I. THE EFFECT OF ULTRAVIOLET RADIATION AND OF SOFT X-RAYS ON THE SEDIMENTATION BEHAVIOUR AND LIGHT ABSORPTION OF PURIFIED HUMAN SERUM ALBUMIN

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PROTEIN solutions are sensitive to ultraviolet light, and are coagulated by irradiation at the isoelectric point.¹ Coagulation can be prevented by irradiation either at reduced temperatures (0–4°) [Bovie, 1913] or in the presence of small amounts of acid or alkali (vide Arnow [1936] for references), but it then occurs if the temperature is raised, or the *pH* brought to the isoelectric point. It appears that coagulation of proteins by u.v. light takes place in at least two stages, a photochemical reaction independent of temperature, and an aggregation of the molecules attacked, a reaction of comparatively high temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935]. The photochemical reaction causes increased light absorption [Spiegel-Adolf & Krumpel, 1927] and cleavage of the molecule, perhaps with the liberation of aromatic amino-acids [Mitchell, 1936].

Pedersen [1931], from preliminary experiments, reported that when serum albumin in solution at *pH* 3.5 was irradiated by u.v. light, the solution remained clear, the sedimentation constant doubled (indicating aggregation) and the diffusion constant showed a strong drift (indicating heterogeneity).

Previous investigations have not produced much information concerning the state of aggregation of the denatured protein molecules remaining in solution, either before or after the production of a precipitate, or concerning the effects of the substances causing the increased light absorption in irradiated protein solutions, or of *pH* upon the course of the reactions which follow the initial photochemical reaction. The ultracentrifugal method of analysis is suited to the exploration of a protein system after denaturation since both cleavage and aggregation of the protein molecules will be revealed on the sedimentation diagrams either as distortion of the curves or as separate maxima.

In view of the above, and of the importance of actinotherapy in modern curative medicine, an ultracentrifugal and light absorption study has been made of the effects of u.v. and soft X-ray irradiations on solutions of purified human serum albumin.

EXPERIMENTAL

Three series of experiments were made: one near the isoelectric point, at *pH* 5.4, where the irradiation, performed at 30°, caused ultimate coagulation of the protein, and two at *pH* 7.4 (normal blood *pH*), one at 30° and the other at 0°

¹ Arnow [1936] has reviewed the literature on the effects of irradiation on proteins and amino-acids.

where all the solutions remained clear. In each series, equal volumes of protein solution were irradiated in a rotating tube for various periods of time, and ultracentrifuged under identical conditions after as nearly as possible the same lapse of time. Experiment showed that rotation without irradiation did not cause surface denaturation. Some stock albumin solution was ultracentrifuged at the beginning and the end of each series to make certain that the solutions were stable.

Preparation of material. Fasting blood, sufficient for Series A and B, was drawn from a healthy subject into sterile centrifuge tubes and the resulting serum heated to 56° for 25 min. for preservative purposes. The heating of serum to 56° for as long as $2\frac{1}{2}$ hr. has been found by McFarlane [1935, 2] to have no detectable effect upon its sedimentation diagram. The globulins were separated by half-saturation with $(\text{NH}_4)_2\text{SO}_4$ according to the directions of Adair & Taylor [1935] and an unsuccessful attempt was made to repeat their preparation of crystalline human serum albumin. When only tiny globules were obtained, the albumin was purified instead by precipitation three times with $(\text{NH}_4)_2\text{SO}_4$ to saturation. It was dialysed for 30 hr. against distilled water, electro-dialysed for 16 hr. and then stored in sterile bottles at 3° . Micro-Kjeldahl N analyses were made using the Parnas & Wagner [1921] distillation apparatus, and the N content converted into concentration of protein by the factor 6.25.

For Series A, 2 ml. stock solution were diluted immediately before irradiation with 0.7 ml. distilled water and 0.3 ml. 10 % NaCl solution, giving an albumin concentration of 0.81 g./100 ml.; sterile technique was followed as far as possible.¹ At the end of the series, a solution so prepared gave a pH of 5.44 (glass electrode).

For Series B, 20 ml. stock solution were diluted with 10 ml. of buffer solution containing 84.1 ml. $M/5$ Na_2HPO_4 and 15.9 ml. $M/5$ KH_2PO_4 per 100 ml.; the pH (glass electrode) was 7.39. 3 ml. samples were withdrawn for each irradiation experiment.

After the completion of these experiments, a paper by Tiselius [1937] appeared which showed that in serum, heated at 56° for even 10 min., the albumin boundary suffers a large reduction in electrophoretic mobility and migrates near the β globulin; this may indicate incipient denaturation. The albumin used in Series A and B had been prepared from serum heated at 56° for 25 min.; consequently the same change in mobility had probably taken place. Our results and the observations of McFarlane [1935, 2] indicated that this change did not affect the sedimentation diagram, but the possibility remained that the heated material might be more susceptible to irradiation, or might react differently.

