STUDIES ON PHOTO-OXIDATION OF ANTIGEN AND ANTIBODIES*†

HANS SMETANA, M.D., AND DAVID SHEMIN, PH.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, and the Physico-Chemical Institute, University of Upsala, Sweden)

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Studies on the photo-oxidation of body fluids have shown that the proteins of blood serum and lymph account almost entirely for the oxygen uptake of blood and lymph during irradiation to light in presence of a sensitizer (1–3).

The present study deals with attempts to analyze the changes occurring in proteins during photo-oxidation by serological, chemical, and physico-chemical methods.

I. Serological Studies on Photo-Oxidized Proteins

Materials, Methods, and Technique

Three times recrystallized egg albumin prepared according to Heidelberger (4) and the specific carbohydrate of pneumococcus Type I (5) were used as antigens. Anti-egg albumin rabbit serum was prepared according to Heidelberger and Kendall (6). Anti-pneumococcus Type I horse serum was obtained from the Department of Health, New York City. Two times recrystallized hematoporphyrin-HCl (Hp) was prepared in the manner described by Kuester (7).

Irradiation was carried out in a constant temperature water bath using Fenn's respirometers for studying the oxygen consumption; the set up was identical with that described in a previous paper (3).

The qualitative precipitin ring tests were performed in the regular manner and dilutions were made with saline. The pH was determined colorimetrically and by means of the glass electrode.

Passive anaphylaxis was tested by injecting 1 cc. of the antiserum intraperitoneally into guinea pigs weighing approximately 350 gm. followed by an intravenous injection of 0.1 mg. of either normal or photo-oxidized egg albumin 48 hours after the preparatory injection. Active anaphylaxis was induced by injecting 1.5 cc. of normal or photo-oxidized egg albumin containing 1 mg. nitrogen per cc. intraperitoneally into guinea

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pigs (350 gm.) three times with a 3 day interval, followed by a final intravenous injection of 0.1 mg. of either normal or photo-oxidized egg albumin 12 days after the last preparatory injection.

Quantitative precipitin estimations were made according to Heidelberger, Kendall, and Soo Hoo (8). The results of these studies are given in the form of curves made by plotting the values for the total N precipitated against the various dilutions of the antigen.

Separation of the protein fractions of anti-egg albumin rabbit serum was carried out in the electrophoresis apparatus of Tiselius (9–12).

1. The Effect of Photo-Oxidation of Egg Albumin on the Quantitative Precipitin Reaction.—To duplicate sets of tubes containing 1 cc. of diluted antiserum (total protein 5.2 mg. N per cc.) varying amounts of either normal or photo-oxidized antigen was added and the total volume of the fluid of each tube made up to 2 cc. using saline as the diluent. The solution of egg albumin which was exposed to light in presence of Hp 0.5×10^{-3} contained 7.14 mg. of N per cc. and samples were taken for precipitin reactions after the substrate had consumed 265, 506, and 675 c.mm. of O_2 per cc. respectively. The results are presented in Fig. 1.

It can be seen that progressive photo-oxidation caused a progressive decline of the slope of the curve; in order to precipitate an equal amount of nitrogen more antigen is needed.

2. The Effect of Photo-Oxidation of Antisera on the Quantitative Precipitin Reaction.—Anti-egg albumin rabbit serum (total protein 10.4 mg. N per cc.) and antipneumococcus Type I horse serum (total protein 12.16 mg. N per cc.) were studied. The sera were irradiated in presence of Hp and quantitative precipitin reactions were made with untreated and photo-oxidized samples. For this purpose samples of photo-oxidized anti-egg albumin rabbit serum were taken after 40 and 122 c.mm. of O_2 per cc. had been consumed and were diluted 1:1 with saline. The photo-oxidized samples of antipneumococcus horse serum had consumed 55.4 and 150 c.mm. of O_2 per cc. respectively and were diluted 1:4 with saline. The results are presented in Figs. 2 and 3.

The shape of the curves of the photo-oxidized samples did not differ materially from that of the normal serum except after prolonged oxidation, but the peaks of the curves of the irradiated material became progressively lower and less antigen was needed to obtain maximum precipitation.

3a. The Effect of Photo-Oxidation of Specific Antibody Fractions on the Quantitative Precipitin Reaction.—Electrophoretic separation of the protein fractions of anti-egg albumin rabbit serum was carried out in the preparation apparatus of Tiselius (12) with a sample capacity of 240 cc. The serum had been dialyzed against phosphate buffer of pH 8.03 and ionic strength 0.1 (163.5 cc. M/5 Na_2HPO_4 + 10 cc. M/5 NaH_2PO_4 per liter). The same buffer was used in the cells and in the electrode vessels, while the bottom of the electrode vessels was covered with saturated NaCl solution. The apparatus was immersed in a constant temperature water bath of 0°C. The Toepler schlieren method was employed for following the movement of the boundaries and compensation

was used to effect good separation of the components. The different fractions of the serum were obtained by means of a sampling device consisting of a special glass filter pipette which could be gradually lowered into the limbs of the U tube by a rack and pinion arrangement. The N content per cc. as well as the globulin content per cc. (Howe's technique) was determined and quantitative precipitin tests were made of each fraction.

No precipitation occurred in any of the isolated fractions except that of globulin gamma, as might have been predicted from the work of Tiselius

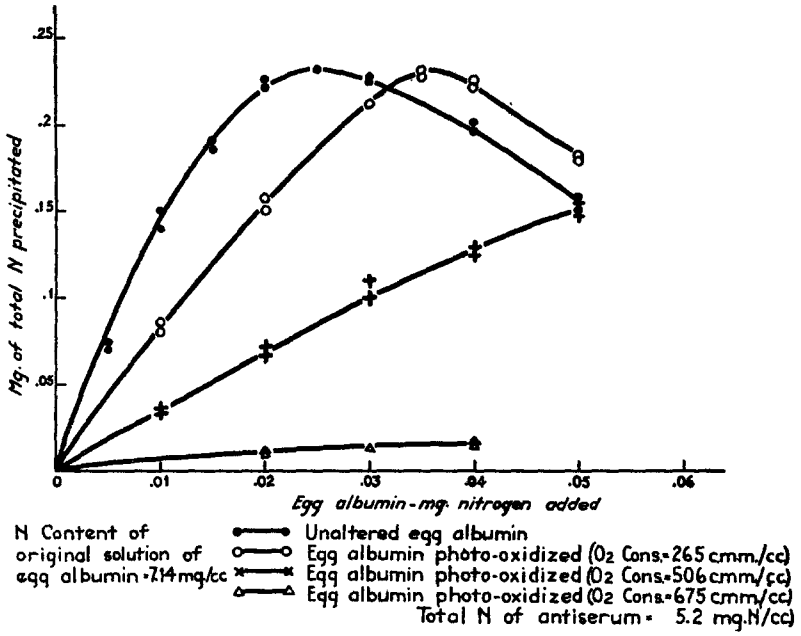


FIG. 1. Precipitin curves of normal and photo-oxidized egg albumin.

