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Intermolecular and Intramolecular Reactions of Human-Serum Albumin after its X-Irradiation in Aqueous Solution

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The work detailed below deals with changes found in the ultraviolet-absorption spectra of aqueous solutions of human-serum albumin after X-irradiation at concentrations where the action of the radiation has been shown to be entirely indirect (Rosen, Alexander & Brohult, 1957). This matter has been variously studied in the past 30 years (Spiegel-Adolf & Krumpel, 1927, 1929; review by Arnow, 1936; Barron & Finkelstein, 1952). The purpose of the present paper is threefold: to demonstrate the important part in the absorption spectra of irradiated solutions played by light scattered from aggregated protein; to show how pH affects the aggregation and consequent light-scattering; and from these results to show how irradiation produces both intermolecular and intramolecular effects on the protein, in a proportion varying with circumstances. Although it is known that serum albumin and other proteins in solution are aggregated by ionizing radiation (Carroll, Mitchell & Callanan, 1952; Barron & Finkelstein, 1952; Rosen *et al.* 1957), the fact that this leads to a greatly enhanced scattering of light has been mentioned only by Passynskii (1955) and by Pavlovskaya & Passynskii (1957). It has been considered, however, in a study of ultraviolet irradiation of proteins (Claesson, 1956).

MATERIALS AND METHODS

Chemical reagents. Freeze-dried human-serum albumin was obtained by courtesy of Dr M. E. Mackay from a pharmaceutical preparation made at the Lister Institute, London; it was pure on examination by electrophoresis and by ultracentrifuge. Water used was redistilled in an all-glass apparatus. Other reagents were of the highest purity available commercially. All glassware before use was

washed in chromic acid, very thoroughly rinsed with tap water and distilled water and then oven-dried. All solutions were prepared on the day of use and were irradiated while open to the air. The concentrations indicated below are with respect to the protein as supplied. The moisture content of the material was 6-8%, but absolute determinations of this figure and of the final concentrations of protein were not attempted.

Radiation source and its calibration. The X-ray machine used was a Newton KX 10 run at 140 kv and 5 ma with no added filtration (half-value layer 2.86 mm. aluminium), run to give a dose rate between 3000 and 3500 rads/min. (The rad is the unit of absorbed dose, 100 ergs/g.) The output of the machine was calibrated chemically, by the oxidation of ferrous sulphate solution, with the solution occupying the same position and volume as the protein solution irradiated later. The *G* value (number of molecules changed per 100 ev of energy deposited by the radiation in the solution) was taken as 15.6 (Lefort, 1958).

Spectrophotometry and light-scattering. Absorption spectra were measured on a Unicam SP. 500 spectrophotometer in 1 cm. quartz cells. The relation between the weight-average molecular weight, M_w , and the light-scattering effect in the transmitted light is given by a modification, due to Debye and Cachin, of the standard Rayleigh formula (see Stacey, 1956). At a concentration c (g./ml.), the turbidity τ (that part of the absorption coefficient due solely to light-scattering) is given by

$$\frac{H_0}{\tau} = \frac{1}{M_w} \cdot \frac{1}{T},$$

where

$$H = \frac{32\pi^2 n_0^2 (dn/dc)^2}{3\lambda^4 N}$$

and

$$\frac{1}{T} = 1 + \frac{16\pi^2 r^2 n^2 n_0^2}{3\lambda^4}.$$

Here n_0 and n are the refractive indices of the solvent and solution, N is Avogadro's number, λ is the wavelength (in vacuo) of the light in which measurement is being made, and r is the radius of gyration of a molecule of the solute.

In the present work, the correction factor T did not depart significantly from unity. A direct measurement of light scattered was made with the apparatus described by Stacey (1956) at λ 4860 Å.

