of precipitating mixtures.--The procedure used was that described by Lanni and Campbell (26). Serial dilutions of antigen with a dilution factor of 2/3 were made with 1% sodium chloride and measured amounts added to Pyrex Wassermann tubes. A constant amount of anti-serum was added to each dilution of antigen and the reagents were thoroughly mixed. The mixtures were maintained at 37°C for two hours, after which they were stored at 5°C for 48 hours. These

tested were always run in duplicate and frequently in triplicate. Serum-saline and antigen-saline controls were also used in all runs, and the nitrogen obtained, if significant, was subtracted from the values of the remaining tubes.

Quantitative analysis of precipitates.--After 48 hours of cold storage the precipitates were centrifuged for 15 minutes at 2000-3000 r.p.m., in an International Refrigerated centrifuge at 4.0°C. Each tube was refilled with ice cold 1% saline and re-centrifuged as described previously. This washing procedure was repeated twice again with thorough draining permitted between washings.

The precipitates were analyzed for total nitrogen by the use of Nessler's reagent (27). Each tube had added 0.5 ml of concentrated sulfuric acid. To insure against inadequate digestion, all tubes were heated with a low flame a minimum of one hour and until a dense white fume was given off. With proper adjustment of the flame, the sulfuric acid would reflux in two thirds of the tube for three quarters of the time of digestion. At the completion of the hour of digestion, the tubes were allowed to cool and a drop of 30% hydrogen peroxide was added. The digestate was reheated for ten minutes, and if complete decoloration had not occurred, the procedure of cooling with the addition of 30% hydrogen peroxide was repeated. Adequately digested samples were transferred quantitatively with distilled water to Folin-Wu tubes calibrated at 35 and 50 mls. All tubes were brought up to the 35 ml mark with distilled water, and to the 50 ml mark with

Nessler's reagent. Thorough mixing was immediately insured by means of a glass rod, and the samples were read 30 minutes later at 420 mu in a Bechman Model B spectrophotometer. Blank samples which consisted of distilled water, sulfuric acid, and Nessler's reagent were analyzed. Ammonium sulfate standards were used in all runs at a concentration of 0.1 mg N per ml. The standard and blank samples were always digested in the Folin-Wu tubes with the procedure otherwise identical.

Qualitative analysis for precipitins.--The technique of layering antigen above anti-serum to give an interfacial precipitate was employed as a qualitative method of ascertaining the presence of precipitins in various chicken and rabbit serums. Every test was observed for a minimum of 20 minutes, and the time required for the development of a precipitate was recorded.

In order to determine the limitations of the interfacial technique, a serum of known antibody concentration was diluted with normal serum up to 0.001 of the original concentration. Various concentrations of the antigen were then layered over the serum and observed for 20 minutes with respect to the presence, the denseness, and the speed of formation of a precipitate. The results of these determinations are summarized in Table 2. The denseness of the precipitate

TABLE 2

DETERMINATION OF SENSITIVITY OF INTERFACIAL TECHNIQUE USING RABBIT ANTI-SERUM

Antigen Dilutions	Dilution of ab Serum 1	1/10	1/100	1/200	1/1000
1/1 T	++++ I	+++ I	++ 1½ min	+ 6 min	-
1/10 T	++++ I	+++ I	++ 2 min	± 5 min	-
1/100 T	++++ I	+++ ½min	++ 2 min	± 5 min	+ 10 min
ab conc. mg/ml	4.000	0.400	0.040	0.020	0.004

I: immediate appearance

is indicated by crosses, with the maximum being four crosses, and immediately following denseness is the time in minutes required for the first appearance of a positive test. From these results it is shown that the sensitivity of the interfacial test by this investigator using rabbit anti-serum is sufficient to detect 0.004 mg of antibody per ml.

CHAPTER III

PHYSICAL CHEMICAL CHARACTERIZATION OF TREATED PROTEINS

Introduction

The principal objective of this investigation was to determine whether the antigenicity of certain proteins would be destroyed by hydrogen peroxide oxidation. Physical chemical characterization was undertaken primarily to aid in the interpretation of immunological changes. Special interest has been directed toward the establishment of the molecular size of the derived proteins, and preliminary studies have been conducted to determine homogeneity and chemical alterations. It was hoped that from this investigation some fundamental knowledge might be obtained relative to protein characteristics essential to antigenicity.

Among the general properties of antigenic proteins, a molecular weight of at least 10,000 to 15,000 appears to be necessary to induce antibody formation.[24] There is also evidence which indicates that antigenic potency increases with molecular size.[28] Knowledge of the molecular weights of the derived proteins was therefore necessary for the interpretation of the antigenic changes induced by hydrogen peroxide oxidation, and for the evaluation of the product as a plasma

expander. Molecular weights were obtained primarily from osmotic pressure determinations. Sedimentation and viscosity studies were carried out to obtain information about the number of components present and the frictional properties of the products.

The study of homogeneity was undertaken with the purpose of gaining some insight in the possible type of size reductions that had occurred with hydrogen peroxide treatment. Highly homogeneous products of reduced molecular size could be indicative of a specific splitting of the molecule while a highly heterogeneous mixture could be interpreted as a random splitting of the molecule. If aggregation of fragments or a survival of the original protein had occurred with the oxidative treatment, detection of such components might be possible with either electrophoretic or sedimentation studies. While a quantitative evaluation of sedimentation patterns for homogeneity is possible, only a qualitative evaluation was made at this stage of development. The degree of spreading of the electrophoretic and sedimentation boundaries was used as criteria in the evaluation of homogeneity.

Changes in the chemical composition of the various proteins oxidized were studied by spectrophotometric analysis, and chromatograms of hydrolyzates. The rationale behind the use of the spectrophotometric analysis is presented in Chapter I, and its main use here was in the investigation of possible changes in the aromatic amino acid composition of the

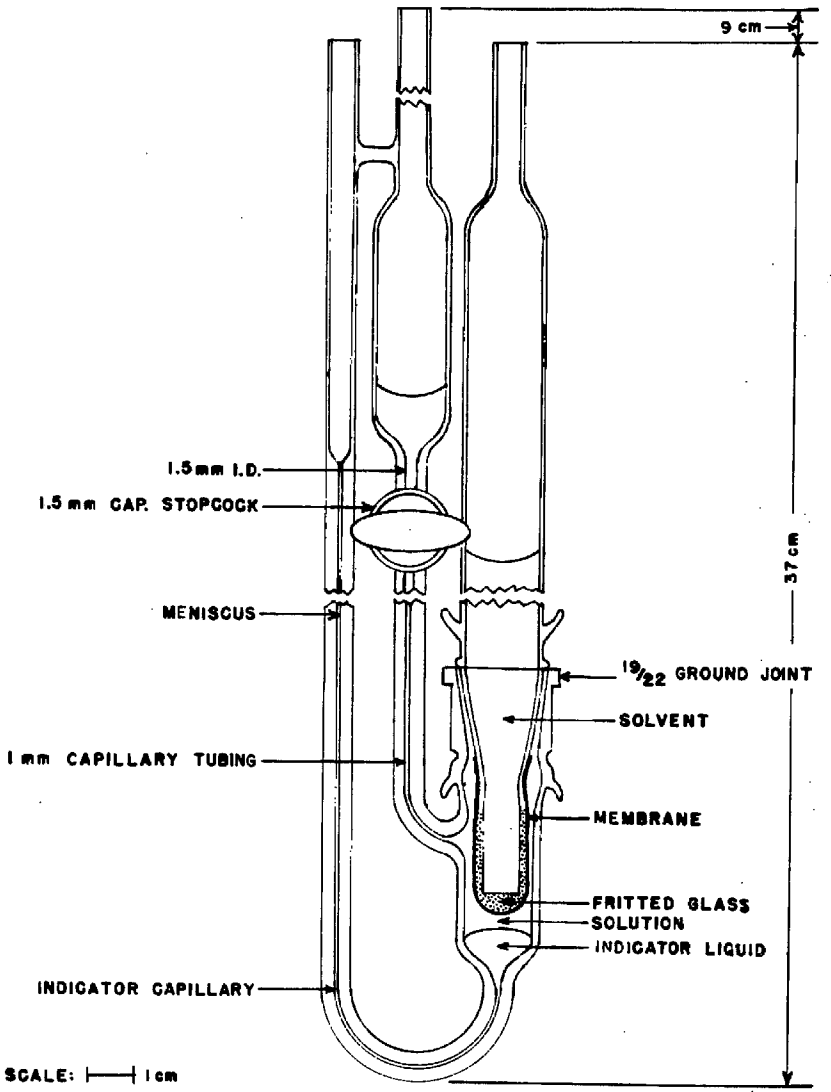
oxidized proteins. Chromatograms of the hydrolyzates of the various proteins were obtained with the purpose of assaying qualitatively how extensively the amino acid composition had been changed by oxidation. A quantitative analysis of the amino acid composition of the derived proteins was not considered within the scope of this investigation.

Methods

Osmotic Pressure Determinations.--The instrument used in the determination of molecular weights was a modification[29] of the Bull osmometer,[30] see figure 1. In contrast to most osmometers, the solvent is placed within an inner compartment, and is surrounded by the protein solution. The semipermeable membrane is cast on a sintered glass gas disperser. The formula for the membrane is taken from one recommended by Adair[31] which allows some degree of freedom in the selection of permeability. The formula is as follows:

4%	Nitrocellulose
50 cc	Absolute alcohol
50 cc	Anhydrous ether
2 cc	Ethylene glycol

The 4.08 grams of nitrocellulose was first mixed with the 50 cc of absolute ethanol, and then the ether and ethylene glycol were added. The procedure accepted for casting was based on the desire to obtain a final osmotic pressure in 24 hours, and therefore consisted of two castings separated by



FRITTED GLASS OSMOMETER

Fig. 1. Osmometer used in the determination of number average molecular weights.

two minutes with a final drying of 30 minutes. The membranes were soaked in distilled water for 24 hours and then equilibrated with the protein solvent.

The differential pressure between solvent and solution is established by the adjustment of the solvent height. After temperature equilibrium is achieved, the stop-cock is closed and there results a solvent flow across the membrane until the osmotic pressure is reached. The solvent flow, and the corresponding change in pressure, is followed by means of an organic fluid in a capillary in direct contact with the protein solution. The osmotic pressure is obtained by adding the indicated change in pressure to the original pressure established by the difference in height between solution and solvent. Since the changes in the indicator fluid occur in a capillary, variations in protein concentration due to solvent flow are markedly reduced while speed in the achievement of equilibrium is accelerated.

The molecular weight is determined by means of Van't Hoff equation (I):

$$\lim_{(c \to 0)} \frac{\pi}{C} = \frac{RT}{M} \qquad (I)$$

$$RT = 253.4 \times 10^{3} \text{ dl x cm } H_2O/g$$

$$T = 298.25^{\circ} \text{ A}$$

Osmotic pressures were obtained at several concentrations and extrapolated to zero concentration. In general the results are linear with a slope greater than zero. The positive slope is a measure of the non-ideality of the solution and includes the effect of the Donnan equilibrium obtained on dialysis.[32] The Donnan effect elevates the osmotic pressure of a solution. The solvent used was an 0.2 M sodium chloride solution which was unbuffered and considered of sufficient ionic strength to minimize the Donnan effect.[32] The protein solution was thoroughly dialyzed against the solvent and subsequent dilutions made using the dialyzate.

The results of a typical run are presented in Table 3. A cathometer reference point, h_r (column 1), was taken at the bottom of the thimble at the start of each run. All heights were measured from the reference point. The height of the organic indicator fluid is h_i and the change from the initial height is indicated by Δh_i. The change in pressure, ΔP_i, is the product of Δh_i and the density of the organic fluid. The initial pressure difference (uncorrected) is equal to Δh, the algebraic sum of the protein solutions height, h_p, and solvent height, h_b. The uncorrected osmotic pressure is the sum of the values in columns 4 and 7. The corrected osmotic pressure, Pc, was obtained by means of the following equation:

TABLE 3

EXAMPLE OF OSMOTIC PRESSURE DATA

OSMOTIC PRESSURE DETERMINATION NO. 6-13

Operator: Terres — Date: 13 May '55

Treated Material: Toval 7-III — Osmometer No.: 2

Conc.: 0.286% — Membrane No.: 2 (24 March '55)

Solvent: 0.2 M NaCl, u = 0.2, pH = 6.9 — Indicator Fluid: TPFBA, d_4^t 1.895

Remarks: 1/10,000 Merthiolate added. Membrane checked prior to run

P_i (averaged) = 1.71

		Time		1	2	3	4	5	6	7	8	9
1	In Bath	14:40										
2	Close	15:25	Temp.	h_r	h_i	Δh_i	ΔP_i	h_p	h_b	Δh	P_u	$P_{u/c}$
3	Open		25.25	16.23	31.36	0	0	43.42	39.00	4.32	4.32	
4		16:00	25.30		32.40	+1.04	+1.97	--	--	--	6.29	
5	14 May	10:30	25.26		32.42	+0.97	+1.84	--	--	--	6.16	
6	"	15:00	25.26		32.46	+1.01	+1.91	--	--	--	6.23	
7	16 May	9:30	25.26		32.19	0.89	+1.68	--	--	--	6.00	
8	"	9:40	25.30		32.20	0.92	+1.74	43.31	39.00	4.31	6.06	21.20
9	open	9:40			31.28	0						

Solution density d_p: 1.0065 — $\Delta h \cdot d_p$: 43.48 — A: 43.78

Solvent density d_b: 1.005 — $(h_b - h_r) \cdot (d_p - d_b)$ 0.03 — P_c: 6.09

$P_{c/c} = 21.28$

$$Pc = \Delta h\, dp + (h_b - h_r)(dp - d_b) + \Delta P_i.$$

$$Pc = A + \Delta Pi.$$

d_p = density of Protein solution.

d_b = density of Solvent.

Corrections for capillary rise were not necessary since only differences in the indicated levels are used.

The proteins used in these experiments were bovine serum albumin, chicken ovalbumin, and rabbit gamma globulin, all before and after hydrogen peroxide treatment.

Sedimentation constant determination.--Sedimentation experiments were performed in the Spinco Model E ultracentrifuge. The same proteins used in the osmotic pressure determinations were run in the analytical ultracentrifuge for the purpose of determining molecular weights and homogeneity.

The sedimentation constant was obtained from the slope of a plot of log x versus t, see fig. 2, in accordance with the integrated form of the equation for the rate of sedimentation, equation II.

$$\log x = \frac{s\omega^2}{2.303}\, t + I \qquad \text{(II)}$$

s = sedimentation constant

t = time in seconds

x = position of boundary at t in cm from the center of rotation

ω = angular velocity in radians per sec.

I = Integration constant.

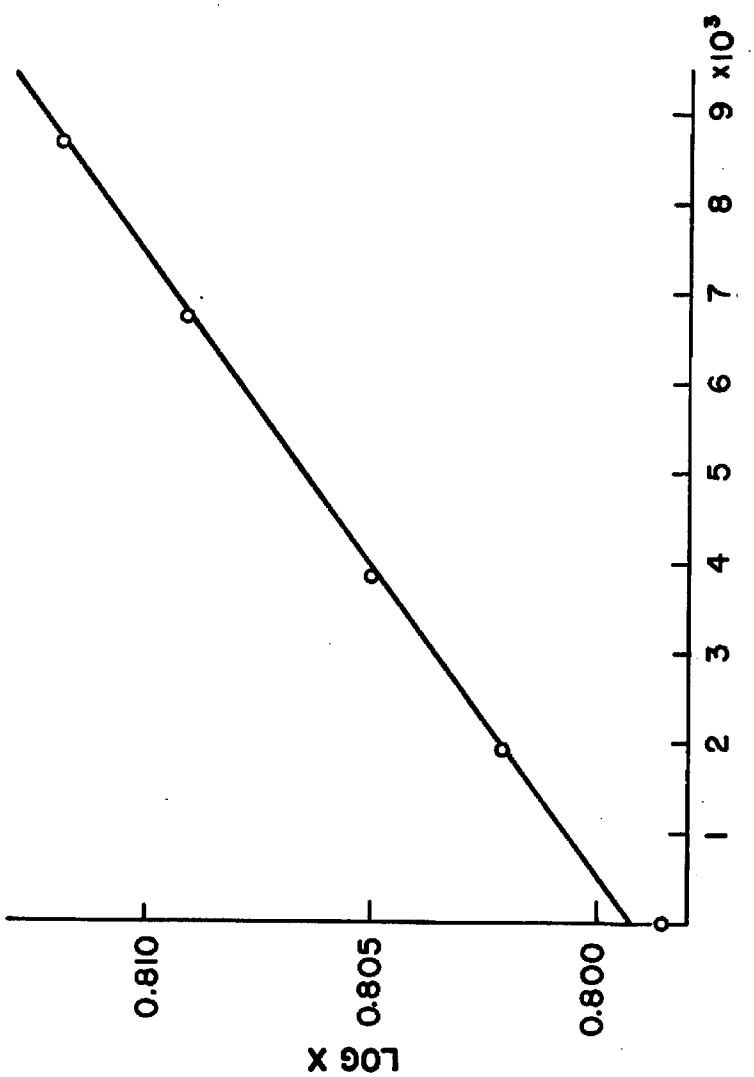


Fig. 2. Sedimentation determination of H_2O_2 treated bovine serum albumin.

Since the molecular weight was known to be low the synthetic boundary cell was used in centrifuge experiments. All the solutions contained 1.0% protein and were completely dialyzed against a solvent containing 0.1 ionic strength phosphate buffer and 0.2 M NaCl. The pH of this solvent was 7.3.

Viscosity studies.--Viscosity studies were undertaken with the various oxidized proteins to obtain information regarding molecular shape. Flow times through the capillary of a modified Ostwald viscometer were determined for both the solution and solvent. From Poiseuille law, equation III, it is seen that flow time is directly proportional to the viscosity and inversely proportional to the density.

$$t = \frac{8\, l\, v}{r^4 l' g} \cdot \frac{\eta}{\rho} = K\, \eta/\rho \qquad \text{(III)}$$

l = length of capillary

v = volume of test fluid

r = radius of capillary

l' = distance between drop in fluid

ρ = density

η = viscosity coefficient

t = flow time, sec.

K = apparatus constant

g = acceleration due to gravity.

The relative viscosity, η_r, which is defined by equation IV, is determined by measuring flow time, and density of a solution and a reference solvent. The specific viscosity, $(\eta_r - 1)$, gives

$$\eta_r = \frac{\eta}{\eta_o} = \frac{t\rho}{t_o\rho_o} \qquad \text{(IV)}$$

η = viscosity of solution

η_o = viscosity of solvent

ρ = density of solution

ρ_o = density of solvent

t = flow time of solution

t_o = flow time of solvent

the contribution of the solute, and the reduced viscosity, $\frac{\eta_r - 1}{c}$, gives the contribution per unit concentration of solute. Intrinsic viscosity, $[\eta]$, is obtained by the extrapolation of the reduced viscosity to zero concentration. As the concentration approaches zero, the contribution of the density term becomes negligible, and was therefore neglected in this work in which our interest is confined to intrinsic viscosity.

Intrinsic viscosities were determined for the following oxidized proteins: BSA, ovalbumin, and rabbit gamma globulin. Normal gamma globulin was also carried through the same procedure as a check on the experimental technique. The data were

plotted as in fig. 3 against concentration according to equation V and equation VI.

$$\lim_{c \to 0} \frac{\ln t/t_0}{c} = [\eta] \qquad (V)$$

$$\lim_{c \to 0} \frac{\frac{t}{t_0} - 1}{c} = [\eta] \qquad (VI)$$

c = grams anhydrous protein per deciliter (dl).