(11) and Tiselius and Kabat (27, 28) following earlier studies of Heidelberger and Pedersen (13) on anti-egg albumin rabbit serum.

3b. The Effect of Photo-Oxidation of the Globulin Gamma Fraction of Anti-Egg Albumin Rabbit Serum on the Quantitative Precipitin Reaction.—The isolated globulin gamma fraction containing 1.02 mg. N per cc. of total protein was exposed to light in presence of Hp 0.5×10^{-3}; samples were taken for precipitin reactions after consumption of 0, 28, 43, and 70 c.mm. of O_2 per cc. and were diluted with saline 1:1. The results are presented in Fig. 4.

It can be seen that the peaks of the curves became progressively lower with increasing photo-oxidation and that less antigen was needed to obtain maximum precipitation.

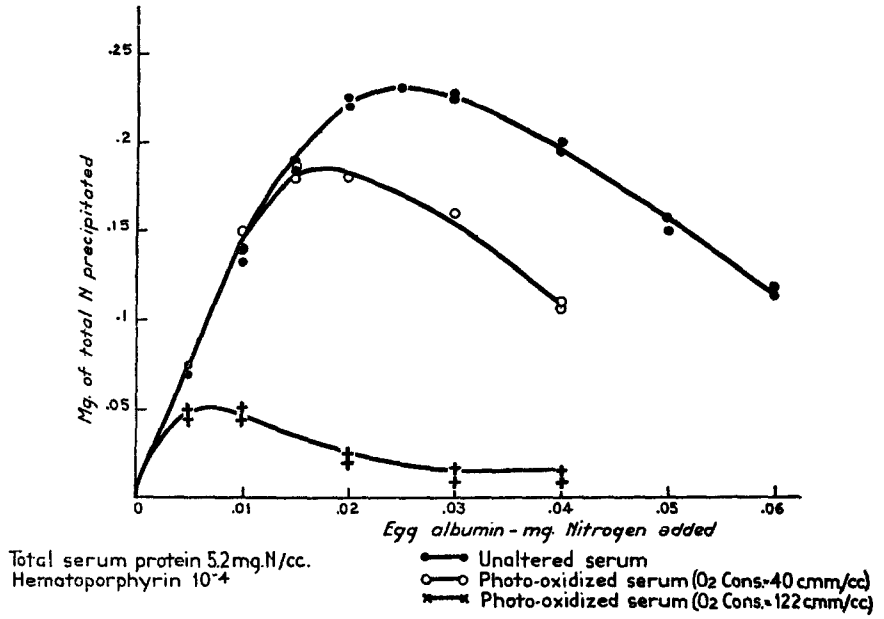


FIG. 2. Precipitin curves of normal and photo-oxidized anti-egg albumin rabbit serum.

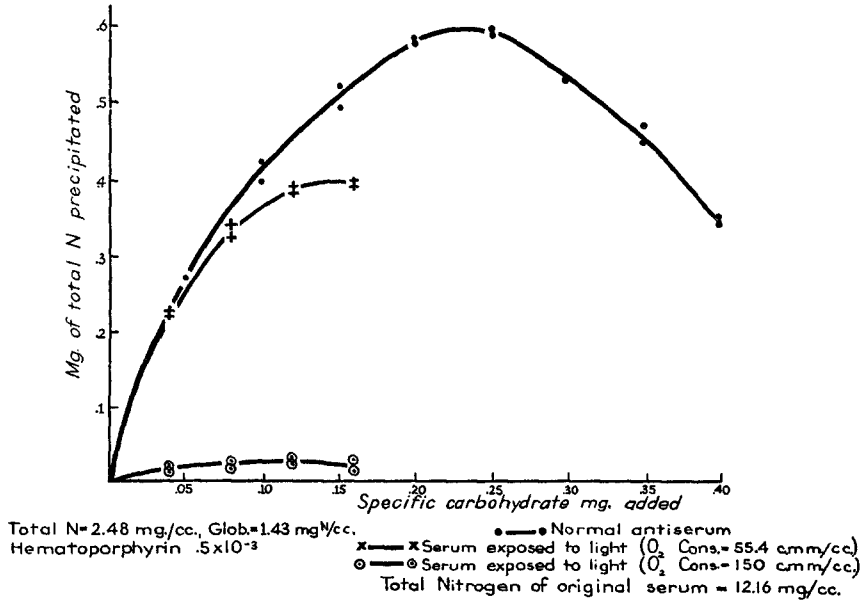


FIG. 3. Precipitin curves of normal and photo-oxidized antipneumococcus horse serum.

3c. The Effect of Photo-Oxidation of Felton Solution from Antipneumococcus Type I Horse Serum on the Quantitative Precipitin Reaction.—Because of the position of the antibody fraction of antipneumococcus horse serum between globulin beta and globulin gamma, which makes it difficult and tedious to separate this fraction from the other components by electrophoresis, a Felton solution (14) of this serum was used for these studies. The solution containing 1.9 mg. N of total protein per cc. was photo-oxidized and quantitative precipitin reactions were performed with samples having consumed 28, 35, and 50 c.mm. of O_2 per cc. respectively in addition to unexposed material. The results are presented in Fig. 5.

