The Dissociation of Histone from Deoxyribonucleohistone by γ -Irradiation

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1. Experiments were carried out to determine the extent of dissociation of histone from deoxyribonucleohistone as a result of irradiation with γ -rays from ^{60}Co . 2. The bulk of the nucleohistone was removed from the irradiated solutions either by sedimentation or by precipitation with dilute sodium chloride solution. The supernatants were then analysed for DNA and histone. 3. The ratios of histone to DNA in these supernatants were less than for the original nucleohistone. This indicated that histone was dissociated by the irradiation, and then aggregated either with itself or with other nucleohistone molecules, and so was removed with the bulk of the nucleohistone during sedimentation or precipitation.

Measurements of the changes in molecular weight and radius of gyration of thymus DNH* after irradiation with γ -rays have indicated that the histone dissociates from the DNA core of the complex as a result of the indirect action of the γ -rays (Peacocke & Preston, 1961; Lloyd & Peacocke, 1963, 1966). This dissociation was also suggested by studies on the priming of RNA polymerase by irradiated DNH (Weiss & Wheeler, 1964; Lloyd, Nicholson & Peacocke, 1967) and by studies on the binding of Ca^{2+} to DNH irradiated with β -rays (Yoshii & Tchoe, 1966). The present paper reports experiments designed to provide further evidence for this dissociation of an analytical nature and to obtain a quantitative measure of it. While this work was in progress, an account of experiments with a similar purpose was published by Robinson, Weiss & Wheeler (1966).

The experiments were designed to separate the dissociated histone from the undissociated DNH by gel filtration on Bio-Gel P-200, chromatography on CM-cellulose, fractional sedimentation, or precipitation of the nucleohistone with sodium chloride, and to detect free histone in the irradiated solutions with 8-anilino-naphthalene-1-sulphonic acid (Shepherd & Nowland, 1965), or by ultraviolet spectrophotometry; and to observe by electrophoresis any change in the net charge on the irradiated nucleohistone that might have resulted from the loss of histone.

* Abbreviation: DNH, original complex of DNA and histone in the proportions as isolated by the method of Zubay & Doty (1959); 'nucleohistone' refers to any such complex, including that produced by irradiation and containing a smaller proportion of histone.

EXPERIMENTAL AND RESULTS

Solutions of DNH in 0.7M-potassium phosphate buffer, pH 6.8, were prepared from calf thymus by the method of Zubay & Doty (1959), and irradiated at a concentration of 0.6g./l. with γ -rays from a ^{60}Co source, as described previously (Lloyd & Peacocke, 1966).

Since histone is a very much smaller molecule than DNA or DNH (mol.wt. about 2×10^4 – 4×10^4 compared with about 10^7 for DNA and 2×10^7 for DNH), it should be possible to separate free histone from DNA or nucleohistone either by fractional sedimentation or by gel filtration. Histone also carries a large positive charge, whereas DNA and DNH are negatively charged. Hence histone is retained by a column of CM-cellulose (whereas DNA and DNH pass through), from which it may be eluted with 0.5M-KCl solution (Robinson *et al.* 1966). Also, if histone were partially removed from DNH the nucleohistone would carry a relatively higher negative charge, and so might be expected to show an increased electrophoretic mobility.

Samples (5 ml.) of irradiated solutions were passed through a 6 cm. column of CM-cellulose and through an 8 cm. column of Bio-Gel P-200. The emergent fractions that contained DNA were tested for loss of histone by measuring the E_{260}/E_{230} ratio, and later fractions were tested for histone by measuring E_{210} . After chromatography on CM-cellulose, E_{210} was measured of fractions obtained by washing the column with 0.5M-KCl solution. No free histone was detected by means of either column after doses up to 16ev/P (83.7 krad).

The free-boundary electrophoresis experiments were made in a Strubin Fokal F electrophoresis apparatus. These experiments were very difficult in practice because of the low concentrations of the solutions and the very low conductivity of the solvent (0.7M-phosphate buffer, pH 6.8). It soon became clear that any change in the electrophoretic mobility of the nucleohistone was being masked by the errors involved in its determination.