RESULTS

Irradiation of isoelectric solutions. For a 0.1% solution of salt-free human-serum albumin at its isoelectric point (pH 5.4) the ultraviolet-absorption spectrum is shown in Fig. 1 (a) (curve A). After irradiation (20 500 rads) the absorption spectrum was that shown in Fig. 1 (a) (curve B). From the two curves, the increase (Δ) in absorption at each wavelength was obtained, and Fig. 1 (b) gives a plot of $\lambda^4\Delta$. It is evident that, except for a slight irregularity at 295–300 m μ , the value of $\lambda^4\Delta$ is constant over the range covered. Such a result is strongly indicative of Rayleigh scattering of the light by aggregated protein. To test this point, direct measurements were made of the light scattered by the protein solutions. Because the

irradiated solutions contained a small amount of material of very high molecular weight, the errors of measurement were particularly large. However, within the limits of the standard error, the average molecular weight from direct measurement of light-scattering and from interpretation of the change in absorption spectrum showed agreement: a typical result is given in Table 1. Previous examination by ultracentrifuge had shown (Rosen *et al.* 1957) that the dose given would have caused about 47% of the protein to aggregate into complexes of two, three, four, etc., protein molecules. The present measurements of average molecular weights gave results entirely compatible with the earlier ultracentrifuge measurements.

Hence it was taken as a working rule that, when the quantity $\lambda^4\Delta$ was constant (within the range of experimental error of up to 15%) over all or nearly all the available wavelength range, then it could be taken as an indication of aggregation of the protein molecules and as a measure of average molecular weight, provided that there was general

Fig. 1. Change in ultraviolet-absorption spectrum of 0.1% salt-free isoelectric solution of human-serum albumin after X-irradiation. (a) Curve A: spectrum before irradiation; curve B: spectrum after a dose of 20 500 rads of 140 kv X-rays. (b) Analysis for Rayleigh scattering where Δ is the difference in extinction between curves A and B at wavelength λ . The ordinate is in units of $10^{-8} \times (\text{wavelength in m}\mu)^4 \times (\text{measured change in extinction})$.

Table 1. Increase of weight-average molecular weight of human-serum albumin when X-irradiated in aqueous solution

Concn. (%)	pH	Dose of X-rays (rads)	Molecular wt.	
			By light-scattering	By u.v. absorption
0.1	5.4	—	94 000 $\pm$ 2 900	70 000 (assumed)*
		20 000	250 000 $\pm$ 50 000	308 000 $\pm$ 29 000
0.1	3.6	—	78 500 $\pm$ 7 100	—
		40 000	170 000 $\pm$ 30 000	—

* The amount of scattering was too small for the molecular weight to be determined by the appearance of the u.v.-absorption spectrum, but the acknowledged molecular weight of the protein was assumed for computing the physical constants involved.

supporting evidence of aggregation from other methods, e.g. sedimentation or orthodox light-scattering measurements. It may be noted that if Δ is known to be produced solely by scattering in the treated solution, then for any particular amount of scattering material Δ will be independent of the concentration of non-scattering material in the original untreated solution. And specifically, a particular dose of radiation, acting indirectly and causing a determinable amount of protein to aggregate (Rosen *et al.* 1957) will lead to a value of Δ for each wavelength independent of the initial protein concentration.

As irradiation of the solution was continued, aggregation of the protein proceeded until the solution became cloudy and the protein was precipitated. In the later stages, the scattering of light was so great that very little passed through the solution. In ultraviolet light, the increase in apparent absorption was initially proportional to the dose, but it became more and more rapid at high doses.

Irradiation of non-isoelectric solutions. Solutions of the protein were brought to various values of pH by the addition of appropriate amounts of hydrochloric acid or sodium hydroxide solution. In Fig. 2 are shown graphs of $\lambda^4\Delta$ against λ (cf. Fig. 1*b*) for two solutions of human-serum albumin X-irradiated at values of pH away from the isoelectric point. In these circumstances Δ was no longer inversely proportional to λ^4 . There was thus no immediate indication of an effect of light-scattering, since Δ might have been entirely the product of a true change in absorption or might have been compounded partly of this and partly of a scattering component. There was, however, a maximum in the curve at $\sim 299 \text{ m}\mu$, as found with isoelectric solutions; yet although they had received a higher dose than the isoelectric solution illustrated in Fig. 1, both the solutions shown in

Fig. 2. Plot of $\lambda^4\Delta$ - λ (cf. legend to Fig. 1*b*) for 0.13% solutions of human-serum albumin at values of pH away from the isoelectric point and irradiated with a dose of 24 600 rads of 140 kv X-rays: O, pH 3.8; ●, pH 10.2.