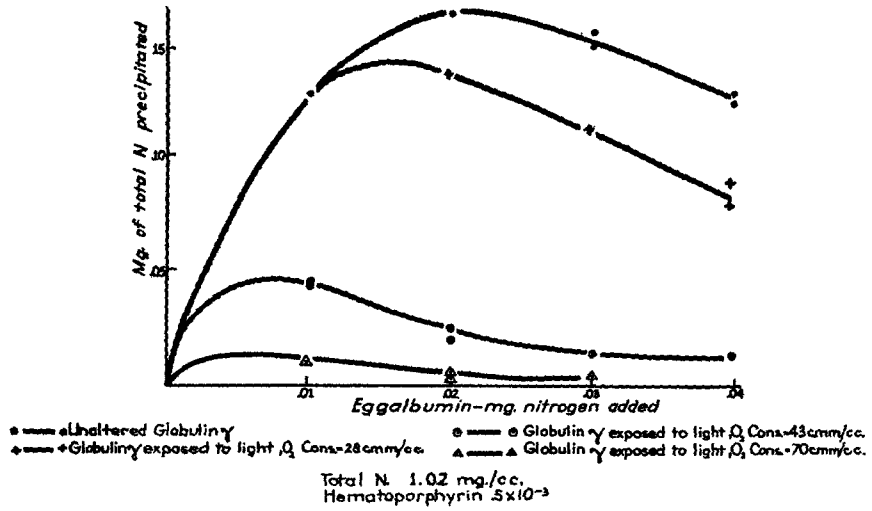


FIG. 4. Precipitin curves of normal and photo-oxidized globulin γ fraction of anti-egg albumin rabbit serum.

There is a lowering of the peaks after progressive photo-oxidation. Due to the flattened top of the curves, the shift of the peaks toward the lesser concentration of antigen is not as clearly seen as in the previous curves.

4. The Effect of Photo-Oxidation of Egg Albumin on Anaphylaxis.—The egg albumin used for these experiments contained 1.65 mg. N per cc. and was exposed to light in presence of Hp 0.5 $\times$ 10^{-3} until it had consumed 600 c.mm. per cc.

While the final injection of 0.1 mg. of normal egg albumin 48 hours after the preparatory injection of 1 cc. of antiserum proved to be fatal, photo-oxidized egg albumin produced no reaction. Likewise, the reinjection of photo-oxidized egg albumin had only a transitory effect on guinea pigs sensitized with normal egg albumin. Photo-oxidized egg albumin had lost its sensitizing action and reinjection of either normal or photo-oxidized material produced no anaphylactic shock.

Rabbits which had been treated with photo-oxidized egg albumin to produce anti-egg albumin serum did not develop precipitins to either normal or photo-oxidized egg albumin. The identical treatment of a control group with normal egg albumin produced a powerful antiserum.

II. Chemical Studies of Normal and Photo-Oxidized Antigen and Antibody Fractions

In 1926, Harris (1) found that when plasma was irradiated with a mixture of visible and ultraviolet light in the presence of hematoporphyrin, the rate of oxygen absorption

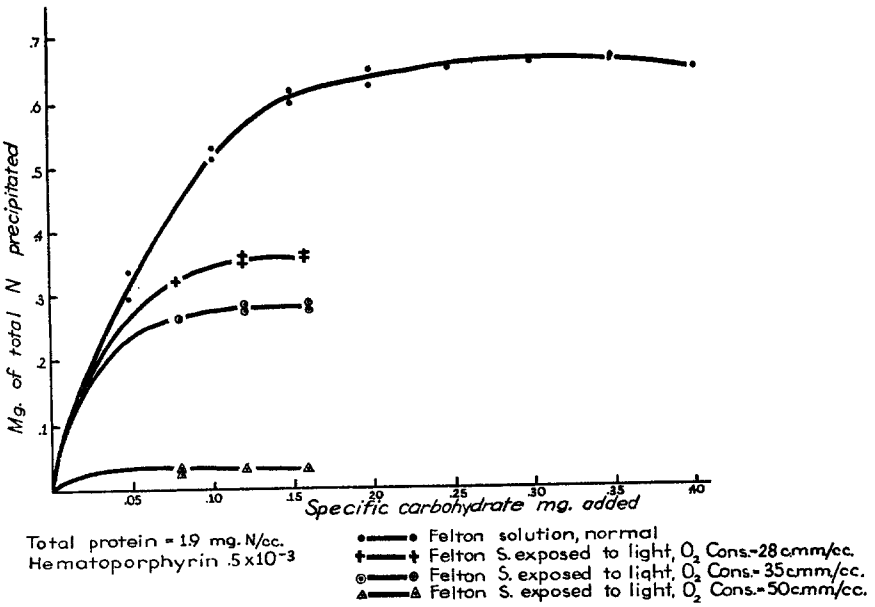


FIG. 5. Precipitin curves of normal and photo-oxidized Felton solution (antipneumococcus Type I horse serum).

was increased 30 to 40 times over that of the oxygen absorption without sensitizer. The increased oxygen uptake was attributed mainly to protein oxidation. He also found that egg albumin, edestin, and other proteins took up an increased amount of O_2 when they were exposed to light in the presence of a sensitizer. However, gelatin differed from the former proteins in that oxygen was not adsorbed on irradiation. The difference was attributed to the deficiency of aromatic amino acids in gelatin. Harris also irradiated amino acids and found that only tyrosine and tryptophane were oxidized. Lieben (15, 16), Gaffron (17), and others confirmed the above results of irradiating proteins and amino acids with only visible light, so that the reaction was truly photodynamic. However, Lieben also found that histidine was oxidized. Carter (18) found that photo-oxidation did not take place with aliphatic compounds, but only with aromatic organic compounds, and of the aromatic compounds only those which

contained a hydroxyl or amino group on the benzene nucleus were oxidized. He also found that compounds containing the purine, the indole, and imidazole groups were oxidized.

The failure of gelatin to give a precipitin reaction with its homologous antiserum has been attributed to the lack of aromatic amino acid residues in its molecule. Since one of the effects of photo-oxidation on protein is to destroy some of the aromatic amino acids, an attempt was made to alter the antigen egg albumin, globulin gamma, and Type I pneumococcus antibody in this manner. The photo-oxidized antigen and antibody were investigated for changes in their immunological behavior and in their content of tyrosine, tryptophane, histidine, and cystine. Determinations of the non-coagulable nitrogen were made in order to find out whether or not splitting of the molecule occurred before the precipitin reaction was lost.

Materials and Methods

Three times recrystallized egg albumin prepared according to Heidelberger (4) was dissolved in 0.1 M phosphate buffer of pH 7.3–7.4 and irradiated with white light in presence of hematoporphyrin (1 mg. per cc.) in an O_2 atmosphere. After varying lengths of time samples of the photo-oxidized egg albumin were removed. Quantitative precipitin curves were made and changes in the amino acid content were studied. Tyrosine and tryptophane were determined by the method of Folin and Marenzi (19). Histidine was determined by a micro method developed in our laboratory (20) and cystine according to the method of Graff *et al.* (21). The non-coagulable nitrogen of the various samples was determined by the method of Sørenson (22) and in some cases the amino nitrogen was studied with the manometric method of Van Slyke (23).