Mathematically in the limit both equations should approach identity and give the intrinsic viscosity as the point of intersection of the lines and the ordinate representing zero concentration, see fig. 3. By using both methods the quality of the data is evaluated on the basis of agreement at the point of interception of the ordinate.

To determine the axial ratio, it is first necessary to establish the viscosity coefficient, ν', which is dependent on both the axial ratio and the degree of hydration of the molecule and is directly accessible as indicated by equation VII. The partial specific volume, $\bar{v}$, was assumed to be 0.75

$$\lim_{c \to 0} \frac{\eta/\eta_0 - 1}{\bar{v} c} = \nu' \qquad (VII)$$

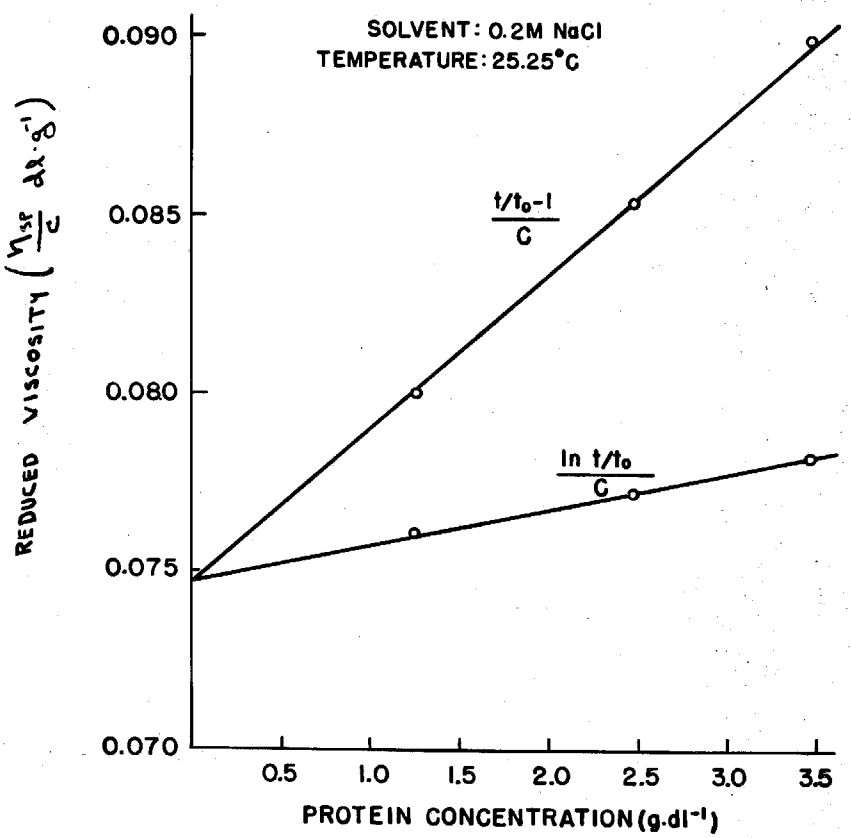


Fig. 3. Intrinsic viscosity determination of normal rabbit gamma globulin.

for the purpose of this calculation. With the aid of Oncley's[33] graph, the viscosity coefficient, and an assumed hydration, it is possible to obtain axial ratios for prolate and oblate ellipsoids of revolution. Finally, these axial ratios can be used to establish frictional ratio, which in conjunction with sedimentation data permit the determination of molecular weight by the following equation:

$$M = 2.45 \times 10^{22} \sqrt{\bar{v}_{20}} \left[\left({}^{f}/_{f_o} \right) S_{20,w} / (1 - 0.9982 \bar{v}_{20}) \right]^{3/2} \qquad \text{(VIII)}$$

f/f_o = frictional ratio

$\bar{v}_{20}$ = partial specific volume at 20° C

$S_{20,w}$ = sedimentation constant at 20° C and in water

Electrophoretic studies.--The Perkin-Elmer electrophoresis apparatus was utilized to obtain patterns for the determination of electrophoretic homogeneity, and mobilities in the characterization of the proteins used in this study. All samples were run at approximately one percent concentration in veronal buffer of 0.1 ionic strength and at pH 8.6. The calculation of mobility was determined from the following equation:

$$\mu \left({}^{cm^2}/_{volt\ sec} \right) = \frac{dx/dt}{E_p} = \frac{dcq}{tIR}$$

d = distance traveled

q = cross sectional area of cell

t = seconds

I = amperes

E_p = electric field strength of protein solution

R = resistance

c = conductivity cell constant

Spectrographic studies.--Absorption spectrums for the various treated and untreated proteins were obtained in the Beckman Model DU or the Cary spectrophotometer. The preparations used in these experiments were dialyzed against a phosphate buffer at pH 7.5. Dilutions necessary to maintain a readable optical density were made with the dialyzate.

Spectrums were investigated in the following treated and untreated proteins: chicken ovalbumin, rabbit gamma globulin, and gelatin. All optical densities were converted to extinction coefficients by the following formula (IX).

$$E = \frac{O.D.}{C \times l} \qquad (IX)$$

E = extinction coefficient

O.D. = optical density

C = mg protein/ml

l = length of cell

The nitrogen concentrations of the various solutions were determined by the Nessler technique, and protein concentrations calculated on the basis of its percentage nitrogen. (See Table 9.)

Chromatographic studies.--The various proteins studied were hydrolyzed by mixing equal volumes of a 1.0% protein solution and 12 N hydrochloric acid. The resulting solution was then sealed in a pyrex glass tube and placed in an oil bath at 105° C from 10 to 50 hours. Paper chromatograms, ascending as well as descending, were obtained with the following solvent: 3 parts n-propanol and 1 part of 1% acetic acid. A second solvent used to differentiate cysteic acid from cystine[22] consisted of 1 part of 50% hydrochloric acid and two parts of n-propanol.

The migrated amino acids were identified by spraying with 0.5% Ninhydrin solution in n-butanol or pyridine and then oven dried at between 75-80° C.

Concentration determinations.--Concentration determinations for the various protein solutions were obtained either by differential refractometery, or by nitrogen determination utilizing the Nesslerization technique. The Brice-Phoenix differential refractometer employs a double prism type cell which by rotating the cell 180° or interchanging the solvent and protein solution, results in a slit image displacement, d. The image displacement, d, is related to concentration by the following equation:

$$d = \frac{c}{K} \, dn/dc \qquad (X)$$

d = image displacement

K = apparatus constant

c = concentration (g/100 ml)

dn/dc = specific refractive increment.

In the case of the unoxidized proteins, the specific refractive increments and percentage nitrogen of these proteins were taken from the literature. The specific refractive increment, dn/dc, have been determined for various proteins and are tabulated by Doty and Geiduschek.[13] It was necessary, in the case of the oxidized proteins, that the specific refractive increment and percentage nitrogen be determined. The procedure used in these determinations was as follows:

(1) The concentration of the various protein solutions was determined by drying known volumes at 115° C for a minimum of 48 hours.

(2) The nitrogen content was determined on a second portion of the same protein solution by a procedure identical to that described for the standards used in the quantitative evaluation of precipitins. One ml of the protein solution was digested with concentrated sulfuric acid in a Folin Wu tube. Subsequent steps were performed as described in Chapter II. The nitrogen content was finally related to the dry weight determinations and a percentage nitrogen obtained.

(3) The image displacement in the differential refractometer was determined on a third portion of the protein solution. Since the concentration of the protein solution and the apparatus constant were known, the specific refractive increment could be calculated by equation (X).

Results

Osmotic Pressure Determination.--The present work constituted the first extensive study employing the modified Bull osmometer. It was found necessary in this work to introduce some modifications in technique. These were pre- and post-operational checking of each membrane for possible leaks, fire polishing of the sintered glass support prior to casting the membranes, and complete temperature equilibration at the anticipated osmotic pressure so as to avoid solvent flow across the membrane and dilution of the protein. It was also found that adding merthiolate to the system resulted in a higher reproducibility, especially the treated ovalbumin.

The results for the various protein solutions are presented in the following figures: normal BSA, Fig. 4, normal ovalbumin, Fig. 5, and the various oxidized proteins, Fig. 6. The molecular weights obtained are summarized in Table 4.

The reliability of the modified Bull osmometer was established by the close agreement between the molecular weights of the un-oxidized proteins reported in the literature and

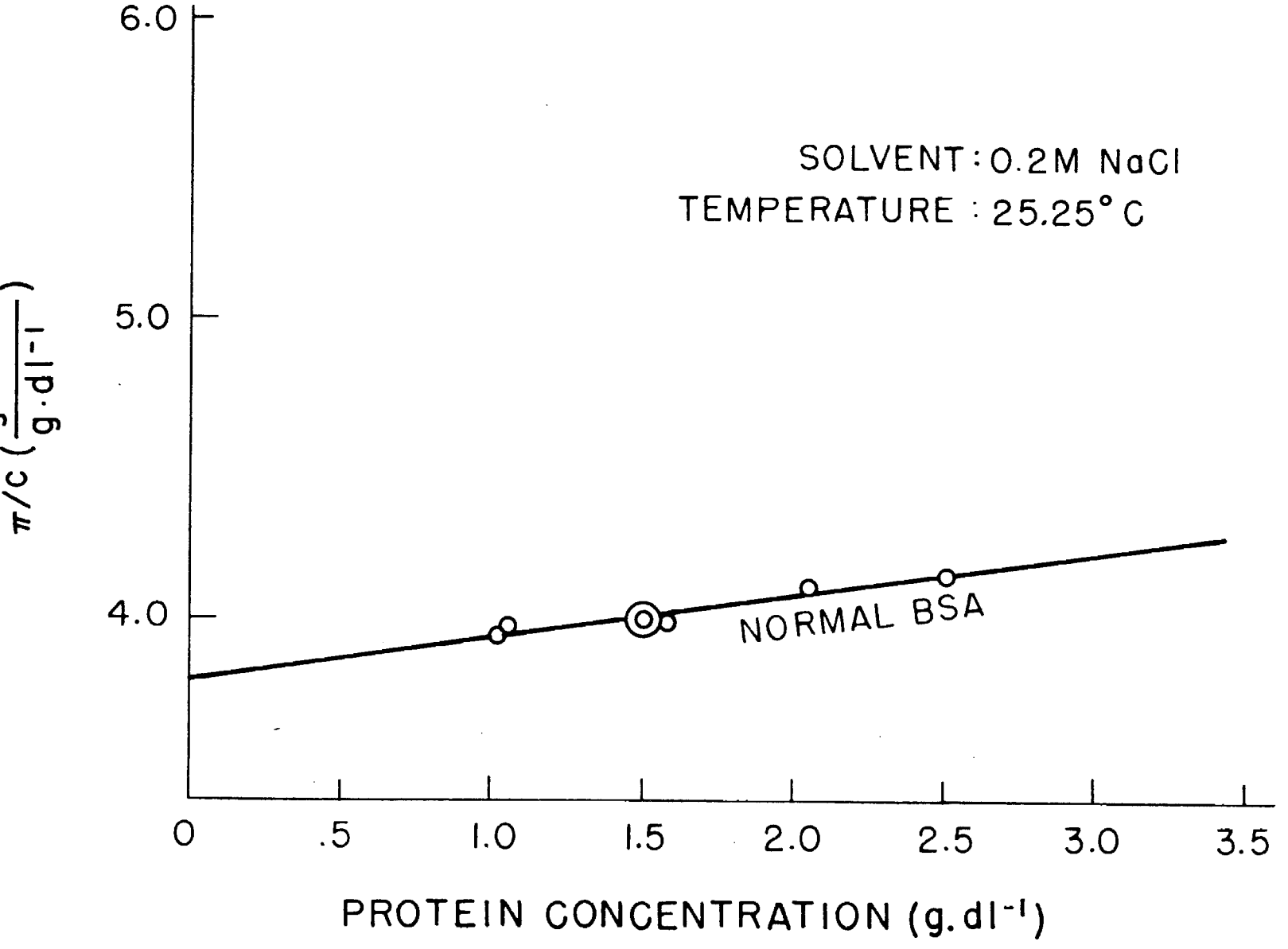


Fig. 4 Osmotic pressure determination of normal serum bovine albumin.

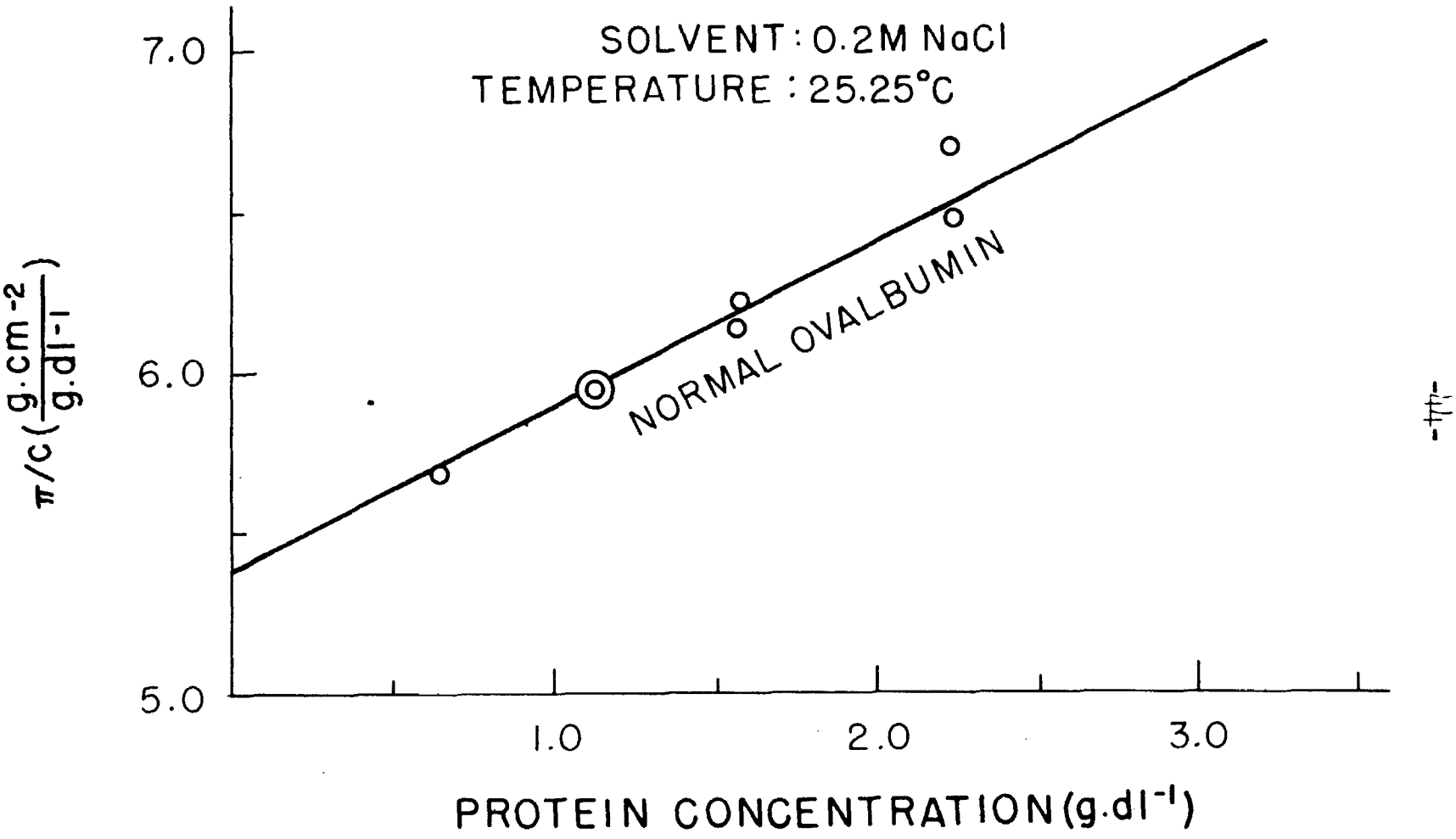


Fig.5 Osmotic pressure determination of normal ovalbumin.

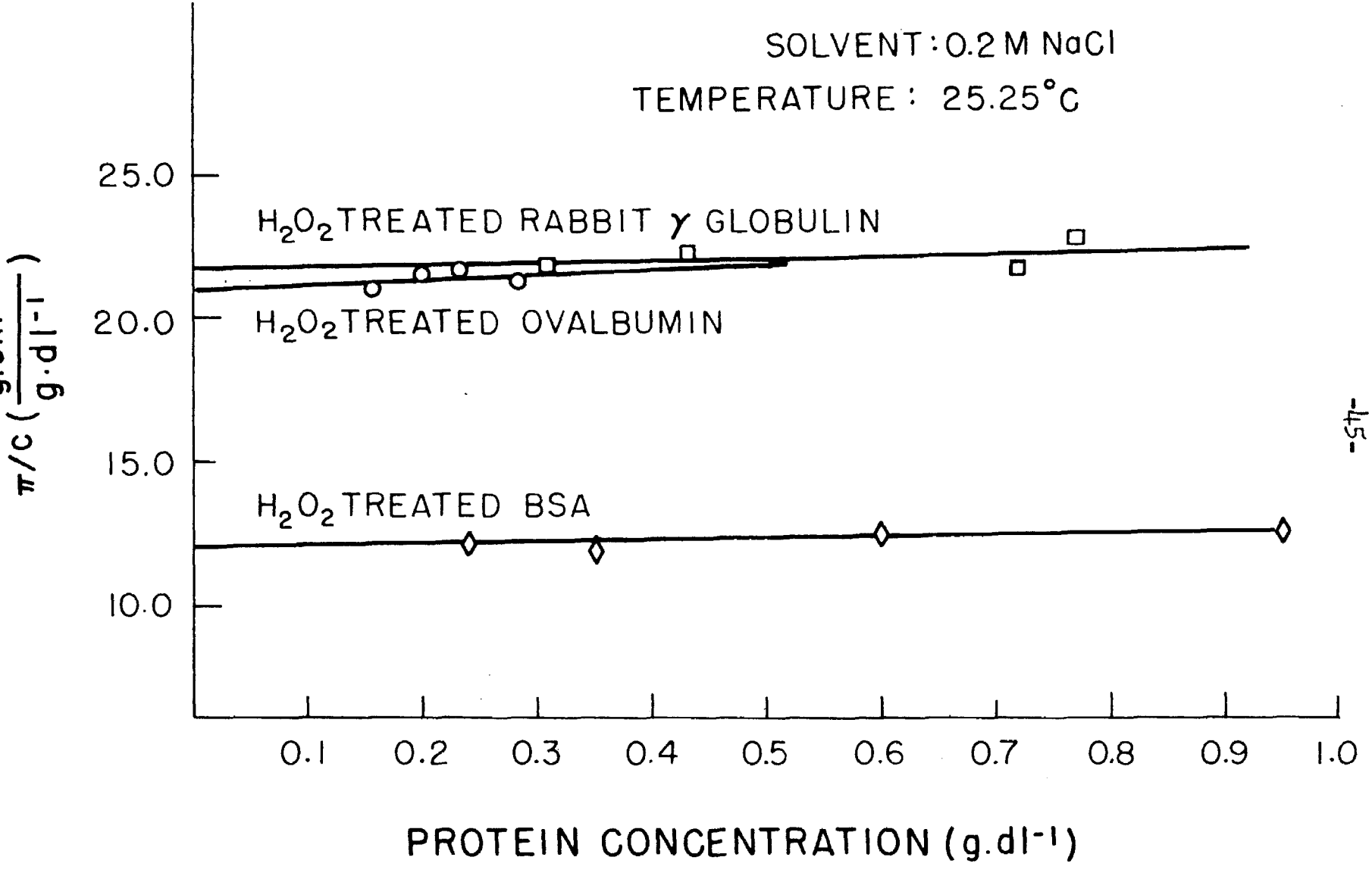


Fig. 6 Osmotic pressure determination of H_2O_2 treated proteins.