For this reason a new stock solution of albumin (for Series C) was made from further blood from the same human; all operations were performed at $0-3^{\circ}$. The globulins were removed by 57 % saturation with $(\text{NH}_4)_2\text{SO}_4$, which was necessary to prevent precipitation during subsequent dialysis. The albumin was precipitated 3 times by saturation with $(\text{NH}_4)_2\text{SO}_4$, dialysed 4 days, electro-dialysed for 50 hr. between 0 and 5° and diluted with phosphate buffer to the same protein and buffer concentrations as for Series B; the pH (glass electrode) was 7.40. This stock solution was frozen for storage to prevent bacterial action, Tiselius [1937] having reported that freezing does not affect the electrophoretic behaviour of serum proteins.

During the experiments in Series C, it was observed that to bring about thorough mixing of the protein-rich layer formed at the bottom of the flask on thawing, more vigorous agitation was necessary than the careful swirling which had purposely been employed to prevent foaming. A number of the samples withdrawn (by pipette, from the bottom of the flask) were more concentrated

¹ Spiegel-Adolf & Pollaczek [1929] found delayed coagulation in bacterially infected solutions.

than 0.81 % protein (see initial areas, Table I, which are proportional to concentrations of centrifugible protein). The concentration differences were not great enough to influence the sedimentation constant or spreading coefficient, which are independent of this factor at low concentration, but did affect all values which are concentration-dependent (curve area, height of sedimentation maximum, extinction coefficient). Analysis showed that the samples irradiated 1 and 2 hr. in Series C contained 0.814 and 0.819 % albumin respectively, so that for these comparison with Series A and B is permissible for all values. The solution used for determination of the sedimentation velocity of the untreated material was about 4 % too concentrated (see initial areas, Table I; no solution available for micro-Kjeldahl analysis). For the relationship shown in Fig. 2, the measured height of the sedimentation maximum for this solution was therefore multiplied by the ratio of the initial areas, 0.0046/0.0048.

Radiation. The actual conditions of irradiation differed somewhat in the three series of experiments, but remained constant during each series. Directly after irradiation, 0.5 ml. of the solution was removed for the immediate determination of its u.v. absorption spectrum; the remainder was placed in a small sterile bottle, stored at 3° overnight and ultracentrifuged the next morning.

Series A. A Uviarc, with a reflector but without a shield in front of the lamp, was the source of u.v. light. The solution was contained in a quartz tube (12.5 cm. long, outer diameter 1.3 cm., inner diameter 1.05 cm.) stoppered with a paraffined cork and placed parallel to the arc, with its axis horizontal, at a distance of 20 cm. The tube was rotated about its axis at *ca.* 80 r.p.m. to ensure uniform exposure of all parts of the solution, and cooled with an electric fan. Under these conditions the temperature rose immediately to 28°, then increased gradually to 33° during the first hour, and to 35° during the next three. The solutions were irradiated for 5, 10, 15, 30 and 55 min. respectively; 30 min. irradiation caused a slight opalescence, and 55 min. a definite cloudiness of too small a particle size to be retained by fine filter paper. Some of this solution was also irradiated under similar conditions in a pyrex tube of the same dimensions; when a slight opalescence appeared after 4½ hr. the irradiation was stopped.

Series B. The same arrangements as in Series A were used, but to minimize the heating of the solution by the arc, and to reduce fluctuations in intensity of irradiation due to erratic cooling of the arc by the electric fan, the lamp was protected by a quartz window. The distance from the arc to the quartz tube was 30 cm. instead of 20 cm. as in Series A. A Photronic cell was placed beneath the rotating tube to detect changes in the amount of radiation falling on the tube.

The solutions in Series B were irradiated for 15 and 30 min., and 1, 2 and 3 hr. The average temperature varied between 27.5 and 31° from experiment to experiment although the maximum deviation during any one irradiation was 2°. The average value of the current given by the Photronic cell was constant from experiment to experiment to within $\pm 2\%$. Because of the greater distance of the arc from the solution, 2 hr. irradiation in this series corresponded very nearly to 55 min. irradiation in Series A, but because of the higher pH, the solutions remained clear even after 3 hr. irradiation.

A portion of the solution used in this series was exposed for 3 hr. to the continuous radiation from a soft X-ray tube (Cu target, 100,000 V.), the total dose used being 29,000 r.u. (measured with a Victoreen R-meter). In the absence of a detectable change in the albumin solution, no further irradiations with X-rays were made (see Table I).

Series C. To separate the effect of irradiation as far as possible from the effect of heat, the solutions in this series were kept below 1° during irradiation by ice-

water flowing over the rotating quartz tube. The irradiated solutions were stored in ice-water (not frozen, to prevent possible aggregation of the denatured protein) until placed in the centrifuge cell, and ultracentrifuged at the lowest convenient temperature (21°), in order to minimize possible aggregation during the run. (Clark [1935] found that coagulation of isoelectric egg albumin irradiated at 4° has a comparatively high temperature coefficient, but occurs only slowly below 20°.) During the 2 hr. irradiation the Photronic cell readings were constant at 70 to within $\pm 2\%$; during the 1 hr. irradiation the average reading dropped to 62, constant to within $\pm 3\%$. The lamp burned out before a third irradiation could be made. The radiation doses in this series are thus not proportional to exposure time; allowance has been made for this variation in the graphical presentation of the data (Fig. 2), on the assumption that the intensities of all wave-lengths were reduced in the same ratio. The uncertainty in this assumption does not seriously affect the conclusions drawn.