The antibody of Type I pneumococcus antisera was concentrated by Felton's method (14). The solution contained 2.2 per cent protein of which 52 per cent of the total protein was antibody protein. Two different samples were photo-oxidized in the regular manner. Sample A consumed 47.8 c.mm. O_2 per cc. in 5.5 hours, while sample B consumed 110.0 c. mm. O_2 per cc. in 20 hours. The globulin gamma fraction of anti-egg albumin rabbit serum was isolated by the electrophoretic method. The pure fraction contained 0.9 mg. N per cc. One sample was photo-oxidized until it had consumed 50 c.mm. O_2 per cc.

Results

1. Egg Albumin.—The changes of the precipitin curves of egg albumin before and during photo-oxidation are given in Fig. 6. The antigen was tested against two different antisera which differed considerably in potency (A and B). It can be seen that the presence of hematoporphyrin does not alter the curve and that exposure to light in an O_2 atmosphere but without sensitizer has no effect. The results of the analyses concerning the amino acid content, non-coagulable nitrogen, and S H groups of normal and photo-oxidized egg albumin are given in Table I. The letters A and B followed by numerals 0 – 3 refer to the corresponding precipitin curves in Fig. 6. It can be seen that there was marked progressive destruction of trypto-

phane and histidine. No destruction of tyrosine took place under the conditions by which photo-oxidation was carried out in these experiments.

Concerning cystine, it was found that by reducing the hydrolysate with zinc for twice the length of time recommended by Graff, the total cystine-cysteine content of the unaltered and photo-oxidized substrate was the same despite the disappearance of the S H group in the photo-oxidized

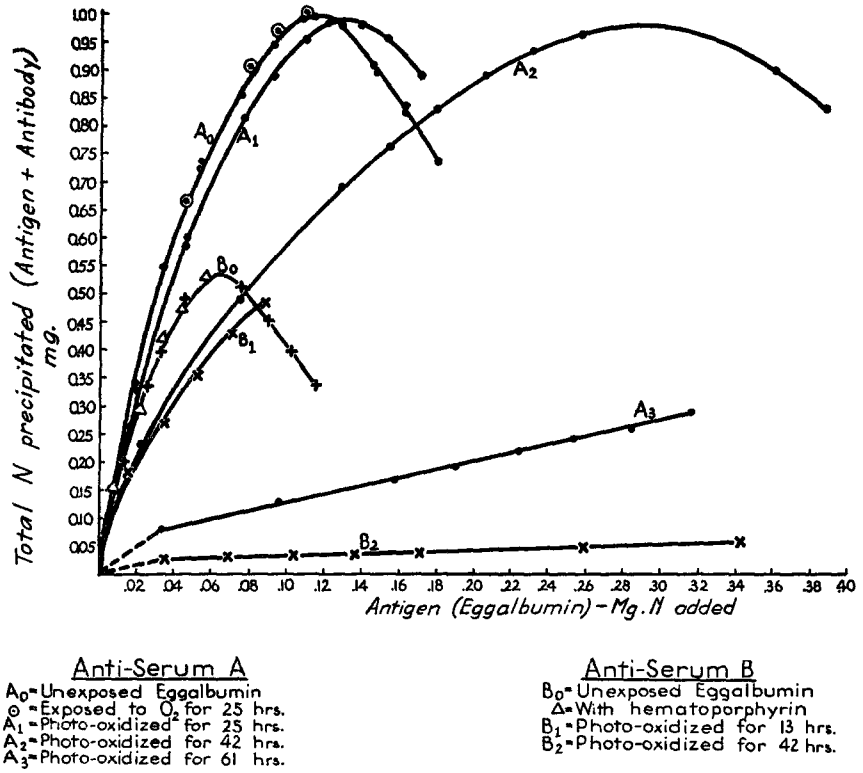


FIG. 6. Precipitin curves of normal and photo-oxidized egg albumin.

protein as shown by the nitroprusside test on the urea denatured protein. Therefore, it seems that the cystine-cysteine system is reversibly oxidized. In Experiment 3, Table I, the cystine contents of the control and photo-oxidized protein were equal but low, for the reduction was only carried out for one hour, so that it would seem that oxidation of cystine proceeded in an atmosphere of O_2 at the same rate, regardless of photo-oxidation.

Non-Coagulable Nitrogen.—There was always a slight increase in non-coagulable nitrogen, but so small as to indicate that no hydrolysis of the protein took place on photo-oxidation.

2. *Felton Solution of Antipneumococcus Type I Horse Serum.*—As seen

in Table I, there was no change in the tyrosine, tryptophane, and cystine content of this antibody fraction as photo-oxidation progressively de-

TABLE I

Chemical Analyses of Normal and Photo-Oxidized Antigen and Antibody Fractions

Substrate	Reference to precipitin curve	Length of exposure		pH	Concentration of protein	Tyrosine	Tryptophane	Histidine	Cystine	Sulfhydryl group	Non-coagulable nitrogen
		To O_2	To light								
		hrs.	*hrs.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*
Egg albumin	A–0	0	0	7.4	3.52	4.1	1.2		1.32	+++	0.56
	A–0	8	0	7.4	3.52		1.2		1.30	+++	
	A–0	25	0	7.4	3.52				1.30	+++	
	A–1	25	25	7.4	3.52		1.06		1.4	++	
	A–2	42	42	7.4	3.52	4.1	0.86		1.4	+	
	A–3	61	61	7.4	3.52	4.1	0.53		1.2	0	2.2
	B–0	0	0	7.35	2.93	4.0	1.2	2.1			1.0
	B–1	13	13	7.35	2.93	4.2	0.96	1.4			1.9
	B–2	42	42	7.35	2.93	4.1	0.41	0.7			5.0
		72	0	7.3	3.23	4.1	1.2	2.0	1.0		
		72	72	7.3	3.23	4.0	0.45	0.63	0.98		
		72	72	7.3	3.36	4.0	0.47				
Felton solution of antipneumococcus horse serum		0	0	8.0	2.2	5.8	2.3		1.9		2.2
		5½	5½ O_2 consumpt. = 47.5 c. mm./cc.	8.0	2.2	5.7	2.4		1.9		3.2
		20	20 O_2 consumpt. = 110 c. mm./cc.	8.0	2.2	5.7	2.2				
Globulin γ of anti-egg albumin rabbit serum		0	0	8.0	0.57	5.8	2.5				2.5
		4	4 O_2 consumpt. = 50 c. mm./cc.	8.0	0.57	5.9	2.2				5.1

stroyed its precipitating properties. The N-C-N and amino N rose but slightly. For comparison between the chemical analyses and the change in the precipitation curves see Fig. 5.