TABLE 4

MOLECULAR WEIGHT OF THE OXIDIZED AND NORMAL PROTEINS FROM OSMOTIC PRESSURE MEASUREMENTS

Protein	Molecular Weights	
	Exp.[a]	Lit.[b]
Bovine serum albumin	67,000	69,000
Oxidized bovine serum albumin	21,000	--
Ovalbumin	47,000	44,800
Oxidized ovalbumin	12,000	--
Rabbit gamma globulin	--	160,000
Oxidized rabbit gamma globulin	11,600	--

(a) Experimentally determined in this investigation.

(b) Reference (32).

those determined experimentally in this investigation. There is only a 2.9% discrepancy between the presently accepted number average molecular weight for bovine serum albumin, 69,000, and that obtained here, 67,000.

Due to the marked reduction in molecular size, difficulty was experienced in obtaining molecular weights for the oxidized proteins. Concentrations were reduced to obtain osmotic pressures within the operating range of the apparatus, and the absorption of the protein to the membrane became significant. It was noted that after several osmotic pressure determinations with the same membrane the results became increasingly reproducible. The attainment of a steady osmotic pressure was further complicated by the gradual loss of small molecules through the membrane. The gradual loss of protein molecules through the membrane was indicated by a slow drop with time in the osmotic pressure. The rate of this leakage was dependent on the characteristics of the individual membrane. In the determination cited, table 3, the drop was very gradual and therefore considered insignificant. With more permeable membranes, the pressure dropped at a greater rate, and in these cases the highest pressure value was accepted as the osmotic pressure. The rationale behind this procedure was (1) that the protein solution was already in dialysis equilibrium with the solvent and therefore little if any solvent flow would be expected across the membrane, and (2) that the flow of water across the membrane was much

faster than the escape of the protein, and therefore the osmotic pressure would be attained before there occurred a significant dilution of the protein solution.

A minimum number average molecular weight of approximately 10,000 would be anticipated if extensive fragmentation had occurred with hydrogen peroxide treatment since in the preparation of the oxidized proteins exhaustive dialysis had been employed. Dialysis bags usually pass materials of less than 10,000, and only slowly those of around 10,000 molecular weight. The molecular weights obtained for oxidized ovalbumin and oxidized rabbit gamma globulin suggest that as a result of the oxidative procedure used, a population of fragments with molecular weights greater and smaller than 10,000 were produced, and that with dialysis a selective retention of those molecules with molecular weights greater than 10,000 occurred. Direct experimental evidence was later found for the existence of dialyzable material which contained 27.5% of the available starting nitrogen (see chromatographic studies). Williams[8] has shown that under milder conditions of hydrogen peroxide oxidation (2% H_2O_2) the reduction in molecular weight was not as pronounced (50,000).

The products of hydrogen peroxide oxidation reported here are not regarded as likely plasma expanders because of

low molecular weight. Such small molecules should be rapidly cleared from circulation. However retention of proteins in circulation is also a function of molecular shape. If the oxidized bovine serum albumin, for example, were rod shaped and of sufficient length, the retention value might be found to be acceptable (25 to 50% for 5 hours)[34] in spite of the low molecular weight.

Ultracentrifuge results.--The results obtained in the determination of sedimentation constants for the various proteins studied are summarized in Table 5. Un-oxidized proteins were also investigated to check the operational techniques. The close agreement between the present results and those reported in the literature established the reliability of the experimental method.

A substantial reduction in the sedimentation rate from that of the unoxidized protein has occurred in all cases studied. The reduction in the sedimentation constant is consistent with the observed decrease in number average molecular weight on one hand and the increased frictional coefficients as shown by the increased intrinsic viscosity.

Sedimentation patterns obtained with treated ovalbumin and gamma globulin (see Figures 7, 8, and 9) indicate that fragmentation is only one process involved in the oxidative treatment. The appearance of a fast moving component in the

TABLE 5

SUMMARY OF SEDIMENTATION CONSTANTS FOR NORMAL AND OXIDIZED PROTEINS AT 1 PERCENT CONCENTRATION

Protein	$S_{20,\omega} \times 10^{+13}$	
	Experimental	Literature
Normal ovalbumin	3.22	3.28
T-Ovalbumin (7-I)	0.87[a]	-
	5.36[b]	-
(7-III)	0.96	
Rabbit globulin	6.17[a]	6 and 7.05
	17.46[b]	-
T-Rabbit globulin	1.00[a]	-
	11.94[b]	-
N-Bovine serum albumin	-	4.27[c], 4.05
T-Bovine serum albumin	0.97	-

[a] Major component.

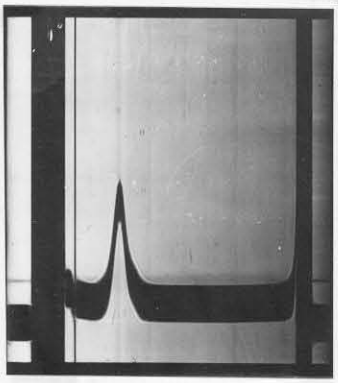
[b] Minor component.

[c] Extrapolated to zero concentration.

[d] $S_{20,\omega}$ is sedimentation constant in c.g.s. unity reduced to water at 20° C.

Oxidized Ovalbumin

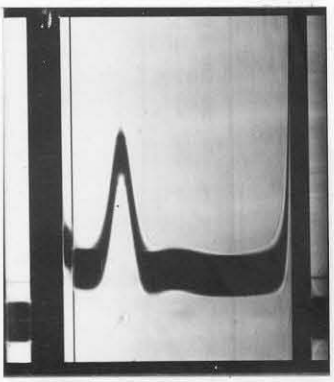
Normal Ovalbumin



Starting

Boundary

59,780 RPM



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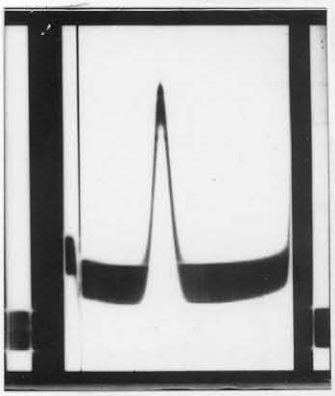

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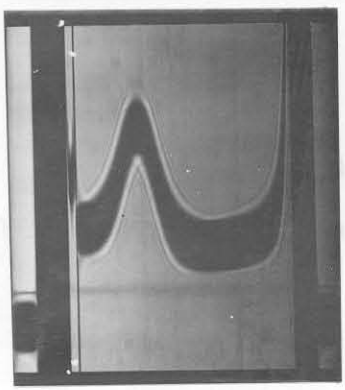

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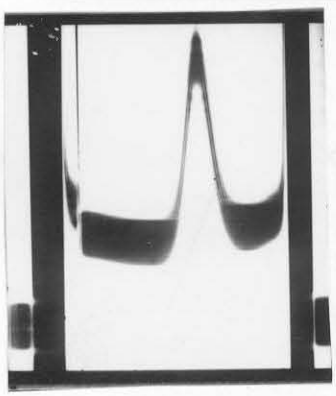

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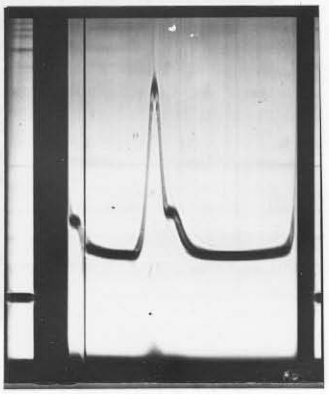
Fig. 7. Sedimentation patterns of normal and oxidized ovalbumin.

Oxidized Rabbit
gamma globulin

Normal Rabbit
gamma globulin



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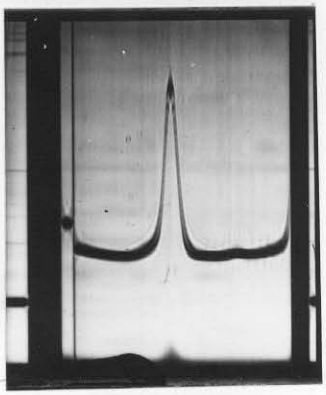

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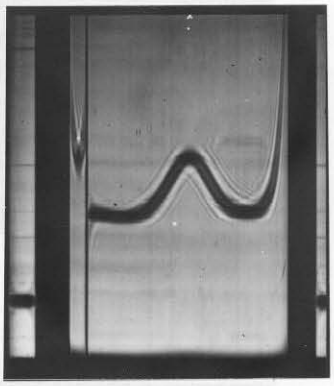
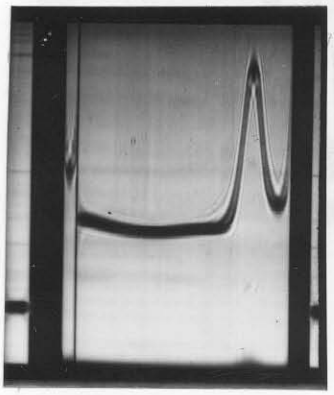




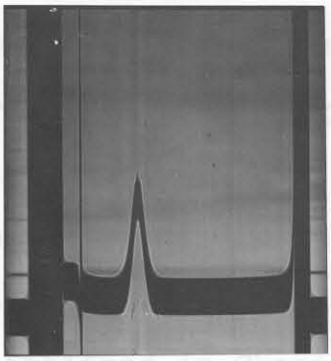
4320 sec.

3840 sec.

Fig. 8. Sedimentation patterns of normal and oxidized rabbit gamma globulin.

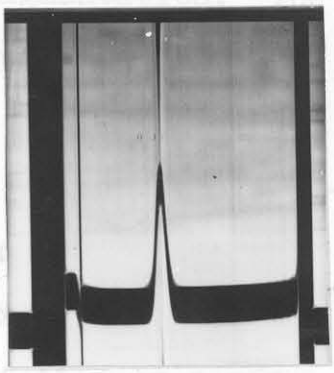
Oxidized Bovine
Serum Albumin

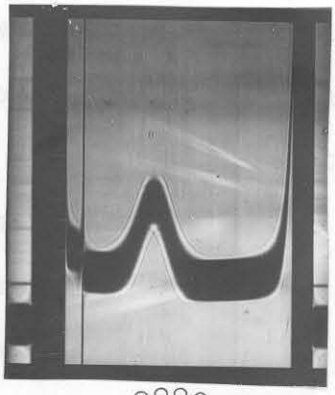
Normal Bovine
Serum Albumin



Starting
Boundary

59,780 RPM





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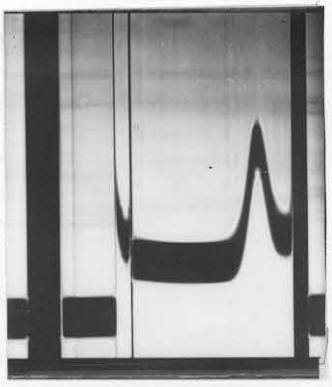

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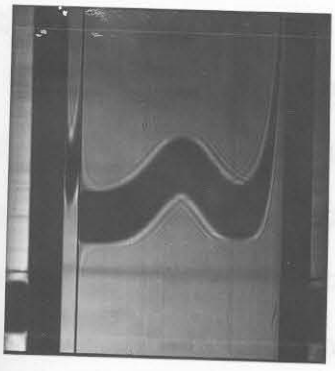


10,560 sec.

Fig. 9. Sedimentation patterns of normal and oxidized bovine serum albumin.

oxidative products of the above mentioned proteins indicates the occurrence of aggregation as well as fragmentation. The possibility exists that with the gamma globulin, partial resistance to fragmentation may account for the survival of a fast component. This, however, cannot be the entire explanation since an increase in the relative concentration of the fast component has also occurred with oxidation. As was mentioned in Chapter I, aggregation was indicated as a possible explanation of both the increased homogeneity associated with photo-oxidation of serums, and with the conversion of precipitating to non-precipitating antibodies.

It is evident that greater spreading of the boundary has occurred with the treated material. Before any conclusion may be drawn as to the homogeneity of the preparation based on boundary spreading, it should be noted that diffusion will also contribute to boundary spreading especially in view of the low molecular weight and the long duration of the run.

Intrinsic viscosity determinations.--The results obtained from the plotted data of determinations of intrinsic viscosity are presented in Fig. 10. The intrinsic viscosity values are tabulated in Table 6 as 100 $[\eta]$ along with values reported in the literature. An inspection of Table 6 shows that in all cases oxidized proteins show larger intrinsic viscosities. The values of intrinsic viscosity are approximately those obtained with gelatin of comparable molecular

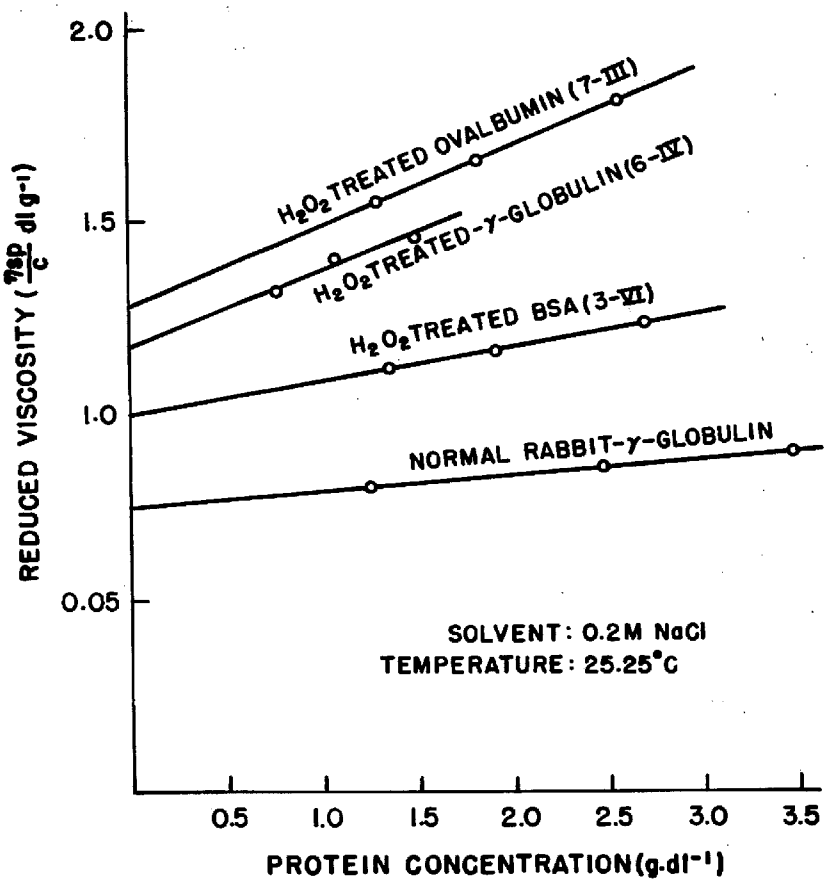


Fig. 10. Intrinsic viscosity determination of the various proteins studied.

TABLE 6

INTRINSIC VISCOSITIES, VISCOSITY INCREMENTS, AND AXIAL RATIOS FOR TREATED AND UNTREATED PROTEINS

Proteins	100 [η]		ν′		a/b Prolate[a]	
	Exp.	Lit	Exp.	Lit.	Exp.	Lit.
Human serum albumin	-	4.2	-	5.6	-	3.3
T-BSA (3-VI)	9.40	-	13.10	-	7.5	-
Ovalbumin	-	4.3	-	5.7	-	3.30
T-Oval. (7-III)	13.00	-	16.80	-	9.08	-
Rabbit globulin	7.47	-	9.95	-	6.0	-
T- -glob. (6-IV)	11.80	-	15.55	-	8.55	-
Human -glob.	-	6.0	-	8.1	-	5.0

[a]Assuming molecules to be rigid ellipsoids.

weight,[35] and of other randomly coiled high polymers in good solvents.[36] It is therefore concluded from these results that a principal effect of H_2O_2 oxidation is the partial uncoiling of the protein molecule. Ovalbumin showed the greatest increase in intrinsic viscosity as a result of oxidation and this is compatible with known tendency of ovalbumin to denature readily. Also included in Table 6 are the viscosity increments and the axial ratios obtained for the various proteins assuming them to be rigid ellipsoids. It was necessary in using Oncley's graphs[33] to assume some arbitrary degree of hydration before axial and frictional ratios could be determined. Thirty percent hydration was chosen for all the proteins.

Weight average molecular weights were determined with the aid of viscosity data and sedimentation constants according to equation VIII. These weight average molecular weights are presented in Table 7 along with the number average molecular weights obtained from osmotic pressure determinations. The results from the two types of determination are in theory only identical when the materials are highly homogeneous. As the heterogeneity increases, a discrepancy results in molecular weight values obtained by the two methods with the sedimentation-viscosity value being theoretically larger. It is apparent from electrophoretic patterns and the large percentage of dialyzable material in the H_2O_2 treated proteins that these products are not homogeneous. When compar-

TABLE 7

FRICTIONAL RATIOS AND MOLECULAR WEIGHTS DETERMINED FROM SEDIMENTATION AND VISCOSITY STUDIES FOR VARIOUS PROTEINS INVESTIGATED ASSUMING RIGID ELLIPSOIDS

Protein	f/f_o	$M_s \times 10^3$	$M_o \times 10^3$
T-BSA			
3-VI	1.60	10.18	21.
N-BSA	1.30	- ,	-
T-Oval.			
7-I	1.70[a]	9.57	-
7-III	1.70	10.78	12.0
N-Oval.	1.16	-	-
T- glob.	1.65	11.08	11.6
N- -glob.	1.45	140.5	158.0

[a]Assumed to be the same as 7-III

[b]The following assumptions were made for all proteins:

hydration = 30%

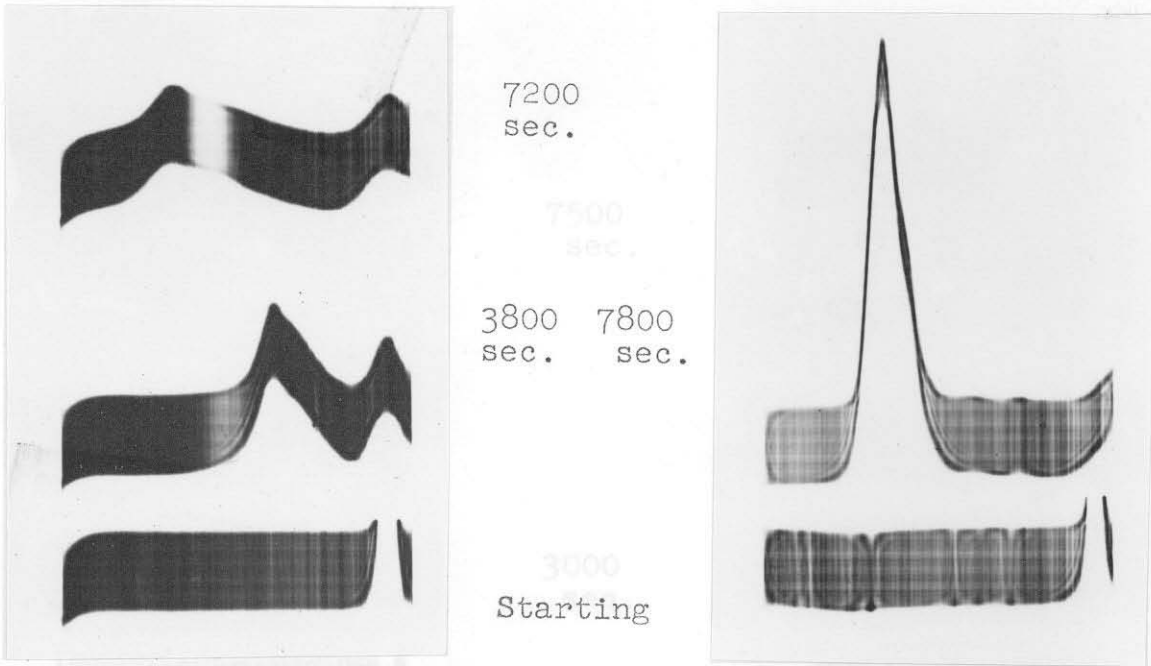
partial specific volume = 0.749.