The use of 8-anilino-naphthalene-1-sulphonic acid enables

free histone in solution to be detected and estimated at concentrations as low as $2\mu\text{g./ml.}$ 8-Anilidonaphthalene-1-sulphonic acid does not bind to DNA nor does it react with intact DNH, which should make it ideal for the detection of small quantities of free histone in the presence of DNH. With this reagent no free histone was detected in an irradiated solution (5ev/P), nor in the supernatant obtained by centrifuging this solution in a partition cell of the analytical ultracentrifuge for such a time that all material absorbing u.v. light at $260\text{m}\mu$ was below the partition. This observation is not inconsistent with dissociation of histone if it was fractions F1 and F2b that had dissociated, since 8-anilidonaphthalene-1-sulphonic acid does not react with these fractions (Laurence, 1966). Nor is it inconsistent with dissociation of histone if the histone forms insoluble aggregates as a result of the irradiation, as will appear below.

Since the sedimentation coefficient of histone (about 1.7 s) is lower than that of DNH (about 7 s at this concentration), any free histone should remain in the supernatant if a solution of DNH is centrifuged for long enough to sediment all the DNH to the bottom of the tube. Samples of DNH solutions in 0.7 mm-phosphate buffer, both irradiated and unirradiated, were centrifuged at 35000 rev./min. in the $3 \times 5\text{ ml.}$ swing-bucket rotor of the MSE Superspeed-50 centrifuge for periods of 1, 2, 4, 8 and 16 hr. and the spectra of the supernatants were measured between 205 and $260\text{m}\mu$. During the first 4 hr. of sedimentation no spectral differences could be detected, and after 16 hr. of centrifugation insufficient DNH remained in the supernatant of the unirradiated solution for spectral measurements. The spectra of the supernatants of solutions irradiated with 0.5, 1.0 and 5 ev/P and then centrifuged for 8 hr., as above, are shown in Fig. 1(a). To show the changes in the spectrum more clearly, they have also been normalized as described in the next paragraph (Fig. 1b).

The extinction of the solution at each wavelength was divided by that at $260\text{m}\mu$ to eliminate differences due to differences in concentration alone. This 'corrected extinction' was then divided by the corresponding 'corrected extinction' for the unirradiated DNH that had been centrifuged in the same way as the irradiated solution. The resulting plot when this ratio (E') is plotted against wavelength (Fig. 1b) is a horizontal straight line at 1.0 for unirradiated DNH. Any supernatant solution that contained more protein per unit weight of DNA than did DNH would have yielded a normalized curve rising above unity at wavelengths below $240\text{m}\mu$; conversely a solution that contained less protein per unit weight of DNA than did the DNH (e.g. DNA) would have yielded a curve falling below unity at low wavelengths. It is evident from the curves in Fig. 1(b) that the supernatants from irradiated solutions, which had been centrifuged for 8 hr. at 35000 rev./min., actually contained less protein per unit weight of DNA than did unirradiated DNH. It is clear that the histone has dissociated from the DNH as a result of the γ -irradiation, and that this histone has not remained free in solution, but has sedimented with the bulk of the nucleohistone (this point is discussed more fully below). The fraction of nucleohistone (expressed as DNA concentration) remaining in the supernatant varied from 0.125 for the unirradiated DNH to 0.214 for the sample irradiated with 3 ev/P. Unfortunately the curves of Fig. 1(b) are inadequate to allow estimation of the amount of protein dissociated.

Fig. 1. (a) Spectra of DNH and of three irradiated samples after centrifugation for 8 hr. at 35000 rev./min. and of DNA in the same solvent (0.7 mm-phosphate buffer). The extinction values refer to the solutions taken from the centrifuge tubes as if measured in a 10 mm. cell. (b) Same spectra as in (a) but normalized as described in the text, to illustrate more clearly the relative changes. 1 (—), Unirradiated DNH; 2 (---), DNH irradiated with 0.5 ev/P; 3 (· · · · ·), DNH irradiated with 3.0 ev/P; 4 (— · — · —), DNH irradiated with 5.0 ev/P; 5 (· · · · ·), DNA.