3. Globulin Gamma Fraction of Anti-Egg Albumin Rabbit Serum.—Loss

of the precipitating action (see Fig. 4) was not accompanied by demonstrable changes in the tyrosine and tryptophane content (Table I). There was a moderate increase in the percentage of N-C-N and of amino N.

Anti-Egg Albumin Rabbit Serum.—After photo-oxidation the non-coagulable N rose from 1.8 to 2.5 mg. per cent and the amino N from 0.67 to 2.5 mg. per cent.

Antipneumococcus Type I Horse Serum.—There was an increase of the N-C-N from 2.0 to 7.4 mg. per cent after photo-oxidation, and an increase of the amino N from 0.2 to 0.9 mg. per cent.

III. Electrophoretic Studies of Normal and Photo-Oxidized Antigen, Antisera, and Antibody Fractions

All experiments were carried out in the electrophoresis apparatus of Tiselius (9–12) and the macro and micro apparatus with a sample capacity of 10 cc. and 2 cc. respectively were used. All solutions had been dialyzed against phosphate buffer of pH 8.03 and ionic strength 0.1 before the runs. The potential gradient F varied in different experiments, but care was taken to keep it constant in individual runs. The temperature of the water bath was kept at 1.5°C. The electrophoretic mobilities of the boundary lines were studied and the pattern was photographically recorded by means of Longsworth curves (24) and cylindrical lens curves (25, 26). The areas under the individual curves were measured by means of a planimeter on enlarged paper copies of the photographic records of Longsworth curves and cylindrical lens curves and the average of 10 measurements was computed. The relative differences in the corresponding areas are given in per cent of the unaltered substrates after due corrections had been made for the varying protein concentrations of the substrates.

The concentration of the hematoporphyrin varied from about 0.1×10^{-3} to 0.5×10^{-3} in different experiments and it was too low to make schlieren observations possible; however, the migration of the Hp boundaries could be recorded because of the strong light absorption.

1. Egg Albumin.—A solution of egg albumin containing 7.14 mg. N per cc. was exposed to light in presence of Hp of concentration 1×10^{-3} and samples were taken after the substrate had consumed 265, 506, and 675 c.mm. of O_2 per cc. respectively. These samples as well as the unaltered egg albumin-Hp were diluted with buffer to a concentration of 1.65 mg. N per cc., and then studied in the electrophoresis apparatus. At the same time precipitin curves of the 4 samples were made.

Results

The Hp of an unaltered egg albumin-Hp mixture moved faster than the egg albumin so that the two substances were separated after a prolonged run. The average mobility of the unexposed Hp in $cm.^2\ volt^{-1}\ sec.^{-1}$ was about $\mu = 10.0 \times 10^{-5}$, while the unaltered egg albumin moved with a speed $\mu = 8.21 \times 10^{-5}$ in the ascending and $\mu = -7.2 \times 10^{-5}$ in the descending limb. After photo-oxidation of the mixture, the Hp became

progressively bound to the egg albumin and finally moved entirely with its boundaries. Similar results were obtained when Hp was substituted by eosin.

Cylindrical lens curves of the ascending limbs of the four samples and their mobilities are given in Fig. 7. The corresponding precipitin curves are those presented in Fig. 1.

No significant difference between the mobilities of non-exposed and photo-oxidized egg albumin was found in the ascending limb, while there was a tendency to slowing down in the descending limb after prolonged photo-oxidation. Longsworth curves and cylindrical lens curves showed

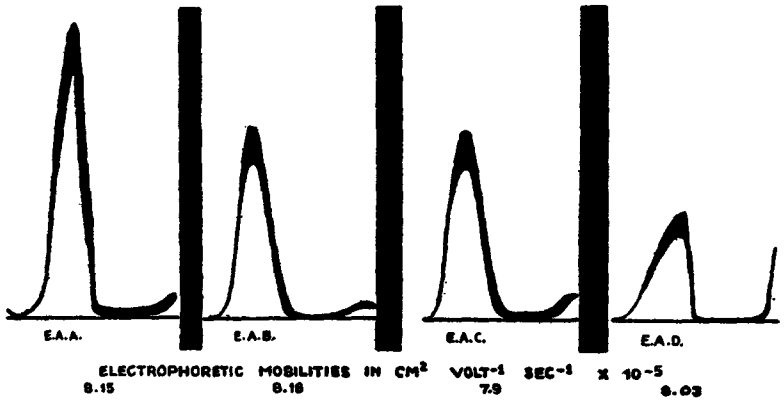


FIG. 7. Cylindrical lens curves (ascending limb) of normal and photo-oxidized egg albumin.

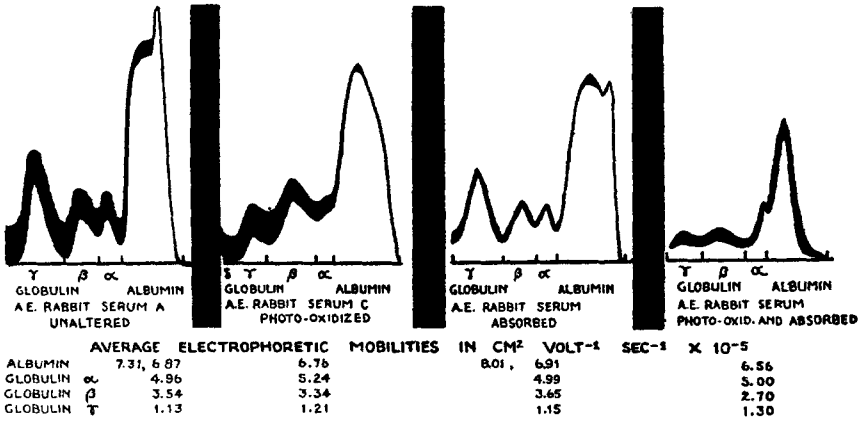
a progressive reduction of 34 per cent (B), 36.7 per cent (C), and 57.5 per cent (D) of the area under the curve representing the egg albumin boundary after progressive photo-oxidation, when compared to unaltered material (A). At the same time there appeared increasing amounts of a very slowly moving, almost stationary component.