[c]M_s = molecular weights calculated from viscosity and sedimentation data.

M_o = molecular weights obtained from osmotic pressure determinations.

ing the molecular weight values obtained by the two methods, it is seen that in all cases the number average molecular weight is larger than the weight average molecular weights. It is therefore apparent that use of sedimentation-intrinsic viscosity data to obtain weight average molecular weights was not valid since derivation of the method was based on rigid ellipsoids and not random coiled molecules. The use of equation VIII was also not valid since sedimentation constants were not extrapolated to zero concentration.

Electrophoretic studies.--Electrophoretic patterns for both the oxidized and unoxidized proteins are presented in Figs. 11, 12 and 13. The principal purpose of this investigation was to determine the degree of homogeneity of the various oxidized proteins. Close inspection of the treated ovalbumin and gamma globulin patterns indicate the occurrence of possible secondary components on the trailing edge that might under different conditions be resolved. The high degree of boundary spreading in the cases of treated bovine serum albumin and ovalbumin is here interpreted as indicating extensive heterogeneity of the preparations. It should however be remembered that under proper conditions (pH, conductivity and buffer) homogeneous substances have been known to spread.[37] This explanation should be kept in mind, but the probability that both preparations were here run under those conditions is low . . . It is concluded that the results obtained suggest a high degree of heterogeneity in the treated bovine serum albumin and treated

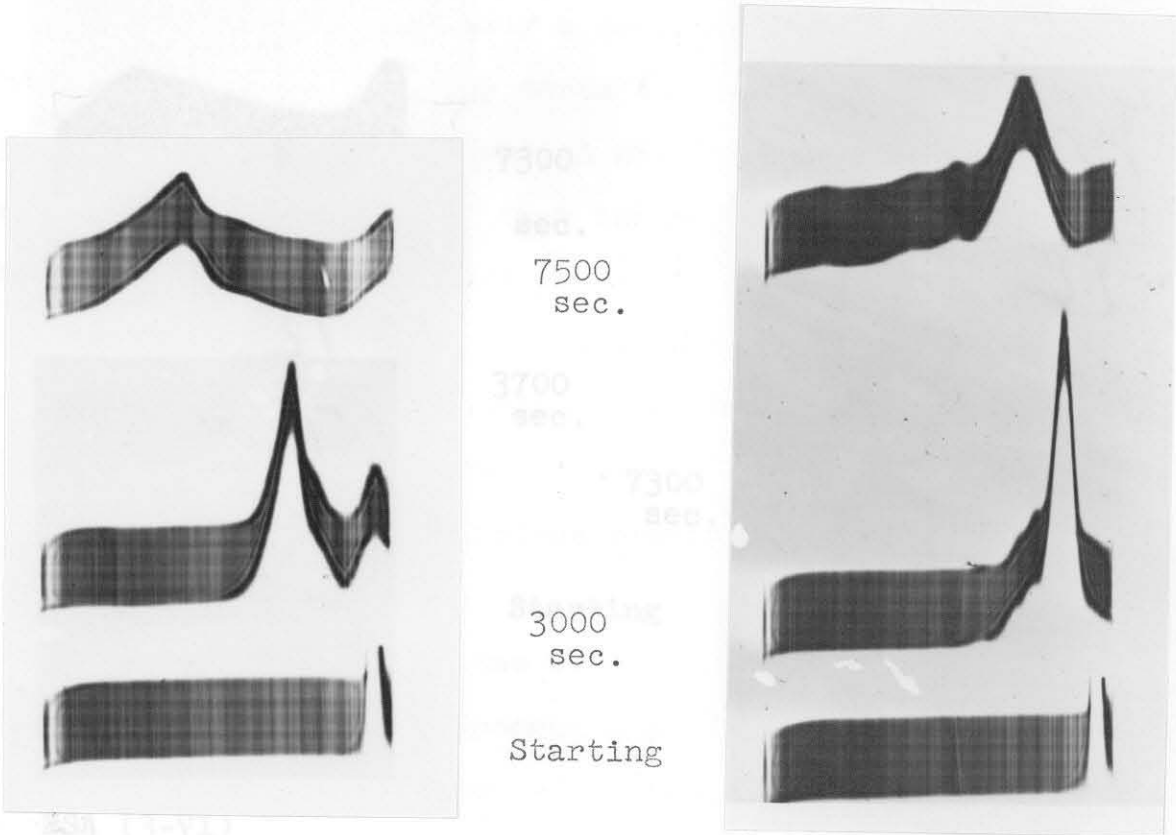


H_2O_2 Treated
Ovalbumin (7-I)
(Descending)

Normal Ovalbumin
(Descending

Veronal buffer
pH 8.6
u = 0.1

Fig. 11. Electrophoretic patterns obtained with normal and oxidized ovalbumin.

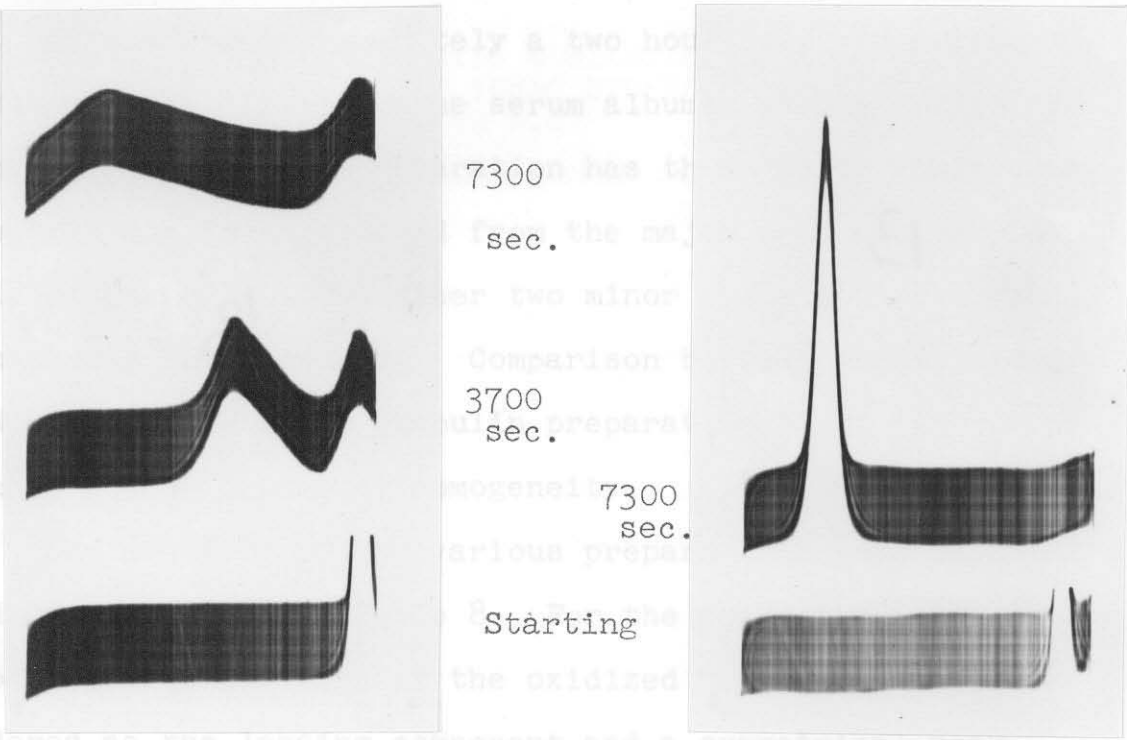


H_2O_2 Treated
rabbit gamma globulin (6-IV)
(Descending)

Normal Rabbit
gamma globulin
(Descending)

Veronal buffer
pH 8.6
u = 0.1

Fig. 12. Electrophoretic patterns obtained with normal and oxidized rabbit gamma globulin.



H_2O_2 Treated
BSA (3-VI)
(Descending)

Normal BSA
(Descending)

Veronal buffer
pH 8.6
u = 0.1

Fig. 13. Electrophoretic patterns obtained with normal and oxidized bovine serum albumin.

ovalbumin.

Even after approximately a two hour run, the pattern obtained with normal bovine serum albumin gave a very sharp peak. The ovalbumin preparation has three minor components. One of these is unresolved from the major peak and is probably plakalbumin. The other two minor components are likely conalbumin and ovomucoid. Comparison between the oxidized and un-oxidized gamma globulin preparation shows little change in the degree of homogeneity.

The mobility of the various preparations were calculated and are presented in Table 8. For the purpose of calculation, the leading edge of the oxidized proteins was considered as the leading component and a symmetrical peak drawn. The mobilities recorded in the above mentioned table are for this assumed leading component. An alternate method would have involved the determination of a coordinate equally dividing the total area. Since a high degree of heterogeneity is apparent, the leading edge was chosen as indicative of the maximum change in electrophoretic mobility induced by H_2O_2 oxidation. All three systems show a fraction of the oxidative product having electrophoretic mobilities greater than the original protein, and a small trailing fraction with a mobility slower than the original protein. If sulfhydryl and possibly disulfide groups were oxidized to sulfonic acids, electrophoretic mobilities should increase due to the lowering of the isoelectric point.

TABLE 8

SUMMARY OF ELECTROPHORETIC MOBILITIES OF NORMAL AND OXIDIZED PROTEINS IN 0.10 IONIC STRENGTH BARBITAL BUFFER (pH 8.6)

Proteins	Mobility $cm^2\ sec^{-1}\ volt^{-1} \times 10^{-5}$	
	Exp.	Lit.
Bovine serum albumin	-6.69	-6.5
Oxidized bovine serum albumin (3-VI)	-7.83	--
Ovalbumin	-5.35	--
Oxidized ovalbumin (7-I)	-6.11	--
Rabbit gamma globulin	-2.24	--
Oxidized rabbit gamma globulin (6-IV)	-5.83	--

Spectographic studies.--The rationale underlying this type of study is presented in the literature review section of this thesis. For comparison, all absorption data are converted to the extinction coefficient as described in the methods and then plotted against the wave length. The following spectrograms are presented: BSA system Fig. 14, ovalbumin system Fig. 15, and gamma globulin system Fig. 16. In each graph are presented the curves representative of the untreated and treated proteins of the system. There is, as a result of treatment, a relative increase in absorption occurring at the previous minimum and a relative decrease in absorption at the previous maximum. It is to be noted that the gross changes in the absorption spectrum as a result of oxidation are essentially the same for each of the proteins studied. Addition of tyrosine to T-BSA in quantities found in normal bovine serum albumin approximately restores the absorption spectrum of T-BSA to that of the unoxidized protein.

The effect of oxidation on a protein (gelatin) with a low percentage of aromatic amino acids is presented in Fig. 16. It is seen from these results that the oxidation of gelatin causes a general increase in its extinction coefficient. This general overall increase in absorption might account for the increase in the extinction coefficient at previous minimum of the other systems studied.

From these results it can be concluded that oxidation of tyrosine and phenylalanine has occurred. Though no informa-

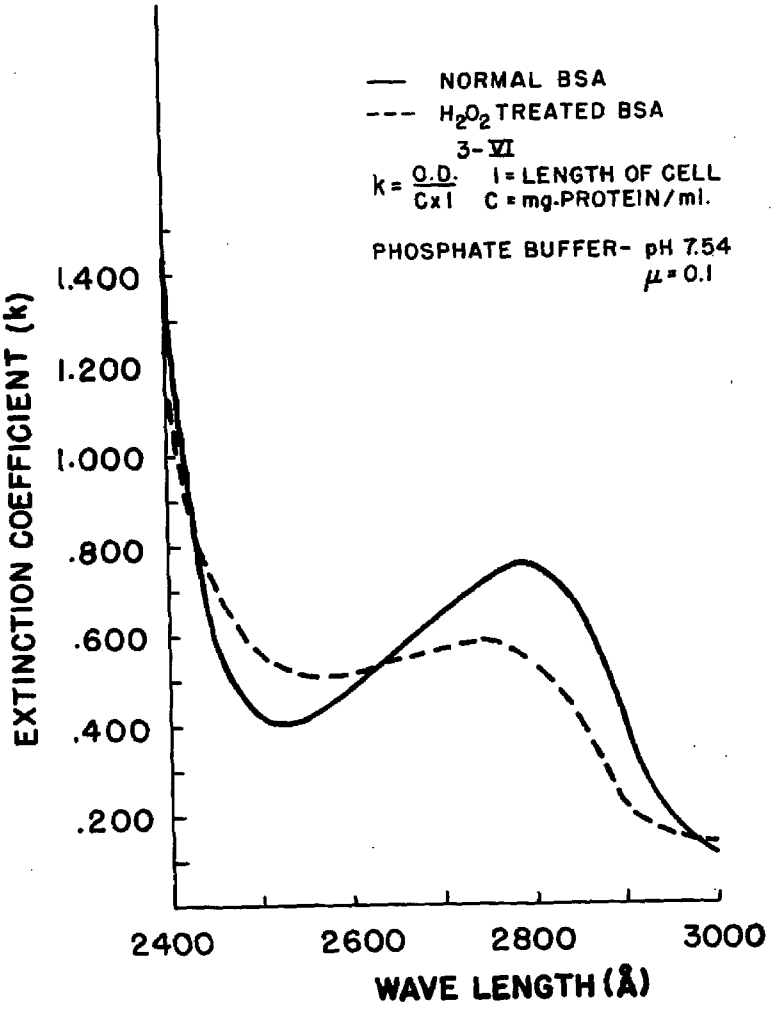


Fig. 14. Absorption spectrum obtained with normal and H_2O_2 treated bovine serum albumin.

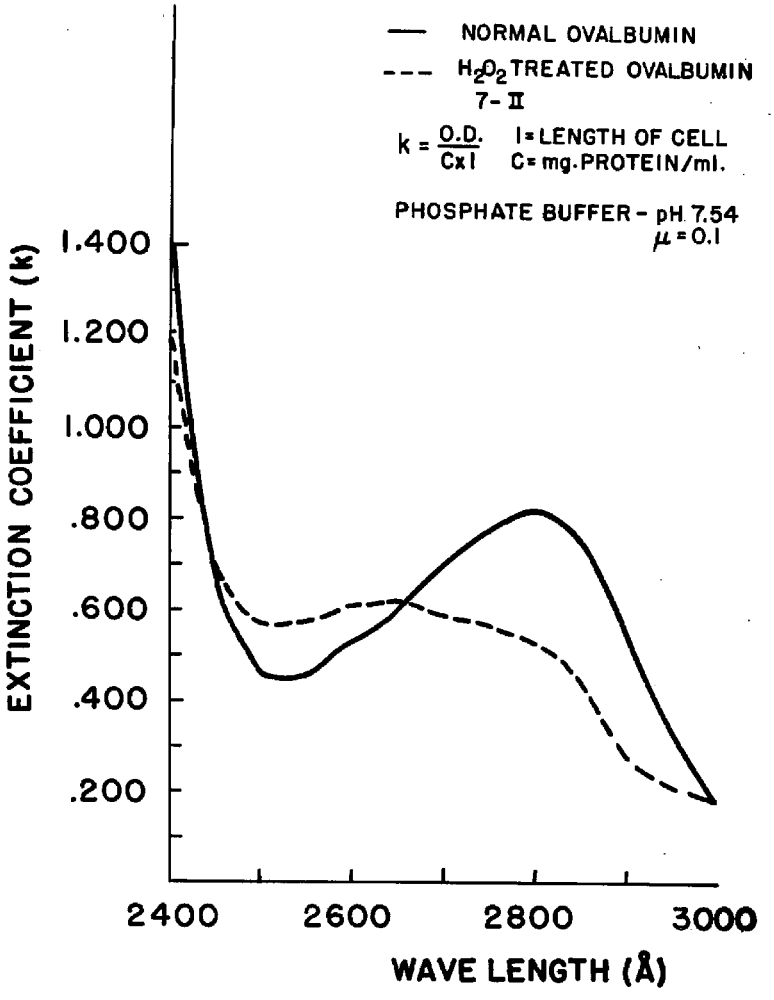


Fig. 15. Absorption spectrum obtained with normal and H_2O_2 treated ovalbumin.

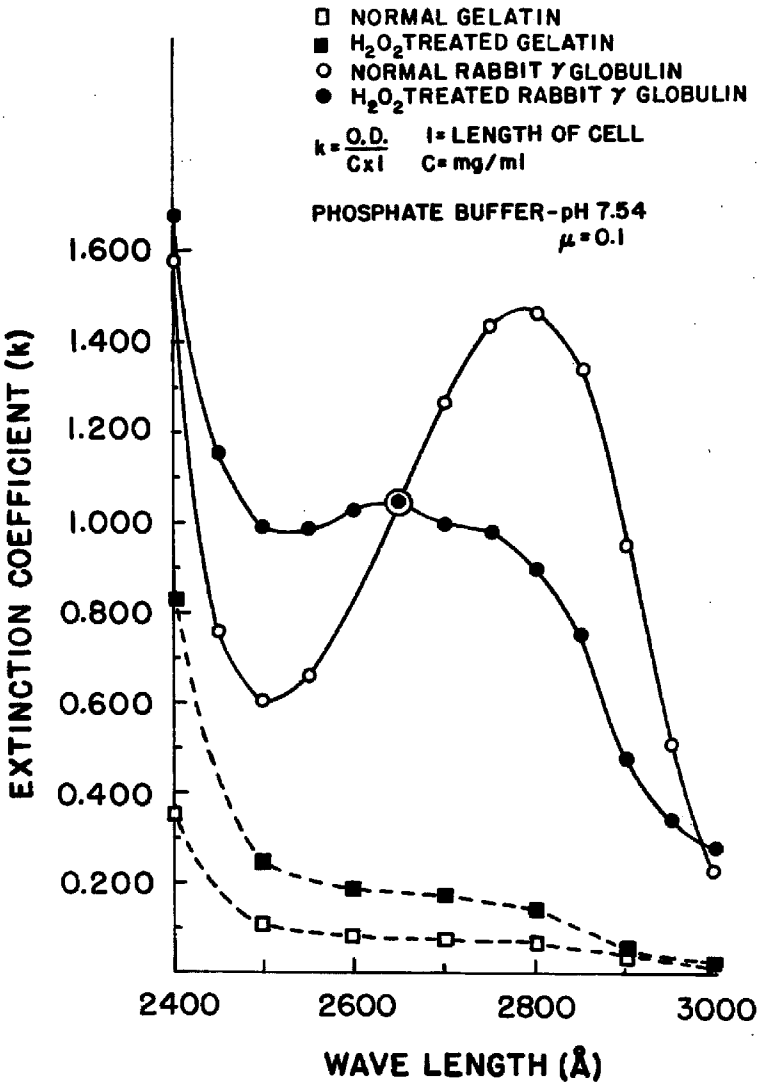


Fig. 16. Absorption spectrum obtained with normal and H_2O_2 treated rabbit gamma globulin and gelatin.

tion is available to suggest the possible end product of this oxidation, the higher extinction coefficient of dopa (3,4 dihydroxyphenylalanine) suggests that the oxidation has proceeded further than dopa and may have resulted in complete destruction of the ring.