Fig. 2 had substantially lower values of $\lambda^4\Delta$ and these values were not sufficiently constant with λ for an average to be determined.

But although Fig. 2 does not demonstrate that part of the increased extinction of the solutions was due to increased light-scattering, it does not preclude the possibility. Ultracentrifuge examination showed aggregation in irradiated non-isoelectric solutions similar to that in isoelectric ones (Rosen *et al.* 1957); and direct measurement of light-scattering showed an increase on irradiation from which average molecular weights could be calculated (Table 1). On moving away from the isoelectric point, the dose of radiation required to produce any particular amount of aggregation (determined by ultracentrifuge measurement of the

Fig. 3. The aggregation and precipitation of X-irradiated solutions of human-serum albumin. In each case the ordinate, F , is the factor by which the dose must be increased over that dose required at the isoelectric point to produce a given amount of aggregation or precipitation. (a) Abscissa: pH. (b) Abscissa: n , the number of positive or negative charges on protein molecules, determined separately from the titration curve. (c) Abscissa: n^2 . O, Determined from sedimentation diagram; ●, determined from the onset of visible precipitation. The arrows indicate points lying outside the scales.

amount of material sedimenting normally) was greater by a factor F than the dose required at the isoelectric point. The same was true of the dose required to produce precipitation (which occurred quite suddenly at a dose determinable with an accuracy of about 5%). The factor F was found to be proportional to the square of the molecular charge, as calculated from the acid-base titration curve in a separate measurement (Fig. 3), and hence proportional to the collision frequency of the protein molecules.

Addition of inorganic salts (NaCl , Na_2HPO_4 – NaH_2PO_4 mixtures, and NaF) in up to half-molar concentrations enhanced the effect of changing the pH.

Thus irradiation of non-isoelectric solutions caused aggregation or intermolecular reaction (revealed by ultracentrifuge examination and light-scattering measurements) to an extent depending on the pH; and also a considerably greater intramolecular change (revealed by changes in absorption spectrum) than occurred in irradiated isoelectric solutions.

Effect of change of pH after irradiation. Addition of hydrochloric acid to an irradiated solution after irradiation to lower the pH to between 2.0 and 2.5 had a profound effect within an hour. Firstly, it dispersed the aggregates formed on irradiation. This could be seen by eye in solutions irradiated heavily enough for a precipitate to have appeared. In less heavily irradiated solutions the same effect could be seen on examination in the ultracentrifuge.

The effect of breaking down the aggregates could also be seen in the decrease of light scattered and the consequent change in the ultraviolet-absorption spectrum. This is shown in Fig. 4.

Fig. 4. (a) Plot of $\lambda^4\Delta$ – λ (cf. legend to Fig. 1b) for a 0.13% solution of human-serum albumin at pH 5.4 irradiated with 15 000 rads of 140 kv X-rays and then diluted (1:1) with water (curve A) and 0.01N-HCl (curve B). For computing curve B, Δ was taken with respect to the unirradiated solution similarly diluted with acid. (b) Plot of $\lambda^4\Delta$ – λ for a 0.13% solution of human-serum albumin at pH 3.9, treated otherwise as the solution in (a). (No measurements were made between 285 and 300 $m\mu$, but the dotted part of curve A has been drawn to accord with Fig. 1b.)

A solution irradiated at the isoelectric point had a change in extinction almost entirely due to Rayleigh scattering (as shown by the $\lambda^4\Delta$ plot in Fig. 4a, curve A; compare Fig. 1). After the pH had been lowered to 2.35 by the addition of hydrochloric acid, the change in extinction was greatly reduced and was of a different character (Fig. 4a, curve B) resembling the change produced when a solution was irradiated away from the isoelectric point; the plot of $\lambda^4\Delta$ against λ shows a sinusosity which did not exist at the isoelectric point. Hence, apart from the breakdown of the aggregates, some further change took place (compared with a similarly treated control on which the addition of hydrochloric acid had no effect) which affected the absorbing groups of the protein.