Light absorption. Absorption spectra were obtained using the Hilger echelon cells [Twyman *et al.* 1931–32; Twyman, 1933] with a medium-sized Bausch & Lomb quartz spectrograph, by technique previously described [McDonald, 1936]. The absorption spectra are shown in Fig. 1. The position of the absorption bands are correctly represented but, because of a 'systematic error in the apparatus [vide Magill *et al.* 1937, Fig. 12], the ordinates do not show the absolute extinction coefficients, but give values which are too high by a factor which varies somewhat from wave-length to wave-length. At wave-lengths corresponding to the absorption maxima the measured values were found to be too large by a factor of 1.1. The corrected heights of the absorption maxima are plotted against the amount of irradiation in Fig. 2 (I, II and III). Using the above correction factor, a value of 6.0 was obtained for the specific extinction coefficient of serum albumin. Svedberg & Sjögren [1930] found a value of *ca.* 5 over the pH range 5–8, and Smith [1929] a value of 5.8 at pH 7.

Sedimentation velocity. Measurements of sedimentation velocity were made in a standard Svedberg oil-turbine ultracentrifuge at a speed of 56,000–58,000 r.p.m., corresponding to a centrifugal force of about 215,000 times gravity at the middle of the cell. The temperature of centrifuging was 24–26° for Series A and B, and 21° for Series C. Sedimentation was followed by the refractive index method of Lamm [1928; 1929] using a cell 3 mm. in thickness and a scale distance of 10 cm. Photographs were taken every 10 min., and the runs were continued for approximately 2 hr. Solvent runs were made from time to time under identical centrifuging conditions [cf. Pedersen, 1936] to provide standards from which the displacements caused by sedimentation of the protein were measured. These displacements were plotted against the distance from the centre of rotation to give the usual sedimentation diagrams.

All experimental conditions were kept as constant as possible from run to run, so that in any series the relative heights of sedimentation curves representing equal times of centrifuging and of diffusion give, to a first approximation, the relative concentrations of albumin of unchanged sedimentation constant in the irradiated solutions. The principal factor reducing the exactness of this relationship is the presence of aggregates incompletely centrifuged from the plane of the boundary, which increase the displacement. This has been minimized by selecting for the comparison a time of centrifuging (50 min.) sufficiently far advanced to ensure fair separation of the heavy particles, but early enough to avoid complications due to reflexion of particles from the bottom of the cell. The relative heights, using 100% for the untreated solution, are given in Fig. 2 (IV and V).

The sedimentation constants were calculated by the usual equation [Svedberg, 1933; 1937]:

$$s_{20} = dx/dt \cdot 1/\omega^2 x_m \cdot \eta_T/\eta_{20} \cdot (1 - V\rho_{20})/(1 - V\rho_T)$$

and are listed in Table I. The sedimentation constant, $s = 4.5 - 4.6$, for the untreated material in Series A and for all but two determinations of Series C, is in good agreement with the published value, $s = 4.5$ [Svedberg, 1937]. For Series B, and two determinations in Series C, the values are lower. This is discussed later.

Representative sedimentation diagrams are shown in Figs. 3 and 4. To avoid repetition, the diagrams for untreated albumin in Series B and C, and for X-ray-treated albumin in Series B, have been omitted; they were similar in appearance and symmetry to that of the untreated solution in Series A.

To estimate the relative amounts of centrifugible material in the various solutions [vide McFarlane, 1935, 1] the areas enclosed by the sedimentation curves were converted into absolute units, and, to correct for the dilution caused by centrifuging in a sector-shaped cell in a field of varying intensity [Svedberg & Rinde, 1924], multiplied by the factor $(x_t/x_o)^2$. Since the refractive indices (measured, at 26°, with an Abbé refractometer using a sodium lamp as light source) of the irradiated serum albumin solutions were identical with those of the unirradiated solutions, the corrected areas so obtained are proportional to the concentrations of centrifugible protein. The initial and final areas for each experiment are listed in Table I.

Spreading coefficients were calculated [Lamm, 1929] from the half-width, u , of the sedimentation curves at a height, h , where

$$h = \text{maximum height}/\sqrt{e} = 0.606 \text{ maximum height.}$$

The values of u^2 were plotted against the time of centrifuging; the spreading coefficients, or "apparent diffusion constants", were given by the slopes of these curves [Loughborough & Stamm, 1936]. Constant values were obtained for the untreated solutions and for those which showed no marked change; these are listed in Table I. The others showed a drift with time, and single values can therefore not be given. In the solutions irradiated for the longest periods of time (A7, A8, B6, B7, B8, C3) there were discontinuities in the u^2 -time curves; these represent the separation of discrete boundaries composed of particles whose concentration is sufficient to cause pronounced widening of the curve at the height considered.