Since the oxidative treatment of proteins might have resulted in the formation of dialyzable reaction products capable of absorbing in the ultra violet range of the spectrum, an oxidized sample not previously dialyzed was dialyzed against a small volume of phosphate buffer, and spectrums obtained both from the dialyzate and from the protein solution. The same sample was exhaustively dialyzed and the experiment repeated. The results of this investigation to determine to what extent fractionation into dialyzable particles occurs with hydrogen peroxide oxidation are presented in Fig. 17. The nitrogen concentration of the dialyzate, non-dialyzable oxidized protein, and untreated protein were determined and revealed that under prolonged dialysis, the amount of N in dialyzable form was found to be 27.5% of that available. The first dialyzate, 68.5 ml, contained 7.85% of the available nitrogen.

The absorption characteristics of the dialyzable particles was found to be quite high in the lower wave lengths studied. The true meaning of this finding is not known, but it is speculated that a selective splitting off of fragments did occur; otherwise an absorption curve would have been obtained similar to the undialyzable oxidized protein

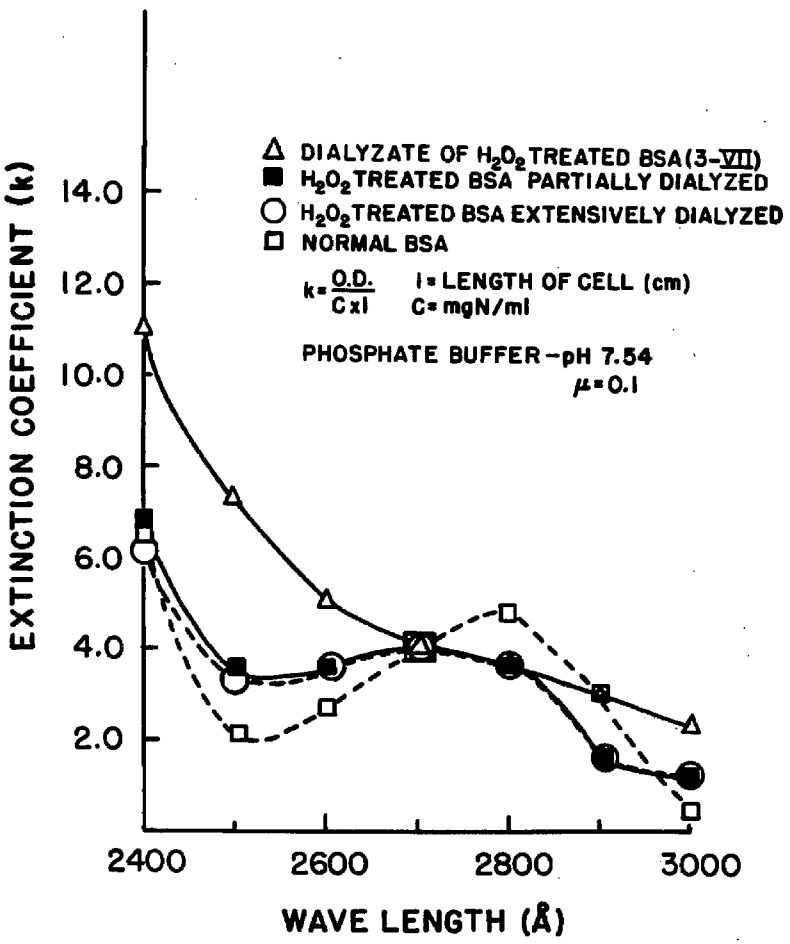
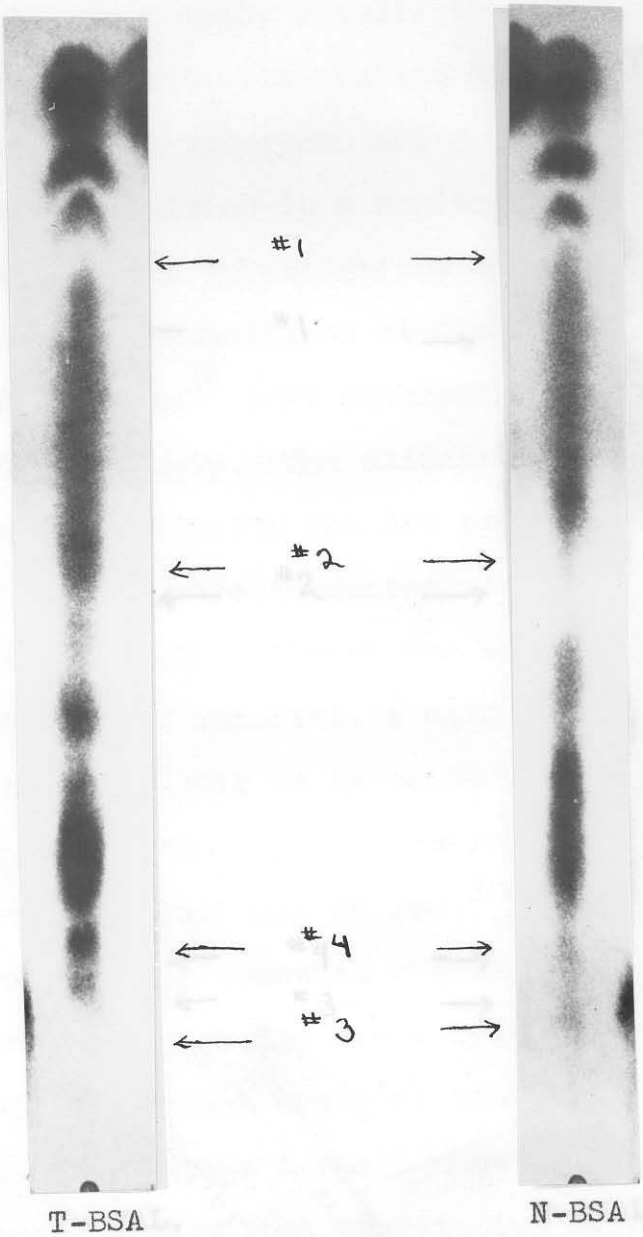


Fig. 17. Absorption spectrum obtained with the dialyzate of oxidized BSA, oxidized BSA partially and extensively dialyzed, and normal BSA.

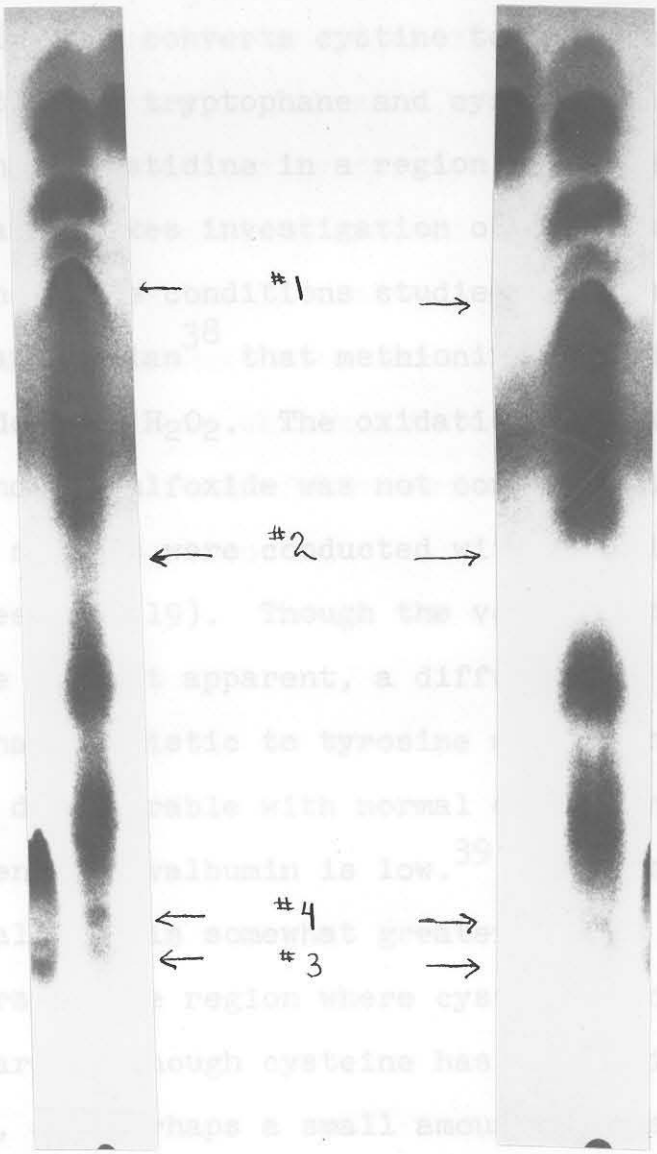
Chromatographic studies.--The records obtained with acid propanol solvent are presented in Figures 18 and 19. The points interpreted as being significantly different are indicated on the chromatographic record.

The comparison of records obtained with treated BSA and normal BSA, see Fig. 18, show four differences. The first difference occurs in the region (1) where phenylalanine is found to migrate. In the H_2O_2 treated BSA there is a void at this point. The second difference is a fill (2) occurring in the region where tyrosine migrates. Tyrosine in this solvent gives a very diffuse spot. These two differences might be interpreted as indicating that phenylalanine was oxidized to tyrosine, however it is felt on the basis of the spectrophotometric studies that the diffuse fill is not tyrosine but some other product of oxidation. The third and fourth differences involve cystine. Earlier studies showed this change much more clearly than the one presented here. The third point (3) is the region where cystine is found to migrate. It can be seen that after oxidation a void occurs in the T-BSA in the region where cystine migrates, and at the same time, a new component occurs in the T-BSA. This new component migrates in the region (4) characteristic of cysteic acid. The disappearance of cystine with oxidation was also demonstrated with a second solvent (1 part 50% HCl - 1 part propanol), but the appearance of cysteic acid with oxidation was not positively demonstrated with this



Solvent: 1 Part 1% Acetic Acid.
3 Parts Propanol.

Fig. 18. Differences obtained with chromatogram of normal and oxidized bovine serum albumin.



Solvent: 1 Part 1% Acetic Acid.
3 Parts Propanol.

Fig. 19. Differences obtained with chromatograms of normal and oxidized ovalbumin.

second solvent. From these results the conclusion is drawn that H_2O_2 oxidation converts cystine to cysteic acid. The small percentage of tryptophane and cysteine in BSA, and the migration of histidine in a region occupied by several other amino acids makes investigation of these amino acids impossible under the conditions studied. It has been shown by Toennies and Callan[38] that methionine is easily oxidized to a sulfoxide with H_2O_2. The oxidation of methionine to its corresponding sulfoxide was not confirmed in this study.

Similar studies were conducted with normal and oxidized ovalbumin (see fig. 19). Though the void in the region of phenylalanine was not apparent, a diffuse fill occurred in region (2) characteristic to tyrosine migration. A cystine spot was not demonstrable with normal ovalbumin, because the cystine content in ovalbumin is low.[39] The cysteine content in normal ovalbumin is somewhat greater. With H_2O_2 oxidation, a spot appears in the region where cysteic acid migrates. Thus it appears as though cysteine has been oxidized to cysteic acid, and perhaps a small amount to cystine.

These results show that qualitative differences in amino acid composition of proteins result from H_2O_2 oxidation. At this time only a few differences are apparent. It is felt that if the hydrolyzates were studied by means of ion exchange column, a greater number of differences would be found. Certainly, the method of choice is an ion exchange column for this type of investigation.

Concentration determination.--The specific refractive increments for the various proteins studied are presented in Table 9. Also included in the same table are the percentages of nitrogen for the various proteins. The differences between the experimentally determined specific refractive increments and those reported in the literature may reside in either an error in the instrument constant or incomplete drying of the protein. The values obtained here were used in determining the concentrations of the various proteins solutions used in this investigation.

TABLE 9

REFRACTIVE INDEX INCREMENT AND PERCENTAGE NITROGEN CONTENT OF PROTEINS STUDIED IN THIS INVESTIGATION

Measurements made in 0.2 M NaCl, pH between 6.5 and 7.0

(C in g/ml)

Protein	dn/dc at 5460 Å		% Nitrogen	
	Exp.[a]	Lit.[b]	Exp.	Lit.[c]
Rabbit gamma globulin	-	-	16.43	-
T- rabbit gamma globulin	0.1735	-	15.13	-
Bovine serum albumin	0.1755	0.1883 0.1854	16.47	16.07
T- bovine serum albumin	0.1765	-	18.20	-
Ovalbumin	0.1755	0.1820 0.1865	-	15.76
T - ovalbumin	0.1745	-	17.48	-

[a] Experimentally determined in this study.

[b] Reported in 13.

[c] Reported in 39.

CHAPTER IV

IMMUNOCHEMICAL CHARACTERIZATION OF TREATED PROTEINS

Antigenicity of Treated Proteins

Test for precipitin formation.--Treated proteins were injected into both rabbits and chickens following the sensitizing procedures described in chapter II, and the serums obtained were tested for the presence of precipitins by means of the interfacial technique (ring test) (see chapter II) against both treated and untreated proteins diluted from 0.001 to 0.00001 gm protein per ml.

Chickens failed to respond with detectable precipitin production after receiving intramuscularly a total of 27 mg of either soluble or alum precipitated (see p. 17) treated bovine serum albumin (T-BSA). However, two control chickens which had received normal bovine serum albumin (N-BSA) in an identical course of injections developed good titres against N-BSA. The results are summarized in Table 10.

Various preparations of soluble T-BSA and one alum precipitated sample were injected three times a week for six weeks (10-9 mg/injection) into a total of eleven rabbits in an effort to induce antibody production against T-BSA. In two cases, the rabbits were first given a complete course with preparation 3-III, rested, and bled, and then rested

TABLE 10

SUMMARY OF RESULTS OBTAINED FROM RING TESTING FOR PRECIPITINS IN SERUM OF CHICKENS INJECTED WITH T-BSA

Sensitizing Antigen	Antigen Used in Ring Test			
	Treated-BSA		Normal-BSA	
	Posit.	Neg.	Posit.	Neg.
T-BSA	0	2	0	2
T-BSA (alum)	0	2	0	2
N-BSA	0	2	2[a]	0

[a] High titer.

for two months. These same animals after the two month rest received a second course of injections of a new preparation of antigen 3-IV, since 3-III had been exhausted.

All serums regardless of antigen preparation or method of injections failed to show antibody to either T-BSA or N-BSA when tested by interfacial precipitin tests. Gamma globulin solutions prepared from serums obtained from animals injected with preparation 3-III were also found to be negative even though the gamma globulin concentration was twice that of the original serum.

Passive sensitization of guinea pigs also was used in an attempt to detect antibodies against T-BSA in serums obtained from rabbits injected with 3-IV. Each guinea pig received 0.5 cc of serum by cardiac puncture and was then challenged the following day with 0.5 cc of 1% T-BSA. All the animals were closely observed for symptoms of anaphylactic shock resulting from the injection of the challenging dose, but in all cases, the results were negative. A control experiment was conducted in which guinea pigs were injected by cardiac puncture with 0.5 ml (0.23 mg of antibody N) of rabbit anti-BSA serum. A second pair of guinea pigs received in a similar manner 0.05 ml serum, and a third pair received 0.005 ml serum. The animals were all challenged the following day with an intravenous injection of 0.5 cc of 1% BSA. It is seen from results summarized in Table 11 that even with 0.005 ml serum, mild anaphylactic shock was

TABLE 11

PASSIVE SENSITIZATION OF GUINEA PIGS WITH VARYING AMOUNTS OF RABBIT BSA ANTIBODY

Antibody N Injected	No. of Guinea Pigs	Results		
		Fatal	Severe	Mild
mg. 0.2300	2	2		
0.0230	2	1	1	
0.0023	2	0	0	2

possible with guinea pigs.

The complete results of sensitization procedures in rabbits with T-BSA are summarized in Table 12. The results from the two animals, #188 and #189, which received a second course of injections, are presented in Table 13.

Although all attempts to elicit precipitin formation in rabbits with T-BSA failed, successful sensitization with T-ovalbumin occurred in one rabbit (#187). The rabbits were injected intravenously with treated ovalbumin 7-II (7 mg/ injection) following the procedure already described (p. 19), and the serums obtained were tested by means of the interfacial method. It is significant to note that the response was weak but specific to the preparation used in sensitization 7-II. Neither preparation 7-I or preparation 7-III gave a positive interfacial test with the serum obtained from rabbit #187. These results are summarized in Table 14 and should be contrasted with the results obtained with guinea pigs using the Schultz-Dale technique in which positive sensitization was demonstrated and cross reactivity occurred between the various preparations, 7-I, -II, and -III.

In vivo anaphylaxis test for the induction of hypersensitivity in guinea pigs.--Three experiments were done for the purpose of investigating gross anaphylaxis by active sensitization of guinea pigs with normal and oxidized bovine serum albumin. In the first experiment T-BSA 3-I was administered intra-peritoneally on three successive days at a concentra-

TABLE 12

SUMMARY OF RESULTS OBTAINED FROM RING TESTING FOR PRECIPITINS AND PASSIVE TRANSFER TO GUINEA PIGS OF SERUM FROM RABBITS INJECTED WITH T-BSA

Antigen Injected	Antigen Used in Ring Test				Passive Sensitization of Guinea Pigs	
	Treated BSA		Normal BSA			
	Posit.	Neg.	Posit.	Neg.	Posit.	Neg.
T-BSA (3-III)	0	9	0	9	0	5
T-BSA (alum 3-III)	0	2	0	2	-	-
BSA	0	2	2	0	-	-

TABLE 13

RESULTS OBTAINED FROM RING TESTING SERUMS FROM RABBITS GIVEN TWO COURSES OF INJECTIONS OF T-BSA

Rabbit No.	Antigen Injected	Antigen Used in Ring Test	
		BSA	T-BSA
188	3-III	0	0
189	3-III	0	0
	Rested two months before second course		
188	3-IV	0	0
189	3-IV	0	0

TABLE 14

SUMMARY OF RESULTS OBTAINED FROM RING TESTING FOR PRECIPITINS IN SERUMS FROM RABBITS INJECTED WITH T-OVALBUMIN

Rabbit No.	Antigen Injected	Antigen Used in Ring Test	
		T-Ovalbumin (7-II)	N-Ovalbumin
186	7-II	0	0
187	7-II	+[a]	0

[a] Weak and only with 7-II and not 7-I or 7-III.

tion of 40 mg per injection, and then challenged intravenously on the eighteenth day, following the last injection. The second experiment was performed by giving a single intraperitoneal injection of 10 mg of preparation 3-IV and allowing 30 days between the injection and the challenging injection. The last experiment was an attempt to elicit a secondary response in the hope that a higher degree of sensitivity would result. Four animals were first given 70 mg of preparation 3-VI intraperitoneally in four injections and then rested 8 months before a fifth injection of 1 mg was given intravenously. The animals were observed for half an hour after the fifth injection but no symptoms of anaphylactic shock developed. Another challenging injection was given seven days after the fifth injection. Animals were also sensitized in all experiments to normal bovine serum albumin to provide controls, and to determine cross reactivity.

The results of these three experiments are summarized in Table 15. Though positive sensitization did occur with both treated and normal bovine serum albumin, the majority of the cases were either very mild or questionable positives. Cross sensitization also apparently occurred since T-BSA sensitized guinea pigs were shocked by N-BSA, and N-BSA sensitized animals were shocked by T-BSA.