When a slightly acidified solution (pH 3.90) was irradiated, the resulting plot (Fig. 4b, curve C) of $\lambda^4\Delta$ against λ is similar to that of the solution irradiated at the isoelectric point and then acidified. However, it is rather flatter, and also the presence of some aggregated protein, known to exist from other studies (see the preceding section), can be inferred from the height of the curve above the base line. When this solution was further acidified (to pH 2.35) it yielded a plot of $\lambda^4\Delta$ (Fig. 4b, curve D) which shows a reduced change in extinction (compared with a similarly treated control) but an enhanced internal change as revealed by the sinusosity of the curve.

The final acidified solutions of the isoelectric and non-isoelectric preparations (curves B and D) suffered, within experimental error, equal amounts of internal intramolecular change. A small parallel upward displacement of curve B relative to curve D suggests that a residue of aggregated protein was left in the solution which was initially at the isoelectric point.

Addition of alkali to the solutions after irradiation produced effects similar to the addition of acid.

Fig. 5. Variation of extinction at 278 $m\mu$ with time after addition of acid to irradiated solutions of human-serum albumin. The original solution was 0.2% of human-serum albumin at pH 5.4 and after irradiation it was diluted (1:1) with 0.01N-HCl. $\circ$, Unirradiated; $\square$, 56 000 rads; $\triangle$, 84 000 rads.

But interpretation of the absorption spectra in these conditions was complicated and made uncertain by the change in extinction coefficient of the aromatic amino acids with ionization at high pH. Back-titration from extreme values of pH towards the isoelectric point did not allow reformation of the aggregated protein nor did it remove the changes in absorption spectra ascribed to intramolecular changes.

The time taken for the changes caused by the addition of acid to an irradiated solution to manifest themselves fully was about half an hour (Fig. 5). The corresponding changes produced by the addition of alkali took somewhat longer to complete (several hours).

Addition of urea after irradiation. Addition of urea to irradiated solutions of human-serum albumin to give a final concentration of 4–6M-urea, and left standing for 2–15 hr., failed to disperse precipitated protein and caused no change in the sedimentation diagram of solutions examined in the ultracentrifuge. Also, within the limits of experimental error, there was no change in the ultraviolet-absorption spectrum when a solution of urea and irradiated protein (measured with respect to a solution of urea and unirradiated protein) was compared with irradiated protein without urea (measured with respect to unirradiated protein without urea).

DISCUSSION

The action of ionizing radiations on serum albumin in dilute aqueous solutions is effected by free radicals produced in the radiolysis of water (Barron & Finkelstein, 1952; Rosen *et al.* 1957). The results presented here show that the reaction between the free radicals and a protein molecule can lead either to a partly reversible intermolecular aggregation of molecules, or to an irreversible intramolecular change in the reacting protein. The extent to which the intermolecular and intramolecular effects separately occur depends on the pH and ionic strength of the solution, and the occurrence of the intermolecular effect appears to be determined by the collision frequency of protein molecules in the solution.

The situation can be explained by the hypothesis that the effect produced by X-rays on proteins in solution is a two-stage process. The first stage is the reaction between the protein molecule and a free radical formed in the radiolysis of the solvent, this yielding a reactive state of the protein molecule. The second stage is the reaction of this compound either inter- or intra-molecularly. It must be supposed that when both paths of reaction are possible, the intermolecular reaction is much the more probable at the concentrations used in the present study; this reaction, however, can take

place only during the moment of contact in a collision. When the collision frequency is reduced (by charged molecules repelling one another), the intramolecular reaction becomes relatively more probable. A similar hypothesis of a two-stage mechanism has been put forward since the present study was completed, by Eidus, Kondakova & Otarova (1958), to explain the effects of oxygen in the irradiation of solutions of myosin.