The spreading coefficients calculated from the curve areas [McFarlane, 1935, 1] are listed in Table I. For characterizing mixtures this method of calculation is preferable to the one given above because it includes the spreading due to all particles present, and not only to those of sufficient concentration to widen the curve appreciably at the height considered. Further, for the untreated solutions, the method gave values which are in better agreement with the published values of the diffusion constant, 6.17 [Svedberg, 1937]. For a heterogeneous mixture the spreading coefficient has no physical meaning, but does provide an index of the range and relative concentrations of particle sizes.

DISCUSSION

Absorption spectra. The absorption spectra (Fig. 1) show that at both pH the light absorption increases regularly as irradiation proceeds; the increase is general for wave-lengths greater than 2400 Å. but most pronounced at the characteristic protein maximum. For the same radiation dose (Fig. 2), the

increase in light absorption is greater at pH 5.4 than at pH 7.4. This suggests that the reactions causing the increase are inhibited at the higher pH .

As a rule the irradiation of amino-acids, dipeptides and related compounds containing only aliphatic radicals causes increased absorption of wave-lengths less than 2500 Å., while irradiation of those containing aromatic nuclei causes increased absorption at wave-lengths greater than 2400 Å. [Allen *et al.* 1937].

Fig. 1. Increase in absorption spectra with irradiation. Irradiation: Series A, 1, untreated; 2, 10 min.; 3, 15 min.; 4, 4.25 hr. in glass; 5, 30 min.; 6, 55 min. Series B, 1, untreated; 2, 15 min.; 3, 30 min.; 4, 1 hr.; 5, 2 hr.; 6, 3 hr. Series C, 1, untreated; 2, 1 hr.; 3, 2 hr.; 4, 2 months after 2 hr. irradiation.

Too little is known about the absorption spectra of individual amino-acids and polypeptides, and of their photolytic products, to ascertain the specific reactions responsible for the increased absorption of irradiated proteins. In general, $u.v.$ light and cathode rays convert primary and secondary amino-acids into aldehydes, and tertiary amino-acids into ketones, by the addition of oxygen and water and the elimination of ammonia, carbon dioxide and water [Allen *et al.* 1937; Neuberg, 1908; 1910; Harris, 1926; Rao & Dhar, 1934; Becker & Szendrő, 1931]. The rate of ammonia cleavage varies from compound to compound, and is more rapid in an acid than in an alkaline medium [Lieben & Urban, 1931]. Peptones and proteins also yield aldehydes, ammonia and carbon dioxide, a fact which can be explained only by assuming that at some stage of the process hydrolysis of the peptide linkage takes place. Mitchell [1936], from a study of the effect of $u.v.$ light on monolayers of egg albumin, has suggested that the primary reaction is the hydrolysis of CONH linkages adjacent to aromatic residues, with liberation of tyrosine, tryptophan, phenylalanine and perhaps histidine, since these groups are responsible for the high light absorption of the protein in the range 2500–2900 Å.

The concentrations of centrifugible material in the irradiated solutions also point to a reaction of this character. Table I gives the areas enclosed by the sedimentation curves, to which the concentrations of centrifugible material are proportional. All solutions which remained clear, and from which, therefore, none of the albumin had been precipitated, gave *initial* areas that were approxi-

mately the same as for unirradiated albumin. This shows that the non-centrifugible material split off by the action of the light constituted only a few % of the total weight. Tyrosine and tryptophan comprise respectively 4.66 and 0.53 % of the weight of serum albumin [Folin & Marenzi, 1929], phenylalanine 2.3 % [Stenström & Rheinhard, 1925], histidine 1.95 % [Block, 1934]; together they amount to about 9.4 %. The sedimentation data (Fig. 2, V) show that 3 hr. irradiation at pH 7.4 caused modification (with respect to sedimentation constant) of about 70 % of the serum albumin. Complete hydrolysis of all aromatic amino-acids in this modified fraction of the albumin would represent, therefore, a loss of about 6.6 % as non-centrifugible material. The observed loss, calculated from decrease in initial area (Table I), is not more than 4.5 % and is probably less, since part of the drop in initial area may be due, to the removal of a small concentration of heavy aggregates. The diminution in area is thus consistent with the view that only aromatic amino-acids are hydrolysed from the molecule.

However, this reaction cannot be responsible for the increased light absorption. Tyrosine, tryptophan and phenylalanine have molecular extinction coefficients of 1576, 5900 and 180 respectively [Gróh & Hánák, 1930]. Calculation, using the above figures, shows that a solution containing as free acids the same concentrations of these amino-acids as are present in a 1 % solution of serum albumin would have an extinction coefficient of 5.83, while the specific extinction coefficient of serum albumin is between 5.0 and 6.0 [Svedberg & Sjögren, 1930; Smith, 1929].

Arnow [1935] has shown that irradiation of egg albumin with α -particles causes increased absorption in isoelectric or acid solutions, decreased absorption in solutions alkaline to the isoelectric point. He has suggested that the increase may result from the photo-oxidation of phenylalanine to tyrosine and of tyrosine to dihydroxyphenylalanine ("dopa") which has a molecular extinction coefficient of 2500 [Abderhalden & Rossner, 1928]. Recently he has demonstrated that low concentrations of "dopa" are formed by the action of u.v. light on tyrosine [Arnow, 1937]. However, the conversion into "dopa" of all the tyrosine and phenylalanine present in serum albumin would cause only a 95 % increase in the extinction coefficient. This mechanism, therefore, even with the improbable assumption of complete conversion, is inadequate, by itself, to account for the increases in light absorption observed in the present investigation.