The antigenic properties of treated and normal rabbit gamma globulin were also investigated by means of <u>in vivo</u>

TABLE 15

SUMMARY OF RESULTS IN DETERMINATION OF SENSITIZATION BY GROSS ANAPHYLAXIS OF GUINEA PIGS INJECTED WITH EITHER NORMAL OR TREATED BOVINE SERUM ALBUMIN

Course of Injections				Challenge	Results			
Material	No. of Injection	Total amt. Protein Injected	Time lapse	Material	Fatal	Severe	Mild	Neg.
T-BSA 3-I	3	120 mg	18 days	T-BSA	0	0	1	0
"	"	"	"	N-BSA	1	0	0	0
N-BSA	"	"	"	T-BSA	1	0	0	0
"	"	"	"	N-BSA	0	0	1	0
T-BSA 3-IV	1	10 mg	30 days	T-BSA	0	0	2	0
"	"	"	"	N-BSA	1	0	1	0
N-BSA	"	"	"	T-BSA	0	0	0	2
"	"	"	"	N-BSA	1	0	1	0
T-BSA	3	72 mg	8 months	T-BSA	0	0	0	2
	1	5 mg	7 days	T-BSA	0	0	0	2
N-BSA	3	72 mg	8 months	N-BSA	0	0	0	2
	1	5 mg	7 days	N-BSA	0	1	0	1

anaphylaxis in guinea pigs. The animals were given a total of 30 mg rabbit gamma globulin intraperitoneally in two injections and were then rested 27 days. The challenging injection was administered intravenously and consisted of 0.5 ml of 1.0% protein. The results with normal material were all fatal, but a gradation in the degree of shock occurred with T- -globulin sensitized guinea pigs. Cross reactivity was demonstrated but only when T- -globulin sensitized guinea pigs were challenged with normal gamma globulin. The guinea pigs that were sensitized to N-gamma globulin but failed to react when challenged with T- -globulin were all fatally shocked the following day with N- -globulin. The results of this experiment are summarized in Table 16.

In vitro anaphylaxis test for the induction of hypersensitivity in guinea pigs.--By means of the Schultz-Dale technique, it is possible to demonstrate the antigenicity of substances with a high degree of sensitivity. The original description[40] of this technique employed in uterine tissue as a source of smooth muscle. In these experiments, intestinal muscle was preferred since as Campbell and Nicoll[41] have pointed out, intestinal tissue has "less tendency to spontaneously contract, more tests per animal, and little if any difference in anaphylactic responsiveness."

The tissue bath used in these experiments was that described by Campbell and McClasland.[42] The bath consisted of

TABLE 16

SUMMARY OF RESULTS IN DETERMINATION OF SENSITIZATION BY GROSS ANAPHYLAXIS OF GUINEA PIGS INJECTED WITH EITHER NORMAL OR TREATED RABBIT GAMMA GLOBULIN

Course of Injections			Challenge[c]	Results			
Material	No. of Injections	Total amt. Injected	Time Lapse	Material	Fatal	Severe	Mild	Neg.
N- -globulin	2	30 mg	27 days	N- -globulin	3	0	0	0
"	"	"	"	T- -globulin	0	0	1[a]	2
T- -globulin	"	"	"	N- -globulin	3	0	0	0
"	"	"	"	T- -globulin	1	1[b]	1[b]	0
Normal Guinea Pigs	-	-	-	N- -globulin	0	0	0	3

[a] Had to resort to cardiac puncture.

[b] All surviving guinea pigs on the following day were fatally shocked with N- -globulin.

[c] All animals challenged intravenously with 5 mg protein.

a heavy glass cylindrical tube 150 mm long, 25 mm in diameter, and rounded and sealed at one end. Three different glass tubes were sealed into the bottom of the bath: (1) A capillary tube through which test solutions were added, (2) a gas disperser for aeration of solution, and (3), a glass tube for adding and removing Tyrode solution. Connected to tube (3) was a three way stop cock which permitted either filling the bath with Tyrode from a large reservoir, or emptying the bath by means of suction from an aspirator.

The tissue was supported in the bath by means of a specially constructed glass unit. Two strips, one a control and one sensitized, were used in every run, and each was suspended by being tied between an ink recording lever and the supporting unit. The tissue supporting unit was in turn held by a partially cut away rubber stopper inserted into the bath. Enough of the stopper was removed so that the threads connecting the muscles to the ink recording levers were free from obstruction. All muscular activity was recorded on a kymograph which was set to rotate at approximately 1 cm per minute. The bath was kept in a large cylindrical glass water bath and thermo-regulated at 37° C.

The physiological solution used in these experiments was a modification of the Tyrode's formula as described by Feigen and Campbell.[43] The salts and the quantity of each per 100 ml of solution are as follows:

$MgCl_2 \cdot 6H_2O$	0.0213 g
KCl	0.0195 g
$CaCl_2 \cdot 2H_2O$	0.0193 g
$NaHCO_3$	0.1015 g
NaCl	0.8000 g
glucose	0.1000 g

The above solution at 37° C had a pH of 8.2. The purity of the reagents used in the preparation of the above solution was considered of utmost importance. In all cases, the salts were of reagent grade and recrystallized two or three times. The water used in the preparation of the physiological solution was conductivity water, prepared by redistillation over alkaline (NaOH) potassium permanganate.

Guinea pigs of either sex were used and varied in weight from 300 to 500 g. After the sensitization, the animals were sacrificed and the lower third of the small intestine removed. The gut obtained was placed in warm physiological solution as defined above and cut in 15 cm strips for the purpose of cleaning. Special care was observed in order to preserve the activity of the sensitized tissue. The tissue was handled as little as possible, and in flushing the gut intrinsic muscular activity was utilized as much as possible. After the gut was thoroughly cleaned, it was cut into sections of 25 to 30 mm and stored in the cold between cotton pads moistened with the physiological solution.

Two strips were simultaneously tested in all experiments, the control and the sensitized gut. When first placed in the bath, the tissue was allowed to equilibrate for 20 minutes, at the end of which time a base line was established. The test antigen was next added and the muscular activity followed by means of the kymograph record. If there occurred a contraction as a result of the addition of antigen, the tissue was allowed to stand in the solution for an additional 20 minutes. The bath was then flushed and fresh physiological solution added. In order to have demonstrated the occurrence of an immunological reaction, desensitization of the gut must follow the original antigen-induced contraction. Therefore, desensitization was shown by the absence of contraction following the addition of a second dose of antigen, while the tissue's ability to contract was subsequently demonstrated by the addition of histamine. In some experiments, both strips were sensitized, but to different proteins, e.g. T-BSA and N-BSA. Thus one strip acted as a control for the other while simultaneously demonstrating the lack of cross reactivity between the two proteins. Since in testing for cross reactivity, it is conceivable that inhibition of contraction of sensitive tissue might occur by the prior exposure of the tissue to the original protein, the sequence of addition of the test antigens in each experiment was reversed with fresh tissue.

The active sensitization of guinea pigs with bovine

serum albumin was attempted using various concentrations, time intervals, and routes of injections. The tissue was challenged with both the treated and untreated bovine serum albumin in each experiment. Normally each animal received just one course, but in one of the latter experiments, (No. 4), a set of guinea pigs was given a second booster shot 25 days after the first course.

The results of the active sensitization of guinea pigs as determined by the Schultz-Dale technique are summarized in Table 17. In the few cases where active sensitization to N-BSA was demonstrated, cross reactivity with T-BSA was always negative, and exposure to T-BSA first did not desensitize the tissue to N-BSA.

In order to determine whether the technique used in the general preparation of the tissue or the sensitization of the animals might be responsible for the large fraction of negative animals, guinea pigs were passively sensitized with rabbit anti-BSA serum. The serum used in these experiments was of high titer and was always given twenty-four hours prior to the experiment. The amounts used and the routes of injections are summarized in Table 18. The procedure of challenging sensitive tissue to both treated and untreated

TABLE 17

SUMMARY OF RESULTS OBTAINED IN THE DETERMINATION OF SENSITIZATION BY THE SCHULTZ-DALE TECHNIQUE OF GUINEA PIGS INJECTED WITH NORMAL BSA

Course of Injections			Results			
			BSA		T-BSA	
Total Amt.	No. Injections	Time Lapse	Posit.	Neg.	Posit.	Neg.
mg		days				
3[a]	3	27	1	1	0	2
48	2	25	0	4	0	4
48	2	48	0	1	0	0
48	2	25	-	-	-	-
24	1	23	0	2	0	0
42	3	45	1	1	0	2
120	3	26	1	0	0	1
			3	9	0	9

[a] Injected subcutaneously. All the rest were injected intraperitoneally.

TABLE 18

SUMMARY OF RESULTS OBTAINED IN THE DETERMINATION OF SENSITIZATION BY SCHULTZ-DALE TECHNIQUE OF GUINEA PIGS INJECTED WITH RABBIT ANTI-BSA SERUM

Passive Transfer of Serum			Results			
			BSA		T-BSA	
Amt.[a]	Route[b]	Time Lapse	Posit.	Neg.	Posit.	Neg.
cc		day				
1.5	I.V.	1	1	0	0	1
3.0	I.P.	1	1	0	0	1
3.0	I.P.	1	2	0	0	2
1.0	I.C.	1	1	1	0	2
2.0	I.P.	1	1	0	0	1

[a]All serums were high titer, greater than 0.9 mg antibody per ml.

[b]I.V. - intravenous
I.P. - intraperitoneally
I.C. - cardiac puncture

protein was extended to these experiments and all subsequent Schultz-Dale experiments.

The results are presented in Table 18. It was concluded that the techniques and the solutions used were adequate since all but one intestine gave a positive contraction when challenged with N-BSA. All the preparations were challenged with T-BSA, but in all cases the results were negative. Also, prior exposure of the tissue to T-BSA did not inhibit the contraction of tissue passively sensitized to N-BSA.

Active sensitization of guinea pigs with various preparations of oxidized proteins was attempted. Details as to the sensitization procedure are summarized in Table 19 along with the results obtained. Only one preparation of treated bovine serum albumin (3-I) was found to be specifically antigenic in guinea pigs as determined by the Schultz-Dale technique. The intestinal strip from the guinea pig sensitized with preparation 3-I gave a definite contraction when 0.1 mg of T-BSA was added to the bath, and it was found that the prior addition of 0.1 mg of N-BSA did not inhibit the contraction induced by T-BSA. The subcutaneous injection of preparation 3-IV surprisingly produced in one guinea pig a definite sensitization to N-BSA. The kymograph record of experiment #54 demonstrating the cross sensitization is presented in Fig. 20.

Six guinea pigs were sensitized to N-ovalbumin

TABLE 19

SUMMARY OF RESULTS OBTAINED IN THE DETERMINATION OF SENSITIZATION BY SCHULTZ-DALE TECHNIQUE OF GUINEA PIGS INJECTED WITH TREATED BSA

Course of Injections					Results			
					BSA		T-BSA	
Material	Amt. Total	No injections	Route	Time Lapse	Posit.	Neg.	Posit.	Neg.
	mg			days				
3-I	120	3	I.P.	26	0	1	1[a]	0
3-II	60	3	I.P.	45	0	2	0	2
3-III	42	3	I.P.	45	0	3	0	3
3-IV	2	2	Subcut.	27	2[b]	2	0	4
3-VI	96	2	I.P.	27	0	2	0	2

[a] Gave positive contraction with 3-IA.

[b] One of these was very weak or questionably sensitive to N-BSA.

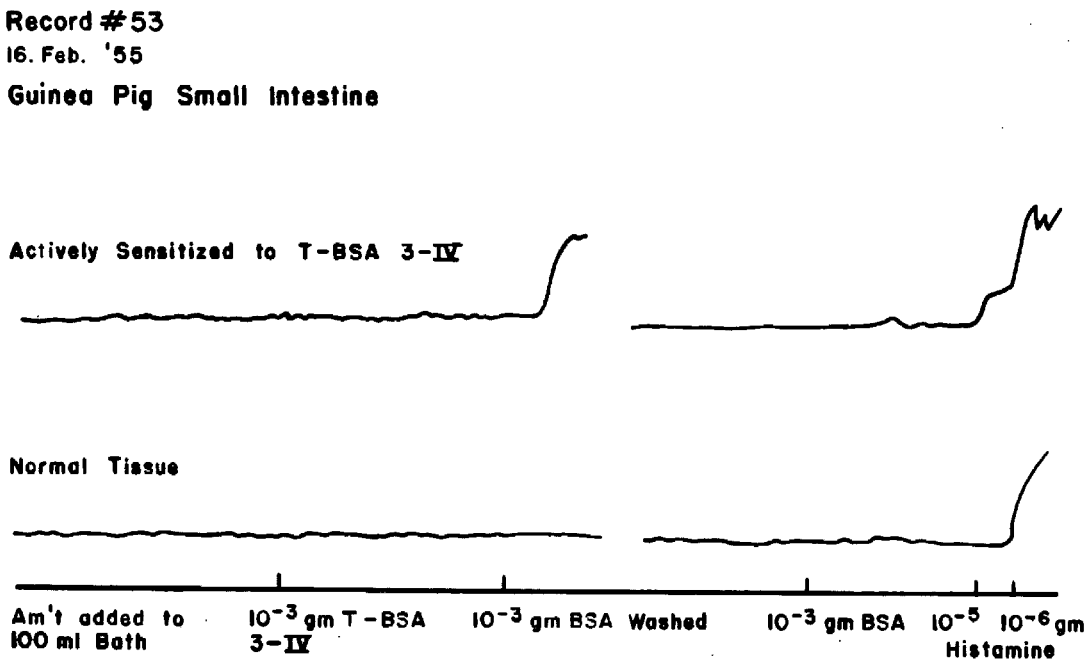


Fig. 20. Kymograph record of a Schultz-Dale experiment with small intestine from a guinea pig injected with T-BSA 3-IV.

by a series of intraperitoneal injections. These N-ovalbumin sensitized guinea pigs were challenged with both normal and treated proteins, and inhibition was investigated by sequence analysis. The details of sensitization are summarized with the results obtained, and appear in Table 20. All the guinea pigs injected with N-ovalbumin were found to be sensitive to N-ovalbumin, and negative to the treated ovalbumin. The degree of sensitivity attained with N-ovalbumin is demonstrated by experiment #26, see Fig. 21, in which a positive response was obtained from an intestinal strip challenged with 0.000,001 grams of normal ovalbumin added to 100 ml muscle bath.

The method employed in the sensitization of guinea pigs to oxidized ovalbumin is identical to that described for the normal ovalbumin. Details and results are to be found in Table 21. The animals sensitized to preparation 7-II were also challenged with preparations 7-I, and 3-IV (Treated BSA).

Typical results from the latter type of experiment are illustrated by the kymograph record of experiment #75, fig. 22. The upper tracing was made by an intestinal strip from a T-ovalbumin 7-II sensitized guinea pig, and the lower tracing by an intestinal strip of a normal guinea pig. The results show that neither N-ovalbumin or T-BSA 3-VI cross reacted with an intestinal strip of a T-ovalbumin 7-II sensitized guinea pig, but that a second preparation of T-ovalbumin 7-I was capable of inducing a contraction and specifically desensitized the strip to 7-II.

TABLE 20

SUMMARY OF RESULTS OBTAINED IN THE DETERMINATION OF SENSITIZATION BY SCHULTZ-DALE TECHNIQUE OF GUINEA PIGS INJECTED WITH NORMAL OVALBUMIN

Course of Injections				Results			
				N-Oval.		T-Oval	
Total Amt.	No. Injections	Route	Time Lapse	Posit.	Neg.	Posit.	Neg.
mg			days				
30	3	I.P.	38	3	0	0	3
60	3	I.P.	21	3	0	0	2

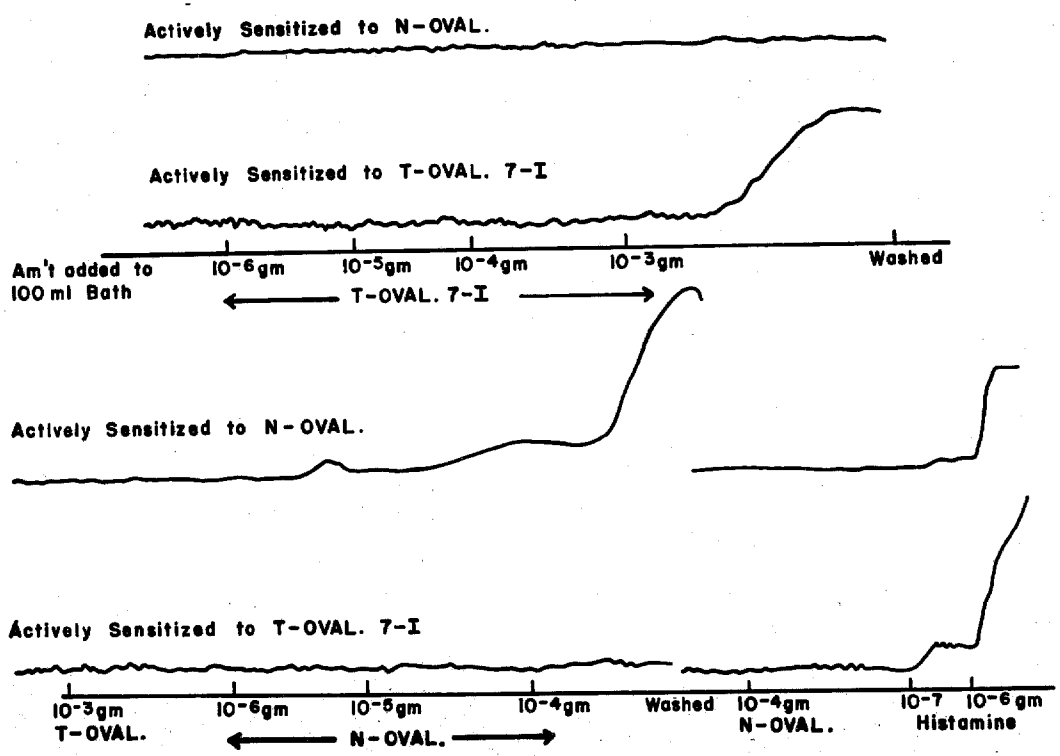


Fig. 21. Kymograph record of a Schultz-Dale experiment with intestinal strips from guinea pigs injected with either normal ovalbumin or T-ovalbumin 7-I.