The absorption spectra give very little indication of the chemical nature of either the intermediate or final products of the denatured protein. Barron & Finkelstein (1952) suggested that the changes at wavelengths greater than 240 m μ in the spectrum of serum albumin after irradiation were entirely due to oxidation of tyrosine, giving dihydroxyphenylalanine. The present results show that this is not the case and even the small part of the increase in absorption which is due to an intramolecular change may not be due to a change in tyrosine. The difference between the absorption spectra of tyrosine and either dihydroxyphenylalanine or irradiated tyrosine shows a strong sharp peak at 285 m μ (D. Rosen, unpublished work), whereas in the irradiated protein a small peak, revealed only by mathematical manipulation, occurs some distance away at 295–300 m μ .

SUMMARY

1. The effect of 140 kv X-radiation on aqueous solutions of human-serum albumin has been studied by ultraviolet-absorption spectroscopy, supplemented by light-scattering measurements and ultracentrifuge examination.
2. It is shown that, depending on the conditions of pH and ionic strength, the radiation can produce either or both of two effects: a partly reversible intermolecular effect of aggregation, and an irreversible intramolecular effect which can be detected spectroscopically.
3. The intermolecular aggregation causes light-scattering, which affects the absorption coefficient in spectroscopic measurements. A method of correcting for this is used which, in isoelectric solutions where the radiation effect is almost entirely intermolecular, yields a value of the average molecular weight in quantitative agreement with direct light-scattering measurement.
4. A two-stage process is proposed for the changes occurring in proteins in these conditions.

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Thetin-Homocysteine Transmethylase

THE DISTRIBUTION OF THE ENZYME, STUDIED WITH THE AID OF TRIMETHYLSULPHONIUM CHLORIDE AS SUBSTRATE

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Thetin-homocysteine transmethylase, which catalyses the transfer of a methyl group from dimethylthetin or dimethyl- β -propiothetin to homocysteine, was first identified in rat, pig and guinea-pig liver and kidney by Dubnoff & Borsook (1948*a, b*). Kensler & Langemann (1954) detected the transmethylase in the livers of a number of other mammalian species, including man, and it was subsequently found in horse liver (Durell & Anderson, 1956) and in pigeon liver (Sloane, Boggiano, Coulomb & Hutchings, 1956). Thoai & Robin (1956) also showed the presence of the enzyme in turtle and mullet liver and produced evidence for its occurrence in several invertebrates. According to Schlenk & DePalma (1956), the yeast *Torulopsis utilis* is unable to utilize the above-mentioned thetins as methyl donors for homocysteine and, presumably, does not possess the enzyme. Shapiro (1958) has recently obtained similar findings for *Aerobacter aerogenes*, *Saccharomyces cerevisiae* and some strains of *Escherichia coli*.

In assays of thetin-homocysteine transmethylase carried out previously by other workers, the activity of the enzyme has been expressed in terms of the amount of methionine formed in a given time, the product being determined colorimetrically. It is not possible to compare these published findings on a quantitative basis, since in most cases optimum substrate concentrations were not

employed, and no attention appears to have been paid to whether methionine formation was linear with respect to time or whether the experimental conditions chosen gave a linear relationship between enzyme activity and amount of tissue preparation.

In this paper a manometric method has been used for the study of the transmethylase. This method measures enzyme activity in terms of the rate of carbon dioxide formation from a bicarbonate medium, since the methylation reaction involves the formation of acid (Maw, 1955). With either dimethylthetin chloride or dimethyl- β -propiothetin chloride as methyl donor, it was found that neither the reaction rates nor activity/concentration relationships obtained with rat-liver thetin-homocysteine transmethylase were linear (Maw, 1956). However, it was shown (Maw, 1958) that trimethylsulphonium chloride acts as a methyl donor for the enzyme and, with this compound as substrate, reaction rates as well as activity/concentration relationships were linear. Trimethylsulphonium chloride was therefore suggested as a possible substrate for the assay of the transmethylase.

Further evidence is now presented that trimethylsulphonium chloride can act as a substrate for the enzyme and can be used in its assay. This compound has been employed in a manometric study of the transmethylase in various tissues of the rat, mouse and guinea pig, and in a survey of its occurrence in twenty-four species of plant and

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