Fig. 2. Correlation between extinction coefficient and relative height of sedimentation maximum. The abscissae represent actual times of irradiation for Series B and C. The times for Series A are the actual irradiation times $\times 9/4$, to compensate for the change in distance of the light source. G_1 and G_2 give the extinction coefficient and relative sedimentation height, respectively, of the solution irradiated 4.25 hr. in glass.

Table I

Treatment	Appearance	$s_{20} \times 10^{13}$	Spreading coefficient		Curve areas	
			Area	Width	Initial sq. cm.	Final
Series A. $pH=5.44$; temp. approx. 30°						
1. Untreated (before)	Clear	4.67	6.53	5.4	0.0047*	
2. Untreated (after)	Clear	4.50	6.15	5.3	0.0047*	
3. Glass 4.25 hr.	Opalescent	4.84	12.8	—	0.0034	0.0034
4. Quartz 5 min.	Clear	4.49	5.59	5.4	0.0047	0.0047
5. Quartz 10 min.	Clear	4.61	7.19	5.8	0.0047	0.0047
6. Quartz 15 min.	Clear	4.89	16.8	—	0.0046	0.0037
7. Quartz 30 min.	Opalescent	4.98	24.8	—	0.0026	0.0020
8. Quartz 55 min.	Cloudy	4.92	18.7	—	0.0016	0.0013
Series B. $pH=7.40$; temp. approx. 30°						
1. Untreated (before)	Clear	4.41	5.33	4.0	0.0046*	
2. Untreated (after)	Clear	4.47	5.25	4.0	0.0046*	
3. Quartz 15 min.	Clear	4.41	7.08	—	0.0046	0.0044†
4. Quartz 30 min.	Clear	4.29	7.44	—	0.0046	0.0043†
5. Quartz 1 hr.	Clear	4.44	16.9	—	0.0046	0.0039‡
6. Quartz 2 hr.	Clear	4.41	48.5	—	0.0046	0.0036‡
7. Quartz 3 hr.	Clear	4.41	73.0	—	0.0044	0.0031§
8. Quartz 3 hr.	Clear	4.33	80.0	—	0.0044	0.0035§
(repeated 6 days later)						
9. X-rays, 29,000 r.u.	Clear	4.39	6.24	3.7	0.0046*	
Series C. $pH=7.40$; temp. 0°						
1. Untreated (before)	Clear	4.63	6.61	3.5	0.0048	
2. Quartz 1 hr.	Clear	4.57	17.1	—	0.0046	0.0040‡
3. Quartz 2 hr.	Clear	4.57	54.6	—	0.0046	0.0035‡
4. Heated, and rotated for 3 hr.	Clear	4.22	4.69	—	0.0071*	
5. Untreated (temp. of centrifuging, $25-26^\circ$)	Clear	4.23	4.72	—	0.0075*	
6. Untreated (temp. of centrifuging, $20-22^\circ$)	Clear	4.53	6.02	—	0.0076*	

* Average values over entire time of centrifuging: † 72 min., ‡ 60 min. and § 50 min. from start of centrifuging.
 || Albumin concentration greater than 0.81%.

Immediately after irradiation, the solutions irradiated at 0° (Series C) showed less increase in absorption at the protein maximum, but more increase on the long-wave side of the protein band, than the solutions of the same pH irradiated for the same periods of time at 30° (Series B) (compare B4 and C2, B5 and C3, Fig. 1). Prolonged standing at 3° led to the disappearance of this difference, resulting in increased absorption below $3000 \text{ \AA}$. and decreased absorption above $3000 \text{ \AA}$. in both series of solutions, but to a far more limited extent in Series B than in Series C (compare B5, C3 and C4, Fig. 1). The effect of the temperature of irradiation was in this way eliminated, and identical spectra were obtained for the two series, a finding which is consistent with the observations of Rajewsky [1929; 1930] and Clark [1935] that the temperature coefficient of the primary photochemical process is unity. Since no changes could be detected in the absorption spectra of the unirradiated solutions which had been stored at 3° for the same lengths of time, it seems possible that part of the absorption at wave-lengths longer than $3000 \text{ \AA}$., observed in the solutions irradiated at 0° , represents an unstable intermediate stage [Mirsky & Pauling, 1936, p. 444] which produces, on warming or on prolonged standing in the cold, the compound(s) responsible for the increased absorption at the protein maximum.

The two solutions irradiated 2 hr. at $pH 7.4$ were brought to $pH 5.4$ by dialysis at 3° through cellophane membranes against large volumes of $M/15$ phos-

phate buffer. The absorption spectra were taken first with the chilled solution, then again after warming to 30° for 20 min. Both solutions remained clear. Each gave the same spectrum before and after warming, but the two differed a little from each other, perhaps because of slight dilution during dialysis. The increased absorption, above that of the native protein, was in both instances reduced to about half what it had been before dialysis, i.e. to an extinction coefficient of 7. This suggests that while part of the increase results from cleavage products small enough to diffuse through cellophane membranes, much of it must arise either from reactions in the protein molecule without cleavage, or from cleavage products too large to diffuse through cellophane.