TABLE 21

SUMMARY OF RESULTS OBTAINED IN THE DETERMINATION OF SENSITIZATION BY SCHULTZ-DALE TECHNIQUE OF GUINEA PIGS INJECTED WITH TREATED OVALBUMIN

Course of Injections				Results			
				-ovalb.		T-ovalb.	
Total Amt.	No Injections	Route	Time Lapse	Posit.	Neg.	Posit.	Neg.
mg.			days				
16	4	I.P.	35	0	3	3	0
28	4	I.P.	33	0	1	2	0
69	3	I.P.	21	0	2	2	1

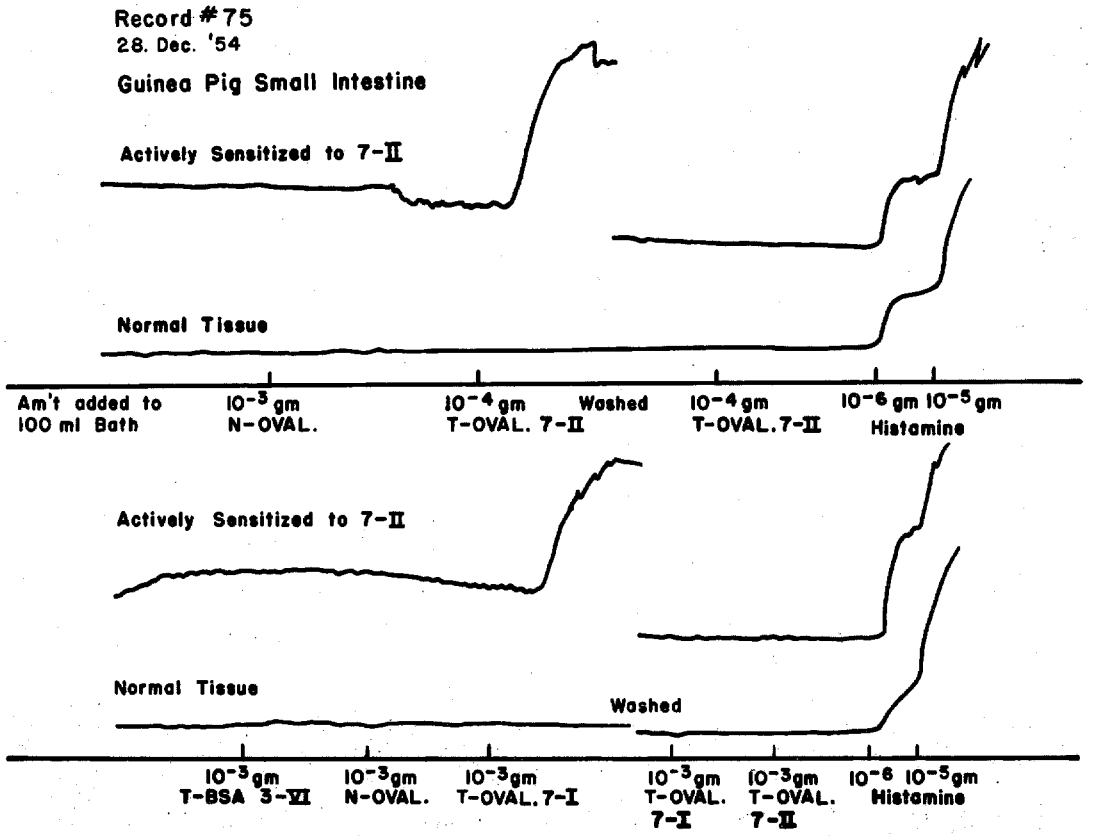


Fig. 22. Kymograph record of a Schultz-Dale experiment with intestinal strips from a guinea pig injected with T-oval 7-II.

Serological Activity of Oxidized Proteins

Test for cross reactivity between rabbit anti BSA and treated BSA.--A gamma globulin preparation of rabbit anti BSA was diluted 1 to 5 with 1.0% saline, and mixed with various dilutions of normal and treated bovine serum albumin 3-I. The results were read qualitatively after two hours incubation at 37° C. The concentration of T-BSA 3-I ranged from 4 mg protein/ml to 0.00063 mg protein/ml without the formation of a detectable precipitate with the rabbit gamma globulin anti BSA which gave a maximum precipitation at 0.025 mg BSA/ml. The experiment was repeated using whole serum, and the results investigated quantitatively as described in Chapter II. The results were similar to those of the qualitative experiments. All the T-BSA concentrations tested gave the blank value with the diluted anti BSA serum which contained 0.2 mg antibody per ml.

Test for inhibition of a normal BSA precipitin system by treated BSA.--It was demonstrated in the above section that precipitation between T-BSA and anti BSA serum does not occur. The possibility still remained that BSA haptenic particles capable of combining with antibody without the formation of a precipitate were in the T-BSA preparations. An experiment was therefore designed based on the effective elimination of precipitating antibody with haptenic groups. Using undiluted rabbit-anti BSA serum, the maximum precipitating ratio was established and used in testing the possible

inhibition of the system with T-BSA 3-IV. Varying concentrations of T-BSA, diluted in a two fold manner and ranging from 0.002 to 0.0000078 gm T-BSA per ml were mixed in equal proportions with the antiserum and incubated at 37° C for a period of one hour. Following the incubation, the predetermined quantity of BSA capable of inducing maximum precipitation was added and reincubated for an additional hour. Quantitative nitrogen analysis of the precipitates was made as described in Chapter II. The results of this experiment are presented in Table 22. In part A of Table 22, the results show that antiserum directed against normal BSA does not precipitate the oxidized protein, indicating that if any BSA combining sites have survived hydrogen peroxide treatment there must effectively be only one per particle. In part B of Table 22, the results are presented of mixing T-BSA first with anti BSA and then the addition of N-BSA. These results show that within the concentration range of T-BSA tested, the resulting precipitates are experimentally equivalent to those obtained only with N-BSA and anti BSA. These results were therefore interpreted as meaning all BSA combining sites were either destroyed or significantly altered by the oxidative treatment employed.

Test for the possible retention of antibody combining activity in treated gamma globulin.--The gamma globulin 6-IV was originally obtained by ammonium sulfate precipitation (see chapter II) from a high titer rabbit anti ovalbumin

TABLE 22

A. RESULTS OF THE DETERMINATION OF PRECIPITABILITY OF T-BSA WITH RABBIT ANTI-BSA

Amt. T-BSA	Anti-BSA Serum	BSA Added	Mg N Precipitated
mg[a]	cc	cc[b]	
10.0000	0.5	0.5 saline	0.002
5.0000	"	"	-
2.5000	"	"	0.001
1.2500	"	"	0.004
0.6250	"	"	0.002
0.3125	"	"	0.002
0.1562	"	"	0.001
0.0781	"	"	0.002
0.0390	"	"	0.003
Saline	"	"	0.003

B. RESULTS FROM THE TEST FOR THE POSSIBLE INHIBITION OF A BSA PRECIPITATING SYSTEM WITH T-BSA 3-IV

Amt T-BSA	Anti-BSA Serum	BSA Added	Mg N Precipitated
mg[a]	cc	mg[a]	
10.0000	0.5	0.6250	0.109
5.0000	"	"	0.111
2.5000	"	"	0.106
1.2500	"	"	0.106
0.6250	"	"	0.107
0.3125	"	"	0.108
0.1562	"	"	0.100
0.0781	"	"	0.104
0.0390	"	"	0.111
Saline	"	"	0.112

[a] Mg proteins in 0.5 cc.

[b] No protein added just 0.5 cc of saline .

serum. The possible retention of precipitating activity was checked by the mixing of 10.5 mg of T gamma globulin in one ml with various 1.0 ml dilutions of N-ovalbumin. Visual observation and subsequent quantitative N analysis failed to reveal any precipitating activity associated with T-gamma globulin, see Fig. 23. Reports by Tyler[20] have shown that as a result of photo-oxidation of anti-serum non-precipitating antibodies have been produced. T-gamma globulin was therefore investigated for the possible content of non-precipitating antibody. The following original anti-ovalbumin gamma globulin, and T-gamma globulin mixtures were made and precipitins curves obtained.

	N globulin	T-γ-globulin	Saline
A.	1 cc	0.0 cc	1.0 cc
B.	1 cc	0.5 cc	0.5 cc
C.	1 cc	1.0 cc	0.0 cc
D.	0.0 cc	1.0 cc	1.0 cc

The results are presented in Fig. 23. If non-precipitating antibody existed in the T-gamma globulin, the amount at the point of maximum precipitation should have increased with an increase in T gamma globulin added. This did not occur, and also since all the curves are approximately identical the results have been interpreted as meaning that T gamma globulin 6-IV was completely devoid of any antibody activity.

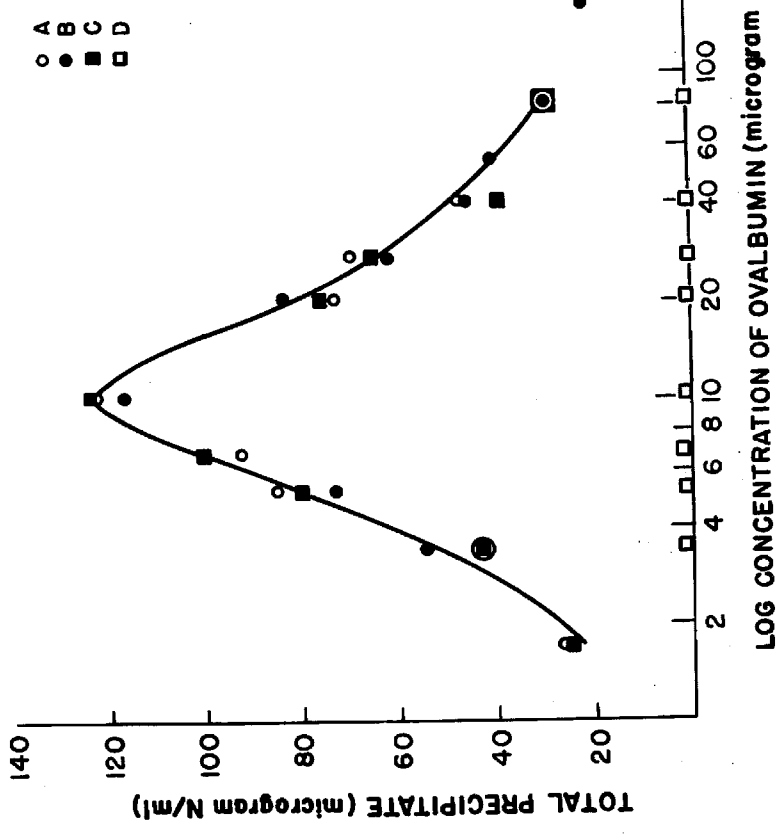


Fig. 23. Precipitation curves obtained in test for possible retention of antibody combining activity in treated gamma globulin.

CHAPTER V

DISCUSSION

The antigenic studies with oxidized ovalbumin and rabbit gamma globulin show quite clearly that a new specificity was acquired as a result of hydrogen peroxide treatment. With treated ovalbumin the old specificity was completely destroyed by oxidation, but in the gamma globulin system enough of the native specificity survived to sensitize guinea pigs. However, the oxidized gamma globulins appear to be less antigenic as judged from the degree of anaphylactic shock elicited. All the antibody precipitating activity of the gamma globulin was eliminated with H_2O_2 oxidation. The possible production of non-precipitating antibodies by the H_2O_2 oxidation of rabbit (anti ovalbumin) gamma globulin was investigated but none was found. The oxidative procedure was severe, and therefore the absences of non-precipitating antibodies should not be considered as eliminating this procedure for the possible production of non-precipitating antibodies. It was reported by Tyler et al.[20b] that photo-oxidation of globulin anti sheep red blood cells produced non-agglutinating antibodies, but with extensive irradiation the sensitizing activity was reduced. The retention of some of the original gamma globulin specificity in the oxidized preparation should allow a Coombs type test for non-precipitating antibodies as has

already been reported by Tyler et al.[20b] using photo-oxidized gamma globulin.

Evaluation of the reduction in the antigenicity of ovalbumin with oxidation is based on a low precipitin response in rabbits. It is interesting to note that in both of these cases a high molecular weight species resulted from oxidation. It is purely speculative whether or not the high molecular weight species was responsible for the newly acquired antigenic activity of treated ovalbumin or treated gamma globulin. Experimentally it would be interesting to isolate the fast-sedimenting component and study its immunological properties. The results obtained with the rabbit anti-T-ovalbumin are suggestive of a special factor in preparation 7-II. It was found that guinea pigs sensitized with either 7-II or 7-I cross reacted with each other when tested by means of the Schultz Dale technique. Serum obtained from rabbits which had been injected with 7-II were tested for precipitins, only 7-II gave a positive test. Preparations 7-I and 7-III both gave negative ring tests with rabbit anti 7-II.

The interpretation of the antigenic activity of H_2O_2 treated BSA in guinea pigs was complicated by the apparent low antigenicity of normal BSA in that animal. It may well be that previous antigenic interpretations of BSA were partly based on impurities such as serum globulins. Taylor[44] has reported that the high antigenic properties of salt precipitated BSA were due to the presence of serum globulin and bacterial contamination. The method of choice in the prepa-

ration of BSA was considered by Taylor to be the procedure utilizing methyl alcohol precipitation. Also Williams[8] has reported that non-crystalline preparations of BSA were more antigenic than crystalline BSA. Control experiments such as passive sensitization of guinea pigs, and the experiments conducted with ovalbumin preclude an explanation based on deficient techniques. The possibility still remained that the right combination of quantity of BSA injected, route of injection and time lapse prior to challenging would produce marked changes in the apparent antigenic potency of crystalline BSA.

Three guinea pigs injected with treated BSA were found to be sensitive to normal BSA. Though the serological results indicate complete destruction of the native specificity, apparently in some preparations enough survived to actively sensitize guinea pigs, but insufficient to shock the animals once sensitized. This finding suggests that perhaps too much BSA was used in the attempted sensitization of the animals.

The antigenicity of H_2O_2 treated BSA in guinea pigs might be slightly less than that of normal BSA in the same animal. The conclusion that treated BSA is less antigenic than the untreated protein is based on the negative results obtained with precipitin formation in chickens and rabbits. The use of alum precipitated treated BSA did not induce precipitin formation in either the rabbit or chicken. Thus the possible explanation based on rapid clearance of treated material by the animals is unlikely, especially in the chickens, where the

alum precipitated treated BSA was injected intramuscularly.

It should be noted that the reduction in antigenicity reported here with oxidation is in agreement with the work of Williams[8] using the same oxidizing agent, and of Smetana and Shemin,[6] Henry[1] and Tyler[20c] using photo-oxidation. Since in the present study no attempt was made to quantitate the reduction in antigenicity, a comparison is not possible of the oxidative method employed in this investigation with those reported earlier.

Of the three proteins oxidized with hydrogen peroxide, bovine serum albumin may be considered the best qualified as a possible plasma expander, however the slight antigenic activity of T-BSA makes its use in such a capacity impossible. The large reduction in molecular size also is an undesirable feature with respect to retention. The obvious increase in asymmetry of the molecule may in part reduce the expected increase in clearance from the circulation associated with a drop in molecular size. The possibility remains that under slightly different oxidative conditions, and with a possible fractionation of the product some portion might be isolated which is non-antigenic and possesses the desired retention characteristics.

The reduction of antigenicity with oxidation may be explained on the basis of a reduced molecular size. The antigenicity of the oxidized ovalbumin and oxidized rabbit gamma globulin could therefore be due to their fast sedimenting components. However, if molecular size is a deter-

minant to antigenicity because of greater retention, then the increased asymmetry of the oxidized proteins should result in functionally larger molecules. Other explanations for the reduced antigenicity could be based on the chemical composition of the proteins. The oxidation of the aromatic amino acids and the oxidation of cystine and cysteine to cysteic acid parallel the reduction in antigenicity, and is therefore suggested that these amino acids might play a role in antigenicity. These results may be considered significant in view of the fact that gelatin which is non-antigenic, or only slightly so, is low in content of aromatic amino acids, and completely void of either cystine or cysteine.[39]

In conclusion, hydrogen peroxide oxidation of the proteins studied resulted in a reduction in antigenicity which was correlated with the oxidation of tyrosine, phenylalanine and tryptophane residues, conversion of cystine and cysteine to cysteic acid, and a reduction in molecular weight. Besides reducing molecular weight, H_2O_2 oxidation of proteins resulted in a higher electrophoretic mobility, increased heterogeneity and greater molecular asymmetry. Due principally to the residual antigenicity and reduced molecular size, the present products of oxidation are eliminated as plasma expanders. The possibility, however, still remains that oxidation under other conditions may result in less molecular splitting and that through proper fractionation selection might be made of non-antigenic species.

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PART TWO

CORTICAL DISCONTINUITY AND PROPAGATION OF SPREADING DEPRESSION

by

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Several investigators have been interested in the mechanism underlying the propagation of Leão's spreading depression (S.D.). This phenomenon has been generally recognized as exclusively cortical (1, 2, 3, 4). In his first paper on S.D., Leão (5) postulated that propagation is due to synaptic activation of elements situated in areas adjacent to the depression. The absence of any electrical sign of this activation was explained by the assumption of random placement of these elements in the cortex. The low rate of propagation (2 - 5 mm/min. for the rabbit) typical for the S.D. would be due to the necessity of a long period of summation before adjacent regions are activated. However, Leão and Morison (1), Leão (6) and later van Harreveld and Stamm (7) found that periods of cortical asphyxiation up to 1 minute in duration did not stop the march of the S.D. Since this resistance to asphyxiation did not support the concept that synaptic activation is the basis for propagation the following explanation was considered by Leão and Morison (1). The stimulus setting up a S.D releases a depressing compound which would stimulate nerve endings in the pial membrane. By an axon reflex these impulses now would set free more of the depressing compound in adjacent regions etc. This theory found support in Leão's finding (5) that cocainization of the cortical surface or damage of the upper cortical layers by thermal or mechanical means stops the S.D.

Sloan and Jasper (2) found that S.D. can invade a corti-

cal area isolated from the rest of the cortex by a cut if the wound surfaces are physically co-apted. This spread was prevented by placing an insulator between the wound edges. Also when the gap between the wound edges was 1 1/2 to 2 mm wide and filled with tissue fluid or blood no propagation of the spreading depression took place. They concluded that only physical contiguity and not neural continuity is required, and the suggestion was made that "steady potential fields" are responsible for the march of the S.D.

Leão (6) described as a concomitant of the S.D. a slow potential change (S.P.C.) which may last 4 - 6 minutes and which consists of an initial surface negativity followed by a positivity, both with respect to an indifferent electrode. The potential of the negative phase may be as large as 10 - 15 mV and is in general larger than that of the positive phase. This S.P.C. has been found to be a more consistent indicator for the S.D. than the electrocorticogram (8, 9). The effects of pentobarbital and ether on the S.P.C. described by van Harreveld and Stamm (10) seemed to support Sloan and Jasper's view that "steady potential fields" may be involved in the propagation of the S.D. Pentobarbital in doses so large that they obliterate the electrocorticogram and can be expected to prevent all synaptic activity, reduces the S.P.C. only moderately and does not prevent its propagation. Ether, on the other hand, causes a more marked reduction of the S.P.C. even at concentrations which still allow some spontaneous

electrocortical activity and stops the march of the S.P.C. before the electrocorticogram is fully extinguished. These experiments seemed to support the postulate that the S.P.C. is the agent of propagation in a similar way as a nerve action potential is instrumental in nerve conduction. Bures (11) supported this concept. A similar mode of propagation has been proposed by Gerard and Libet (12) for "caffein waves" in the frog's cortex.

Marshall and his coworkers (13, 14) suggested that a chemical substance might be involved in the propagation of the S.D. Burns and Grafstein (4) proposed the following mechanism of the propagation. The S.P.C. would be due to depolarization of cortical elements causing rapid spike activity with consequent loss of potassium ions. This K would diffuse into adjacent areas causing depolarization etc.

The propagation of the S.D. across a cut in the cortex observed by Sloan and Jasper is perhaps the most direct evidence for the postulate that the S.P.C. is the agent of transmission, although, as pointed out by Marshall et al. (14), this observation does not exclude the possibility that a compound is involved in the propagation. Sloan and Jasper's observations were made in acute experiments in which it must be assumed that abnormal steady potential fields are present in the cortex due to injury potentials. Also injuries have been found to be potential sources of "spontaneous" waves of S.D. (3) which may be mistaken for S.D. transmitted across

a cut. To circumvent these complications it seemed of interest to investigate the possibility of propagation of S.D. across a neuronal discontinuity in chronic experiments.

Method

At first it was attempted to cut the rabbit's cortex sub-pially in the usual fashion by exposing the dorsal surface of one hemisphere. After a few weeks firm adhesions had developed between the cortex and the overlying tissue which hampered greatly the exposure of the cortex in the final experiment in which transmission of the S.D. across the scar was to be examined.