Studies of the sedimentation rate of unaltered and photo-oxidized egg albumin likewise showed progressively increasing amounts of particles of a greater sedimentation rate in the photo-oxidized material. This study, which was undertaken in collaboration with Dr. K. O. Pedersen of the Physico-Chemical Institute of the University of Upsala, Sweden, will appear elsewhere.

2. Anti-Egg Albumin Rabbit Serum.—Rabbit serum containing 10.4 mg. N per cc. was exposed to light in presence of Hp of concentration 1×10^{-3} and samples were taken for electrophoretic studies after the substrate had consumed 122 c.mm. of O_2 per cc. In addition, unaltered serum, partly absorbed with antigen as well as photo-oxidized and absorbed serum, was studied. All samples were diluted with buffer and the total N of

serum A (normal) was 5.2 mg. N per cc., of serum C (photo-oxidized) 5.18 mg. N per cc., of the partially absorbed serum 4.5 mg. N per cc. and of the photo-oxidized and absorbed rabbit serum 2.0 mg. N per cc. At the same time quantitative precipitin curves were made with samples of unaltered and photo-oxidized material. Cylindrical lens curves of the descending limb of the different samples and the mobilities of the components are shown in Fig. 8. The corresponding precipitin curves are presented in Fig. 2.

There was a marked alteration of the pattern of anti-egg albumin rabbit serum after prolonged photo-oxidation (serum C), affecting all components. Aside from the disappearance of the double boundary of the albumin, its area increased 7.4 per cent. The area under the curve of globulin alpha



AVERAGE ELECTROPHORETIC MOBILITIES IN CM^2 $VOLT^{-1}$ SEC^{-1} × 10^{-5}

ALBUMIN	7.31, 6.87	6.76	8.01, 6.91	6.56
GLOBULIN α	4.96	5.24	4.99	5.00
GLOBULIN β	3.54	3.34	3.65	2.70
GLOBULIN γ	1.13	1.21	1.15	1.30

FIG. 8. Cylindrical lens curves (descending limb) of normal, photo-oxidized, absorbed, and photo-oxidized and absorbed anti-egg albumin rabbit serum.

decreased 25 per cent, while that of globulin beta increased 81.0 per cent; globulin gamma showed a reduction of 48.4 per cent. The total area representing photo-oxidized rabbit serum decreased 12 per cent when compared with that of the unaltered serum. This loss could be partly accounted for by a new, stationary component in the region of the delta boundary. For comparison cylindrical lens curves of partially absorbed and photo-oxidized and absorbed anti-egg albumin rabbit serum are shown in Fig. 8. The double boundary of the albumin of the partially absorbed serum was probably due to the presence of antigen, since the mobility of the first peak corresponded to that of egg albumin. The area representing albumin shows an increase of 12.3 per cent, while all others are slightly reduced. The most marked reduction was that of globulin gamma (18.6 per cent) due to the loss of antibody.

Analyses of the diagram of the photo-oxidized and absorbed antiserum showed a relatively small increase (3.7 per cent) of the area representing albumin, an increase of 71 per cent of the area of globulin alpha and of 48.5 per cent of globulin beta, while that of globulin gamma was diminished 55 per cent. Except for the increase in the area of globulin alpha, the pattern was similar to that of serum C.

The Hp of an unaltered serum-Hp mixture did not move independently but was fixed to the albumin fraction and moved with its boundary; only a small portion of the dye migrated with the descending boundary of globulin alpha. Photo-oxidation did not change this relationship. Serum albumin, isolated by electrophoresis, behaved similarly in that the Hp migrated with the boundaries of this protein.

3. Antipneumococcus Type I Horse Serum.—The serum containing 12.16 mg. N per cc. was photo-oxidized in the presence of Hp 0.1×10^{-3} until it had consumed 150 c.mm. of O_2 per cc. Samples of normal, photo-oxidized, absorbed, as well as photo-oxidized and absorbed serum were studied in the electrophoresis apparatus and precipitin curves were made.

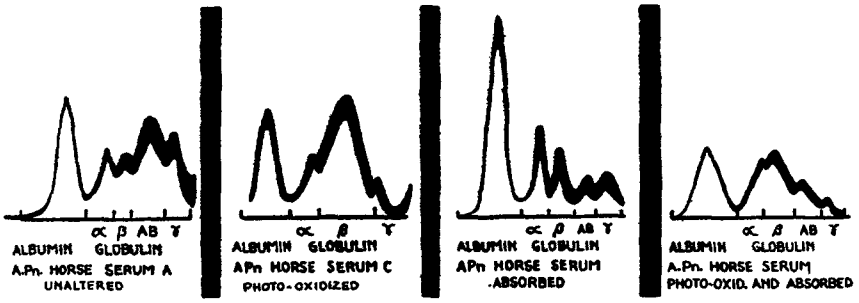
For the electrophoretic studies the serum-Hp was diluted with buffer and the total N of serum A was 3.08 mg. N per cc., that of serum C 3.06 mg. N per cc., of the absorbed serum 1.69 mg. N per cc., and of the photo-oxidized and absorbed serum 1.93 mg. N per cc.

Cylindrical lens curves of the different samples are shown in Fig. 9 where also the mobilities of the serum components are given. The corresponding precipitin curves are presented in Fig. 3.

It can be seen that the loss of the precipitin action due to photo-oxidation was accompanied by a marked change in the electrophoretic pattern of the antiserum. No boundary of the antibody fraction was seen in the curve representing serum C and the field normally occupied by it was taken up by an abnormally large fraction which had no antibody properties, but moved with the speed of globulin beta. There was an increase of 405 per cent of the area under the curve of globulin beta and a reduction of 67.4 per cent of that of globulin gamma and a complete disappearance of the AB fraction. The other components remained essentially unchanged. The total area representing photo-oxidized horse serum remained about the same.