The solution of pH 7.4 irradiated 1 hr. at 30° was brought to pH 5.4 by the addition at 0° of *N* HCl; this caused a 4% increase in volume. Within the limits of error of the method, the absorption spectra measured before and after warming were identical with each other and with that of the solution before dialysis. Since the solution which was given approximately the same radiation dosage at pH 5.4 (Series A, 30 min.) gave much greater absorption, it appears that the action of the light is inhibited at pH 7.4.

Sedimentation velocity measurements. The most obvious result of irradiation is the progressive reduction in the heights of the sedimentation curves (Figs. 3 and 4); but in each series the sedimentation constants remain the same as for the native protein (Table I), except for a slight increase at pH 5.4 (Series A). These facts show that in the irradiated solutions the boundaries represent albumin of unchanged sedimentation constant, and that the concentration of such albumin is diminished by successively longer periods of irradiation. Irradiation also causes an asymmetrical broadening of the sedimentation curves to the right of the maximum, which demonstrates the presence of particles heavier than native albumin, and suggests that the protein molecules which have been attacked by the light coalesce to form aggregates. Resolution of the faster-moving boundaries was not sufficient for calculation of the sedimentation constants, but qualitative information concerning aggregate sizes can be gained from the curve areas and curve spreading.

For solutions irradiated longest, the curve areas (Table I) diminish as centrifuging proceeds, because the heavy aggregates, in order of size, have been centrifuged to the bottom of the cell and removed from the field of observation. At pH 7.4 the rate of this reduction increases with increase in irradiation time; this shows that as irradiation proceeds not only the concentrations but also the average sizes of the aggregates become greater. At pH 5.4 the separation of a visible precipitate causes a reduction in *initial* area, the greater the longer the effective period of irradiation; the further reduction in area during centrifuging shows that the heavy aggregates characteristic of the solutions irradiated 15 and 30 min. are present in lower absolute concentration in the solution irradiated 55 min., and are almost entirely absent from the solution irradiated in pyrex glass for 4½ hr. The spreading coefficients show the same trend, a continuous increase at pH 7.4, and at pH 5.4 an increase to a maximum near 30 min. irradiation; but the greater magnitude of the variations emphasizes the differences in average particle size. These differences were also clearly shown by the variation of u^2 , the square of the half-width of the curve at 0.606 times the maximum height, with time of centrifuging: at pH 7.4, each solution irradiated for 30 min. or more gave curves which indicated progressively diminished spreading, the result of the removal of the heaviest aggregates as the centrifuging proceeded, while at pH 5.4 the solutions irradiated for 30 and 55 min. both gave curves which indicated the gradual separation of particles sedimenting with velocities of the

same order of magnitude, and the absence of appreciable concentrations of heavy particles. The discontinuities in the u^2 -time curves for the solutions irradiated longest (A7, A8, B6, B7, B8, C3) represent the separation of discrete boundaries each composed of particles whose concentration is sufficient to cause marked

Fig. 3. Sedimentation diagrams. Series A. pH 5-44. Abscissae, distance from centre of rotation (X in cm.); ordinates, scale-line displacement (Z in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of u.v. irradiation. Temperature of irradiation *ca.* 30° .

broadening of the curve at the height considered. The approximate sedimentation constants for these boundaries, estimated from the discontinuities, fell into two groups, one about 6, the other a little more than 7, and probably correspond to aggregates composed of two or three albumin residues.

The results as a whole indicate that in solutions sufficiently alkaline to prevent precipitation of the denatured protein, irradiation leads to the formation of increasing amounts of aggregated product of successively greater particle size,

Fig. 4. Sedimentation diagrams. Series B and C. *pH* 7.40. Abscissae, distance from centre of rotation (*X* in cm.); ordinates, scale-line displacement (*Z* in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of u.v. irradiation. Temperature of irradiation: Series B, *ca.* 30°; Series C, 0°.

but there appears to be a limit to the size developed. This type of aggregation seems to take place even at low temperatures, or else very rapidly at about 20°, because the precaution, observed in Series C, of irradiating and storing at 0° until the beginning of the ultracentrifuge run, had negligible effect on the aggre-

gation; this can be seen from the similarity of the sedimentation curves (Fig. 4), of the relative heights of the sedimentation maxima (Fig. 2, V) and of the spreading coefficients (Table I), to those of the solutions irradiated for the same times at 30°.

On the other hand, in solutions nearer to the isoelectric point there is no limit to the particle size, and a visible precipitate is formed at the expense of aggregates of intermediate size. Here the comparatively high temperature of irradiation (30°) has definitely influenced the particle size distribution. Because the coagulation process has a rather large temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935], the solutions irradiated the longest were given the most favourable conditions for coagulation, and became most impoverished with respect to soluble aggregates, especially those of large size. The maximum in the spreading coefficient after about 30 min. irradiation in quartz, therefore, probably represents the stage of aggregation which immediately precedes the formation of a visible precipitate, first seen after 30 min. irradiation.