Another method for cutting the cortex was therefore adopted. Using aseptic precautions an oblong hole was made in the skull, and after incising the dura, a curved knife (Figure 1, A) was passed into the brain cavity pressing lightly with the blunt part (X) against the underside of the pia. In this way a cut was made through the cortex and underlying white matter. In most of the experiments the hole in the skull was made over a frontal lobe and the cut extended from there in a caudo-lateral direction (Figure 1, B). The term longitudinal cut will be used to indicate a cortical cut of this kind. In other experiments the hole was made over the parietal lobe and the cortex was cut in a transverse direction (Figure 1, C). When the skull was opened 3 weeks to 3 months later a slight indentation of the cortex indicated the location of the scar. The visible pial vessels

passed over this indentation without interruption. After an interval of 3 weeks there were in some preparations fine adhesions between the dura and pia, which never caused serious difficulties. In the 3 month preparations the adhesions were more troublesome.

Silver-silverchloride electrodes mounted on springs were placed on the cortex in the pattern shown in Figure 1, D. The distances between these electrodes was of the order of 2 to 2 1/2 mm. Either electrode 1 (or 2, when the location of the scar prevented the placement of all 4 electrodes on one side of the scar) or 8 (respectively 7) were used as stimulating electrodes. Bipolar electrocorticograms were led off from electrodes 2 - 3, 3 - 4, 5 - 6 and 6 - 7 with a 4 channel Offner electroencephalograph. Two slow potential change records were led off from two electrodes facing each other across the cut (3 and 5, or 4 and 6), using an electrode on the ear as an indifferent electrode. The amplifiers and galvanometers used have been described previously (15). In the course of an experiment the S.D. was made to pass across the cut in both directions by alternately stimulating through electrode 1 (2) and 8 (7).

After the experiment the electrode placement was marked by depositing electrolytically some silver from the electrodes on the cortex. Then the brain was removed and fixed in alcohol. The region investigated physiologically was embedded in paraffin and sectioned (15 μ thick). Each 20th section was mounted and stained with gallocyanin.

Results

Histological findings.

Nine rabbits were examined 3 weeks after a longitudinal cut was made in the cortex; 4 animals were investigated after an interval of 3 months. Seven preparations were made 3 weeks after cutting the cortex in a transverse direction. The histological preparations of the group of experiments in which the cortex was cut longitudinally showed the following features. The cortex had been cut completely in all experiments over the entire region investigated, histologically (10 - 12 mm). After an interval of 3 weeks blood and necrotic tissue had been absorbed, and replaced by a scar consisting of cellular elements (neuroglia, connective tissue). In some preparations there was a decrease in neuronal and an increase in glial elements in the cortex immediately adjoining the scar. In most preparations this was not the case. The actual width of the scar was from 0.3 to 0.1 mm. In general the wound edges were well approximated although in a number of preparations the deeper cortical layers were not in direct contact, a spur of the lateral ventricle separating them. Figure 2 shows a narrow scar (less than 0.1 mm wide), with well approximated wound edges. The preparations of experiments in which the interval had been 3 months did not differ essentially from those of the 3 weeks experiments.

In 5 of the 7 experiments in which a transverse cut was applied, the cortex was cut completely in the investigated

region. In 2 experiments, however, a bridge of undamaged cortical tissue connected the cortical areas on both sides of the cut. In one instance this bridge was 2.1 mm wide, in the other 2.7 mm. In both cases the upper 2/3 of the cortex was undamaged.

Electrophysiological findings.

Figure 3 shows part of the results of an experiment performed 3 weeks after the cortex was completely transected longitudinally. A reasonably normal electrocorticogram is led off from electrodes situated about 1 mm from the scar (channel A and B). The cortex lateral of the scar was stimulated by a 4 1/2 V. direct current stimulus of 3 sec. duration. After about 1 minute a very complete depression develops in trace A, which is recorded from cortex on the stimulated (lateral) side of the cut. This depression does not appear in the channels B and C leading off from cortex medial of the scar. The slow potential change (S.P.C.) record led off from electrodes facing each other across the cut shows a typical slow potential change of the usual magnitude on the stimulated side (Figure 3, A'). However, a small S.P.C. starting about 1 minute later is recorded from an electrode located on the other side of the scar (Figure 3, B'). The failure of the depression to cross the scar is not due to an inability ofthe cortex medial of the scar to be depressed and to produce a large S.P.C., since about 15 minutes before the traces shown in Figure 3 were recorded a very complete depres-

sion was produced in channels B and C accompanied by a sizeable S.P.C. led off from one of the electrodes recording the corticogram B. This depression was not observed in channel A and therefore did not cross the scar from medial to lateral.

In all experiments--whether the cut was longitudinal or transverse--in which subsequent histological examination showed that the cortex was completely severed, the results were the same as in the above example. In none of them did a clearcut depression of spontaneous electrical activity appear in the cortex across the scar within a few minutes after the depression in the cortex on the stimulated side became apparent. It was possible to show that the cortex on both sides of the scar was capable of depression and of producing sizeable slow potential changes by alternating the side of stimulation. The small S.P.C. in the cortex on the nonstimulated side was recorded only from electrodes close to the scar and was observed in only part of the preparations. It became apparent 1/2 to 1 minute after the start of the S.P.C. on the stimulated side. However, the time interval between the deflection maxima was usually smaller (between 20 and 30 seconds).

Since the area of cortex available for experimentation is small, it is necessary to avoid using strong stimuli. If stimuli are used which are considerably above threshold the physical spread of the current may produce stimulation of cortex across the scar and in this way imitate transmission

of S.D. This occurrence can easily be recognized by the fact that the depression then starts on both sides of the scar simultaneously and with a very short latent period. A requirement for a significant experiment is that the depression starts on the stimulated side after a period which is commensurate with the distance between stimulating and leading off electrodes and with the low rate of propagation of the S.D.

In a few experiments in which the cortex was cut longitudinally a S.D. appeared across the scar 10 - 15 minutes after the stimulus. This occurred too regularly to be explained by fortuitous stimulation. It seemed quite unlikely that such late depressions would have been transmitted across the scar; at least not by a potential field since by that time the slow potential change and even the depression of the corticogram on the stimulated side are over. A more likely possibility is that the depression went around the scar, using as a bridge the caudal and basal parts of the occipital cortex where the cutting knife did not reach. The distances involved are such that they can be traversed in 10 to 15 minutes by the spreading depression at its typical low rate of propagation (2 - 5 mm/min.).

In the two experiments in which the cortex was not severed completely the S.D. was clearly propagated across the cut. Large slow potential changes were recorded on both sides of the scar; first the S.P.C. on the stimulated side which was followed after 1/2 to 1 minute by a S.P.C.

on the opposite side of the scar. These experiments are of interest since they show that the damage to the subcortical structures is immaterial for the arrest of the S.D. by a cut severing the cortex completely. Furthermore they show that S.D. can be conducted by a relatively small bridge of intact cortical tissue.

Discussion

Leão (5) found that S.D. does not penetrate into the area retrosplenialis granularis dorsalis (Rose, 16). In the experiments in which the cortex was cut longitudinally the electrode system often approaches this area and in some instances the most medial electrode had even to be placed on this region. Although the S.D. was demonstrated in the cortex medial of the scar in all experiments, the possibility existed that the cortex in the neighborhood of the area retrosplenialis granularis has unusual properties which would prevent the S.D. from crossing the cut. It was for this reason that the cortex was severed in a transverse direction in a number of instances. In these experiments it was possible to place the electrode system at considerable distance from the area retrosplenialis. The disadvantages of the latter variation of the experiment are that it is more difficult to cut the cortex completely and also that the electrogram of the cortex caudal of the scar sometimes is of low potential.

From the various mechanisms proposed for the propagation of the spreading depression some can be eliminated with a degree of probability. That synaptic transmission (5) would be involved is made unlikely by the considerable resistivity of spreading depression to acute asphyxiation (1, 6, 7) and to large doses of pentobarbital (10). The importance of pial structures (7) is denied by Grafstein's experiments in which the deeper cortical layers were found to be essential for the horizontal transmission of the spreading depression. Leão (18), on the other hand found that the S.P.C. travels downwards from the superficial layers of the cortex, which would indicate that normally horizontal propagation takes place in the superficial layers.

The small S.P.C. across the scar recorded in part of the experiments can be considered as evidence for the physical spread of the potential field produced by the S.P.C. on the stimulated side. The time difference of 20 - 30 seconds between the maxima of these potentials may represent the time it takes for the S.P.C. to spread from the electrode recording the S.P.C. on the stimulated side to the scar, a distance of 1 to 1 1/2 mm.

The concept that the slow potential change would be the agent of propagation does not find much support in the experiments described in the present paper. From the fact that slow potential changes of about the usual magnitude can be led off from electrodes placed at a distance of 1 to 1 1/2 mm

from the scar, it can be concluded that the processes underlying the spreading depression approach the scar very closely. The scar which in some experiments was less than 0.1 mm in width cannot have been an important obstacle to the potential field of the slow potential change. Indeed, the small slow potential changes which sometimes can be recorded across the scar are evidence for the physical spread of this potential field. Nevertheless in none of the experiments was an unequivocal crossing of the spreading depression observed, although the conditions for such a transmission must have been more favorable in the present chronic experiments than in Sloan and Jasper's (2) acute experiments, since the cortical wound edges have been brought together more closely by the absorption of blood and necrotic material. The steady potential fields due to injury potentials which can be expected to be present in the acute experiments will have disappeared in the chronic ones. It is conceivable that these have facilitated the transmission across the cut as observed by Sloan and Jasper. Although the present experimental evidence speaks against the concept that the slow potential change is the agent for transmission, the possibility remains that the probably much more intense fields in the immediate neighborhood of cortical elements which produce the slow potential change are instrumental in the transmission of the spreading depression by an action on similar elements in their immediate neighborhood. Even a relatively narrow

scar would seriously interfere with such "micro fields" and might very well arrest the march of the spreading depression. An alternate explanation is that the scar interferes with the diffusion of a chemical substance, postulated as the agent involved in propagation of the spreading depression (13, 14, 4).

Summary

The mechanism of the propagation of the spreading depression was discussed. Especially the possibility that the accompanying slow potential change is the agent instrumental in the propagation was examined. The transmissibility of the spreading depression across a cut severing all layers of the cortex was investigated in preparations in which 3 weeks to 3 months was allowed for healing of such an injury. No unequivocal signs of transmission of the spreading depression across the scar were observed, although in some instances the scar was less than 0.1 mm wide. In some experiments a small slow potential change was led off from the cortex on the nonstimulated side by an electrode placed in the immediate vicinity of the scar. This potential was considered as evidence for the physical spread across the scar of the potential field produced by the slow potential change on the stimulated side. The failure of transmission of the spreading depression across a scar does not support the concept that the slow potential change is the agent involved in the propagation of this phenomenon.

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Figure 1. A, shows the knife used for the transection of the cortex. The X marks the blunt part which is kept in contact with the skull. B, location of the hole in the skull and direction of the longitudinal cut. C, location of the hole in the skull and direction of the transverse cut. D, scheme of the electrode placement. The horizontal line indicates the scar. The distance between individual electrodes is 2 to 2 1/2 mm.

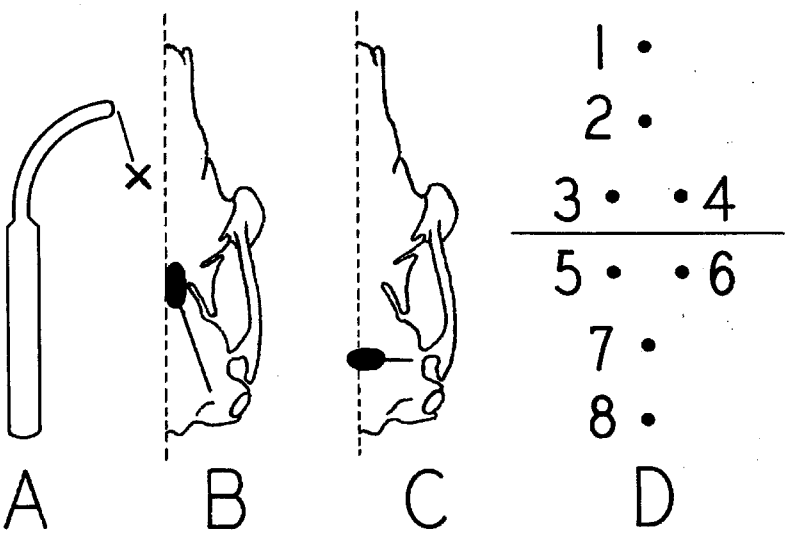
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Figure 2. Photomicrograph of a scar in the cortex 3 weeks after the injury was sustained. A, indicates the scar. B is a blood clot in the lateral ventricle. C, indicates the thickness of the cortex. The scale is 0.5 mm divided in 5 parts of 0.1 mm each.

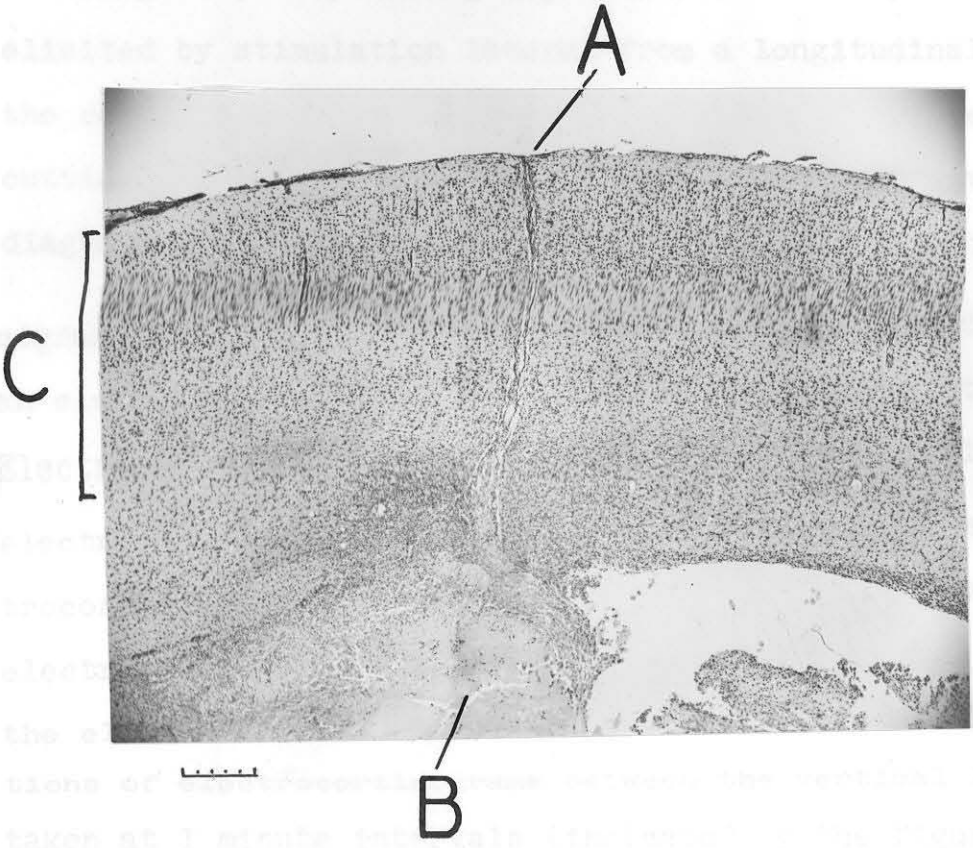
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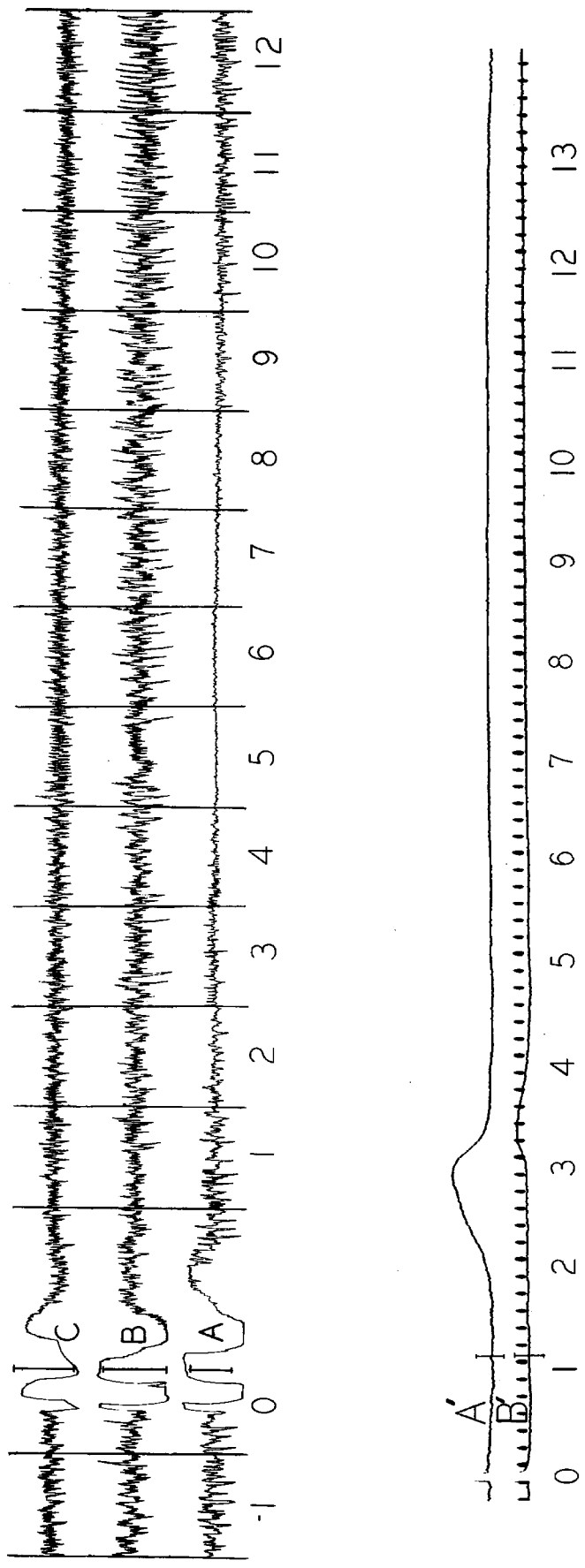
Figure 3. Spreading depression and slow potential change elicited by stimulation lateral from a longitudinal scar in the cortex. The experiment was performed three weeks after cutting the cortex. The electrode placement is given in the diagram. S is the stimulating electrode. The electrocorticogram A is led off from the stimulated (lateral) cortex by an electrode pair parallel to and about 1 mm from the scar. Electrocorticogram B is led off from a similarly placed electrode pair on the non-stimulated (medial) cortex. Electrocorticogram C is led also from the medial cortex by an electrode placed about 2 mm medial of the scar and one of the electrodes of the pair parallel to the scar. The sections of electrocorticograms between the vertical lines are taken at 1 minute intervals (indicated by the figures under the electrocorticograms). The stimulus (4 1/2 Volts d.c.) is applied during the section marked O. The horizontal line indicates 10 sec. The vertical calibration lines in the section marked O indicate 1 mV.

The two slow potential changes are taken from two electrodes facing each other across the scar (A and B). Trace A' is from an electrode on the stimulated side, trace B', from the non-stimulated cortex. The time signal indicates 10 seconds intervals. The figures under the record indicate minutes and correspond with the figures under the electrocorticograms. The stimulus artifact is at time O. The vertical calibration lines show 5 mV potentials.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