Fig. 2. Normalized spectra of DNH (five irradiated samples) and of DNA in 0.15 M-NaCl solution. 1 (—), unirradiated DNH; 2 (---), DNH irradiated with 0.5 ev/P; 3 (· · · · ·), DNH irradiated with 1.0 ev/P; 4 (— · — · —), DNH irradiated with 2.0 ev/P; 5 (· · · · ·), DNH irradiated with 3.0 ev/P; 6 (— · — · —), DNH irradiated with 5.0 ev/P; 7 (· · · · ·), DNA.

Spectral shifts similar to those shown in Fig. 1 might result from conformational changes. However, these spectral shifts were not observed with the solutions immediately after irradiation, and when any consequent conformational change caused by γ -rays would already have occurred, but only after they had been subjected to prolonged centrifugation. This point is discussed below, but it seems that dissociation of histone from the DNH, followed by reassociation either with itself or with other nucleohistone molecules, is the only satisfactory explanation of these observations.

Since DNH shows a minimum solubility at an NaCl concentration of 0.15M, increase of ionic strength can be used to remove DNH from solution. The NaCl concentration of DNH solutions irradiated with various doses of

Fig. 3. Extent of dissociation (α) of histone from DNH (as deduced from the curves in Fig. 2), and the solubility (s) of DNH in 0.15M-NaCl solution, as a function of the radiation dose (R , ev/P). ●, α ; ▲, s .

Fig. 4. Normalized spectra of DNH, four irradiated samples, and of DNA, in 0.05M-NaCl solution. 1 (—), unirradiated DNH; 2 (---), DNH irradiated with 0.5 ev/P; 3 (.....), DNH irradiated with 1.0 ev/P; 4 (-.-.-), DNH irradiated with 2.0 ev/P; 5 (- - - -), DNH irradiated with 3.0 ev/P; 6 (.....), DNA.

γ -rays was raised to 0.15M and the precipitate removed by a light centrifugation (104000g, 10min. at 35000 rev./min. in the 3 × 5 ml. swing-out rotor of the MSE Superspeed 50 centrifuge). The spectra of the supernatants were measured and normalized with respect to the spectrum of unirradiated DNH in the same 0.15M-NaCl solution (Fig. 2). The same sequence of changes was observed with this more extended series of irradiated DNH and it is again concluded that histone had dissociated from the DNH to an extent which increased with the radiation dose, and that this histone was precipitated with the bulk of the nucleohistone. The apparent degree of dissociation (α) of the histone from the DNH may be estimated as $(1 - E')_{\text{DNH}} / (1 - E')_{\text{DNA}}$, where E' is the relative extinction of the solution after the normalization procedure. This ratio was found to be almost independent of wavelength between 205 and 240 mμ, and an average was taken of four measurements in this range. From the curves of Fig. 2 an apparent degree of dissociation of the histone from the DNH was calculated and is shown in Fig. 3, together with a curve representing the solubility of irradiated nucleohistone in 0.15M-NaCl solution. However, comparison of the spectrum of DNH in 0.15M-NaCl solution with that in 0.7M-phosphate buffer indicated that the former solvent caused some dissociation of histone even from unirradiated DNH (to an extent of about 5%). This can be deduced from the downward displacement of the curve for DNA in Fig. 2 with respect to the curve for DNA in Fig. 1(b). Since the DNA samples were identical, this means that the histone content of the solution with respect to whose spectrum the curve of Fig. 2 was normalized was greater than that of the solution with respect to whose spectrum the curve of Fig. 1(b) was normalized. This implies that in 0.15M-NaCl solution histone dissociated from the DNH and that this histone remained free in solution. If the fraction of histone dissociated in 0.15M-NaCl solution was independent of the dose of radiation given to the DNH solution, the values of α derived from the curves of Fig. 2 are valid. Application of the criterion above to the spectra of unirradiated DNH in 0.1M- and 0.05M-NaCl solutions indicated that these solvents did not cause any dissociation of histone from the DNH. Fig. 4 shows the normalized spectra of some irradiated DNH solutions in 0.05M-NaCl solution (after removal of the precipitated nucleohistone by

Table 1. Apparent degree of dissociation (α) of histone from DNH as a function of radiation dose (R)

The values in parentheses are the amounts of DNA left in the supernatants, expressed as percentages of the original DNA concentration.