The absence of heavy soluble aggregates from the solution irradiated for 4½ hr. in pyrex glass is undoubtedly the result of prolonged high temperature. The absorption spectrum of this solution is in all respects similar to those of the solutions irradiated in quartz. Since, as can be seen from Fig. 2 (I and IV), the heights of the sedimentation and absorption maxima correspond to about the same degree of change as that to be expected from approximately 20 min. irradiation in quartz, it seems reasonable to suppose that the reaction is the same in both quartz and pyrex, but that the difference in extent results from the greater transparency of quartz to the effective wave-lengths [Rajewsky, 1930].

The greater uniformity of the sedimentation constants for the observed boundary in solutions irradiated at pH 7.4 can probably be attributed to the preponderance among the aggregates of heavy particles which quickly separate from the main boundary; the upward trend at pH 5.4, to the higher relative concentrations of small aggregates, which, being incompletely resolved from the main boundary, cause an apparent increase in the sedimentation constant.

For equal irradiation at 30°, the reduction in the concentration of albumin of unchanged sedimentation constant is greater at pH 5.4 than at pH 7.4; this is shown in Fig. 2 (IV and V). It appears, therefore, that the aggregation process is inhibited at the higher pH. Gross coagulation, which is also inhibited at pH removed from the isoelectric point, is known to be a process distinct from the light reaction [Rajewsky, 1929; 1930; Clark, 1935]. Doubtless, therefore, even the simplest stages of aggregation must also be considered separate from it, though it is scarcely justifiable to conclude on this basis alone that the initial photochemical process, which may be considered to render the molecules susceptible to aggregation, is also inhibited at the higher pH. However, the correlation between reduction in albumin concentration and increase of light absorption, shown in Fig. 2, is fairly convincing evidence that the two are manifestations of the same fundamental change. It has already been shown that the reaction causing increase of light absorption is inhibited at pH 7.4; it thus seems likely that the photochemical reaction causing aggregation may also be repressed. This conclusion is supported by the fact that while solutions irradiated at pH 5.4 for 30 and 55 min. respectively showed opalescence and turbidity, the solutions which were adjusted to pH 5.4 *after* receiving equivalent amounts of radiation (1 and 2 hr.) at pH 7.4 all remained clear.

Young [1922], from measurements of the amounts of coagulum formed at the isoelectric point after irradiation at various pH, reached the opposite conclusion, that the reaction was progressively more rapid on both sides of the isoelectric

point. However, his solutions were not rotated during irradiation, and since the increase of light absorption has been shown to be less at pH 7.4 than at pH 5.4, a possible reason for his results may be the lower screening effect, at pH removed from the isoelectric point, of the irradiated layers nearest the light source.

Because of the uncertainty of the protein concentrations and radiation doses in Series C, a detailed quantitative comparison cannot be made with the results of Series A and B. However, the relative heights of the sedimentation maxima are, as far as can be ascertained, the same as for the solutions of the same pH irradiated at 30° (Fig. 2, V), while the maximum extinction coefficients are definitely lower (Fig. 2, III). The correlation between rise of absorption maximum and drop in sedimentation maximum is thus not apparent for Series C, the reason probably being that the absorption spectra, taken within 5 min. of the removal of the solution from the ice-bath, represent quite accurately the unheated solutions, while the ultracentrifuge data refer to the solutions warmed to 20 – 22° for about an hour during the loading and accelerating of the ultracentrifuge. After prolonged standing, during which the temperature had at one time risen to approximately 20° for about an hour, the solution irradiated 2 hr. gave an absorption spectrum identical with that of the solution irradiated 2 hr. at 30° . It appears that solutions of pH 7.4 irradiated at 0° tend very readily to assume the same state, with respect to both aggregation and light absorption, as those irradiated at 30° . It would thus seem, too, that the preliminary heating to 56° of the albumin solutions in Series A and B did not affect the reactions caused by the u.v. light.

Pedersen [1931] found that an irradiated solution of serum albumin of pH 3.5 gave a sedimentation constant about twice that of native albumin, presumably about 9. The apparent discrepancy with the present results can most probably be explained either by the difference in pH or by the greater boundary-resolving power of the refractive index method as compared with the light absorption method used by Pedersen, especially in the presence of non-centrifugible material of high light absorption. It is possible that in his experiments the unaltered albumin did not appear as a separate boundary, but was completely merged with the aggregation products, so that the measured sedimentation constant represented an average for the mixture as a whole.

The characteristic odour which has been mentioned by several investigators [Pedersen, 1931; Stedman & Mendel, 1926] was absent from our solutions. We are inclined to attribute this to the precaution of rotating the irradiation tube, which ensured more equal exposure of all parts of the solution, and prevented the accumulation of degradation products of high light absorption in the layers nearest to the source of illumination.