The diagram representing horse serum partly absorbed with antigen shows very clearly that the antibody fraction of the antipneumococcus Type I horse serum used is situated between globulin beta and gamma as found by Tiselius and Kabat (27, 28); allowing for the different protein concentrations due to precipitation, there was a reduction of 71.9 per cent of the area under the curve of the AB fraction accompanied by a loss of 27

per cent of the area representing globulin gamma. There was an increase of 69 per cent of the fraction which migrated at the speed of albumin and increases of the areas of globulin alpha (12.3 per cent) and beta (40 per cent). The diagram representing partly photo-oxidized and absorbed serum shows an increase of 196 per cent of the globulin beta area and loss of 61.4 per cent of the area of the antibody fraction and loss of 58.5 per cent of the globulin gamma fraction. There was an increase of 16.4 per cent in the area representing globulin alpha, and of 18.75 per cent of albumin. There were no significant differences between the mobilities of the components of normal and photo-oxidized serum, while the fractions of ab-



AVERAGE ELECTROPHORETIC MOBILITIES IN CM^2 $VOLT^{-1}$ SEC^{-1} $\times 10^{-5}$				
ALBUMIN	8.00	8.34	8.16	9.05
GLOBULIN α	5.75	5.88	5.94	5.95
GLOBULIN β	4.28	4.12	4.63	3.55
ANTIBODY	3.02	—	2.25	? 2.0
GLOBULIN γ	2.2	2.2	1.40	? 1.0

FIG. 9. Cylindrical lens curves (ascending limb) of unaltered, photo-oxidized, absorbed, and photo-oxidized and absorbed antipneumococcus horse serum.

sorbed and photo-oxidized and absorbed serum moved with a different speed from those of normal serum, except for globulin alpha.

Similar to rabbit serum, the Hp was bound to the albumin fraction; a small portion moved with the descending boundary of globulin alpha. This relationship remained the same after photo-oxidation.

4. Globulin Gamma Fraction of Anti-Egg Albumin Rabbit Serum.—The globulin gamma fraction which had been separated electrophoretically and contained 1.02 mg. N per cc. was exposed to light in presence of Hp 0.5×10^{-3} until the substrate had consumed 43 and 140 c. mm. of O_2 per cc.; it was then studied in the electrophoresis apparatus.

The mobility of the boundary of the photo-oxidized globulin gamma fraction in $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-5}$ was 2.04 and did not significantly differ from that of the unaltered fraction; there was, however, a marked tendency

to splitting of the boundary after advanced oxidation. Similarly to experiments with egg albumin, the Hp which moved independently in the unaltered globulin gamma-Hp mixture became progressively bound to the protein. Ultracentrifugal studies of a normal and progressively oxidized globulin gamma fraction again showed progressively increasing amounts of particles having a greater sedimentation rate than unaltered globulin gamma.

5. Felton Solution of Antipneumococcus Type I Horse Serum.—The solution containing 1.9 mg. N per cc. was exposed to light in the presence of Hp of concentration 0.5×10^{-3} and samples of unaltered and photo-oxidized material after having consumed 50.4 and 160 c. mm. of O_2 per cc., respectively, were studied.

The average mobility of the boundary in $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-5}$ was 4.9 and remained the same within limits of error after photo-oxidation. Splitting of the boundaries occurred after prolonged photo-oxidation. The behavior of the Hp was identical to that of an egg albumin-Hp mixture.

DISCUSSION

In 1905 Fleischmann (29) published a study on the effect of photodynamic substances on the precipitin reaction and reported slowing up the reaction after 2 hours of exposure of antisera to light in presence of eosin and complete disappearance after 8 hours. He also studied the effect of photodynamic substances on antigen and found that much more time is required to destroy its antigenic properties.

It was the objective of the present study to analyze the changes of certain proteins during photo-oxidation and to correlate these changes with some aspects concerning the nature of antigenicity.

The magnitude of the oxygen consumption provided a convenient way for measuring the degree of oxidation, so that experiments could easily be duplicated with the same materials; at the same time the measurements of the O_2 uptake during photo-oxidation revealed certain differences between the character of antigen and antibody proteins regarding the amount of O_2 necessary to weaken or destroy their respective antigenic characteristics: It took a great deal more of O_2 to alter the antigen than the antisera or their specific antibody fractions, if the respective protein concentrations are taken into consideration.

The alteration of the antigenicity due to photo-oxidation is, however, by no means selective, but merely incidental. All proteins—except gelatin—take up oxygen during photo-oxidation (1–3, 15–18) and it seems safe to assume that they are changed by this procedure. The change in the pat-

tern of the sera after prolonged photo-oxidation as reported above in part III, 2 and 3, of this study, would substantiate this assumption. All components of a protein mixture, such as serum, are probably attacked in a similar manner, but some, probably more labile components, are sooner destroyed than others. In this respect it is significant that the boundary of globulin gamma—the carrier of the precipitin reaction of anti-egg albumin rabbit serum—remains visible even after the serum has lost its precipitating action. Obviously only a part of this fraction is concerned with the precipitin reaction. On the other hand the disappearance of the separate boundary of the specific antibody fraction of antipneumococcus Type I horse serum coincided with the loss of the precipitin reaction of this serum. Both findings confirm the work of Tiselius and Kabat (27, 28). The disappearance of this boundary after comparatively short photo-oxidation suggests this component to be of labile character. It is destroyed after about the same degree of oxidation necessary to destroy the antibody component of globulin gamma of anti-egg albumin rabbit serum. Since there occurs but an insignificant increase in the non-coagulable nitrogen after photo-oxidation of both rabbit serum and horse serum, or their specific antibody fractions, even though the precipitating action had been lost, destruction of protein molecules does not account for the loss of the reaction.

The splitting of the boundary of globulin gamma after photo-oxidation occurred only in the isolated material and not of globulin gamma fraction of whole serum. The greater lability of this heterogeneous fraction after separation from the rest of the serum may account for it. The same holds true for Felton solution whose boundary shows a tendency to splitting after prolonged photo-oxidation.

Egg albumin showed a progressive diminution of tryptophane and histidine in progressively photo-oxidized material. This finding differs from reports of other workers, especially in respect to tyrosine (1). The difference may be due to the fact that the photo-oxidation of the protein was carried out at a lower pH (7.3–7.4). Most of the isolated amino acids, however, can be photo-oxidized (1, 15–18). Similarly the oxidation of the sulfhydryl groups of egg albumin during photo-oxidation may alter the antigenicity of the protein. However, on photo-oxidation of the globulin gamma fraction of anti-egg albumin rabbit serum and of Felton solution of antipneumococcus Type I horse serum, the precipitin reaction was destroyed without any noticeable destruction in the amino acids investigated. It would appear, therefore, that other non-detectable alterations occurred in the antibody molecule which were responsible for the loss of its im-

munological properties. The destruction of tryptophane and histidine in egg albumin may be of significance in regard to its antigenicity.