To determine whether rotation of the albumin solution caused any surface denaturation contributing to the observed results, especially in the solutions which had been heated to 56° , and which might, therefore, be more susceptible to denaturation, some of the solution used for Series C was heated to 56° for 25 min., rotated without irradiation for 3 hr. at 26 – 29° and ultracentrifuged at 25° . The sedimentation curves were similar in appearance and symmetry to those given by the untreated albumin, and showed no evidence of the aggregation which might be expected to result from denaturation. There was a drop in the sedimentation constant, beyond the limits of experimental error, from 4.6 to 4.2, and a drop in spreading coefficient, D , from 6.6 to 4.7 (see Table I), but further experiments showed that heating was the cause, not rotation. A sample of the untreated solution ultracentrifuged at a cell temperature of 25 – 26° gave the same

low constants, $s=4.2$, $D=4.7$; while a further sample centrifuged at 20–22° gave higher values, $s=4.5$, $D=6.0$, approximately those originally observed. The conclusion was drawn, therefore, that in the irradiation experiments the results were not affected by the rotation, and were due solely to the u.v. light.

The sedimentation constant of human serum albumin appears to be definitely lower at pH 7.4 than in the region of the isoelectric point, as can be seen by comparison of Series B with the unirradiated solutions of Series A (Table I). In the solution used for Series C, the change to pH 7.4 from the isoelectric point was made at a temperature which certainly did not exceed 15°; the solution was subsequently kept frozen and thawed only for removal of samples. In all the solutions of this series, whether irradiated or not, the temperature of which had never risen above 22°, the sedimentation constant was that of isoelectric serum albumin, $s=4.5$ – 4.6 , also observed in Series A, instead of the lower value which would be expected from the increase in pH; for the unirradiated solutions of this group, the spreading coefficients, too, were the same as in Series A, $D=6.0$ – 6.6 . The lower values appeared only in the two samples which had been heated to 56° and 25–26° respectively. It is believed, therefore, that whatever the nature of the change producing these differences, it must occur at a negligible rate below 22–23°, but rapidly at 25° and above. In view of the very high temperature coefficients of many protein reactions, such a hypothesis is not theoretically unreasonable.

At pH 9.5 the molecular weight of lactoglobulin is the same as at the isoelectric point, but both the sedimentation constant and diffusion constant are lower by about 6% [Lamm & Polson, 1936]; this difference has been attributed to a change in the shape of the molecule, resulting in a higher frictional constant. Human serum albumin probably also acquires a higher frictional constant without change in molecular weight when brought to pH 7.4 from the isoelectric point, either because of a change in shape or because of a change in the degree of hydration.

While the values for the sedimentation constant and spreading coefficient for Series A are in good agreement with those of the unheated solutions in Series C, those of Series B, instead of agreeing with those of the heated solutions in Series C, lie intermediate between them and the isoelectric values. A possible reason for this discrepancy may be the greater age of the stock solution used for Series B, but the more probable explanation is the greater concentration of the heated solutions in Series C. The diffusion constant of serum albumin near the isoelectric point is substantially independent of concentration up to 2% [Lamm & Polson, 1936]; but it is possible that at pH 7.4, where the frictional constant is higher, increased concentration may more definitely repress both sedimentation and diffusion.

SUMMARY

1. The effect of u.v. irradiation on 0.8% solutions of purified human serum albumin at pH 5.4 (1% NaCl) at about 30°, and at pH 7.4 (*M*/15 phosphate buffer) at 30 and 0°, was studied by means of sedimentation velocity and light absorption measurements.

2. Irradiation caused increased light absorption at both pH, general for wave-lengths longer than 2400 Å., but greatest at the protein band; the increase was greater at pH 5.4 than at pH 7.4. At pH 7.4, irradiation at 0° caused more increase for wave-lengths above 3000 Å., but less increase for wave-lengths below 3000 Å., than at 30°; after prolonged standing in the cold, the two gave identical spectra.

3. The results indicate that irradiation causes the liberation of substances of small molecular weight, and that it also leads to ill-defined photo-oxidation reactions which result in increased light absorption. It is possible that these reactions may involve both the albumin residues and the low-molecular cleavage products.

4. Though visible coagulation occurred only at pH 5.4, irradiation caused aggregation of the protein at both pH, and gradual reduction in the concentration of unchanged albumin. Aggregation was less pronounced at pH 7.4 than at pH 5.4, and a larger concentration of light-denatured molecules remained unchanged with respect to sedimentation constant. In the absence of a visible precipitate, irradiation led to the formation of successively heavier soluble aggregates, while when visible coagulation occurred the solution was impoverished with respect to soluble aggregates of large size.

5. A correlation was observed between increase of light absorption and decrease in concentration of unaggregated albumin in the irradiated solutions.

6. The sedimentation constant of the unaggregated protein in the irradiated solutions remained essentially that of native albumin.

7. Three hours' irradiation with soft X-rays (29,000 r.u.) caused no detectable change in the sedimentation behaviour or the absorption spectrum.

8. The sedimentation constant of serum albumin is lower at pH 7.4 than near the isoelectric point; the change (probably in the shape or degree of hydration of the molecule) which causes this difference, appears to be prevented at low temperatures and to occur rapidly only at temperatures above 23°.

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