The rather insignificant increase of the non-coagulable and of the amino nitrogen in the photo-oxidized egg albumin indicates that egg albumin is not hydrolyzed on photo-oxidation and that the protein molecule as such is still intact. Judging from the results of the chemical analyses, the effect of enzymes on antibodies as reported by Rosenheim (30) appears to be entirely different from that of photo-oxidation.

The difference between antigen and antibody concerning the change of the precipitin curves due to photo-oxidation lies in the difference of function of each of these components taking part in the precipitin reaction. Since the maximum precipitation depends on the potency of the antiserum, this maximum may be reached with partially damaged antigen if enough of the latter is supplied. Therefore, the peaks of the curves representing partially photo-oxidized antigen move toward higher concentrations of antigen but eventually attain the same height as the ones representing unaltered antigen.

On the other hand photo-oxidation of the antisera causes a reduction of their maximum precipitability so that less antigen is required to reach this maximum. Consequently the peaks of the curves become progressively lower and move toward lower concentrations of the antigen.

The experiments on the effect of photo-oxidation on anaphylaxis indicate that egg albumin, photo-oxidized to a degree where the precipitin reaction is destroyed, has lost its antigenicity and no longer produces anaphylactic shock; similarly such photo-oxidized egg albumin does not cause the formation of precipitins in the rabbit. Photo-oxidation of egg albumin does not produce a different antigen but destroys the antigenicity of this protein.

Studies on racemized egg albumin were made by TenBroeck (31) who reported loss of ability to stimulate precipitin formation and other antigenic properties after treating the egg albumin with N/2 NaOH for 3 weeks at 37°C. In addition to racemization, hydrolysis of the protein must have occurred by this procedure, while there was no evidence of hydrolysis of egg albumin by photo-oxidation.

The electrophoretic studies of the various substrates show that although no profound alteration of the protein molecule could be detected chemically, the area under the curves of the boundaries of globulin gamma and Felton solution gradually diminished, as did the area under the curve of egg albumin. At the same time there appeared new boundaries which remained practically stationary. Corresponding ultracentrifugal studies substantiated this finding and demonstrated the progressive change of the original

materials to substances of a greater sedimentation rate. These were interpreted as aggregates indicating denaturation of the proteins.

The electrochemical changes which occur in the sera during photo-oxidation appear to be different from those of isolated protein fractions. If the mobilities of the boundaries are used as a basis for the identification of the serum components and the areas under the curves as representing the relative concentrations of the components, there occurs a marked shift especially of slower moving components to faster moving boundaries. This shift is accompanied by the loss of the precipitating property, while there are but insignificant changes in the non-coagulable nitrogen and the amino nitrogen. The significance of this change in the pattern cannot be evaluated at present.

Hp migrates almost entirely with the serum albumin of whole serum as well as with material isolated by electrophoresis and this relationship remains unchanged after photo-oxidation. The affinity of serum albumin to various chemical dyes and to bilirubin has been observed previously (32, 33, 11) and further investigations of the binding capacities of the various serum components will prove very instructive. In mixtures of Hp and isolated proteins such as egg albumin, globulin gamma, or Felton solution, however, the Hp migrates independently and can be entirely dissociated from the proteins, if runs are continued sufficiently long. This may prove useful to rid proteins from undesirable coloring matter or other components. After photo-oxidation, however, the Hp no longer migrates independently but becomes progressively fixed to the protein. All degrees from partial to complete fixation can be observed, depending on the extent of photo-oxidation. The decided change of the mobility of the Hp was usually observed after the original bright red color of the sensitizer had turned into a brownish red color following prolonged irradiation of the dye-protein mixture. The nature of this fixation is not known; it is, however, conceivable that chains of the dye attach themselves to groups of the aggregates of the denatured proteins formed during photo-oxidation.

Because of the identical effect of photo-oxidation on an eosin-egg albumin mixture, despite the different chemical nature of these two sensitizers, it may be suggested that this phenomenon of fixation is a characteristic feature of photo-oxidation.

CONCLUSIONS

1. Quantitative precipitin studies indicate that progressive photo-oxidation progressively destroys the antigenic function of egg albumin.
2. Quantitative precipitin reactions of antisera (anti-egg albumin rabbit

serum and antipneumococcus Type I horse serum) demonstrate that progressive photo-oxidation causes progressive lowering of the potency of the sera.

3. Quantitative precipitin reactions of the photo-oxidized globulin gamma fraction of anti-egg albumin rabbit serum and of Felton solution of antipneumococcus Type I horse serum show that these specific antibody fractions behave similarly to antibodies in whole sera.

4. Egg albumin whose precipitin reaction is destroyed by photo-oxidation no longer causes anaphylaxis in guinea pigs and does not produce precipitins in rabbits.

5. Chemical studies of progressively photo-oxidized egg albumin show a progressive destruction of tryptophane and histidine while tyrosine remains intact and cystine is reversibly oxidized. Sulfhydryl groups can no longer be demonstrated in photo-oxidized egg albumin whose antigenic characteristics are greatly weakened.

6. Similar studies on the globulin gamma fraction of anti-egg albumin rabbit serum and on Felton solution show no diminution of these amino acids in photo-oxidized material whose antigenic properties are destroyed.

7. The non-coagulable nitrogen and the amino nitrogen of egg albumin, antisera, and their specific antibody fractions show but an insignificant increase during photo-oxidation, indicating that the loss of the precipitin reaction is not due to splitting of the respective protein molecules.

8. Electrophoretic studies of egg albumin, antisera, and their specific antibody fractions show that photo-oxidation causes a marked alteration of the pattern of these substrates.

9. Photo-oxidation of proteins causes the formation of aggregates, indicating denaturation.

10. Hematoporphyrin migrates with the albumin fraction of unaltered as well as the photo-oxidized anti-egg albumin rabbit serum and pneumococcus Type I horse serum; in isolated proteins such as egg albumin, globulin gamma, or Felton solution, etc., the dye moves independently of the protein; after progressive photo-oxidation Hp becomes progressively fixed to the protein. Eosin behaves similarly to hematoporphyrin.

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