By addition of NaCl to*	α		
	0.05 M	0.1 M	0.15 M
R (ev/P)			
0	— (4.9)	— (4.0)	— (0.9)
0.5	0.10 (6.2)	0.10 (5.8)	0.09 (1.1)
1.0	0.14 (6.4)	0.16 (5.9)	0.15 (1.8)
2.0	0.19 (8.7)	0.25 (10.6)	0.26 (1.5)
3.0	0.28 (10.9)	0.39 (9.2)	0.39 (3.1)
4.0	—	0.58 (8.7)	0.58 (3.4)

* Followed by centrifugation at 35000 rev./min. for 30 min. to remove the nucleohistone precipitate.

centrifugation at 104 000g for 30 min.). Very similar results were obtained from solutions in 0.1 M-NaCl solution. The apparent degree of dissociation of the histone was calculated for each NaCl concentration (Table 1).

DISCUSSION

It is well known that conformational changes in DNA can give rise to changes in its absorption spectrum. However, it is not possible to attribute the changes reported in Figs. 1, 2 and 4 to such conformational changes for the following reasons. At the maximum dose given to these solutions (5 ev/P, 29.3 krad) the molecular weight of the DNH had fallen to 10% of its original value (Lloyd & Peacocke, 1966). At this considerable extent of degradation all molecules present must be degraded, and hence any conformational change, of a kind to give rise to a change in optical properties, should be detectable in the solutions immediately after irradiation. In fact changes in the E_{260} of DNH solutions on irradiation are very small at these dose levels (Lloyd & Peacocke, 1966) and no change in the ratio of the extinction at any other wavelength to that at 260 m μ could be detected in the present experiments before prolonged centrifugation or precipitation by sodium chloride. This means that, whatever changes may have occurred during irradiation in the mode of interaction of histone and DNA or in their structures, these changes were not of a kind which altered their absorption between 210 and 260 m μ . Hence the differences in spectra observed (Figs. 1, 2 and 4) between the unfractionated irradiated material and the supernatants obtained after prolonged sedimentation or precipitation of the irradiated solutions represent a real discrimination that had occurred between products of the irradiation of the DNH. These different products became apparent only after their separation by centrifugation or precipitation. The spectral changes, especially at 210–230 m μ , indicate directly that the nucleohistone in the supernatant from irradiated DNH solutions is depleted of histone. Such nucleohistone has therefore formed as a result of the irradiation and its presence has been detected by differential sedimentation. There are two possible interpretations of this observation. First, the DNH molecules may contain, along their length, regions that are relatively free of histone (about 88% of the DNA phosphate groups are neutralized by the histone), and these regions may be attacked preferentially since DNA is more radiosensitive than DNH (Lloyd & Peacocke, 1966; Emmerson, Scholes, Thompson, Ward & Weiss, 1960). However, if this attack was sufficient to release fragments of DNA into solution, small enough to be left in the supernatant after prolonged centrifugation or precipitation of the nucleohistone

by salt, then the molecular weight and the radius of gyration of the nucleohistone should both fall together during irradiation. Indeed, because of the averaging processes involved in light-scattering measurements, the radius of gyration might be expected to fall with irradiation more steeply than the molecular weight. Exactly the opposite effect is in fact observed: the radius of gyration falls much more slowly than the molecular weight, especially at low doses (Lloyd & Peacocke, 1966). Thus this possible explanation of the sedimentation results presented here is quite inconsistent with our previous light-scattering measurements (Lloyd & Peacocke, 1966). Secondly, the only other process that would satisfactorily explain these results is that the histone molecules had dissociated from the DNH complex and had then reassociated in a random way either with themselves or with other nucleohistone molecules. These aggregates, containing more histone per unit weight of DNA than did the original DNH, were removed by centrifugation or precipitation, leaving in the supernatant nucleohistone molecules relatively deficient in histone. These molecules would have spectra similar to those shown in Figs. 1, 2 and 4.

These results, though not in exact quantitative agreement, therefore indicate consistently a loss of histone from the DNH, and that this increases with radiation dose. In every experiment in which the DNH was separated by centrifugation or by precipitation, the supernatant of the irradiated solution contained a smaller amount of histone relative to DNA than did the unirradiated solution similarly treated. Thus, although this work has not yielded an exact quantitative measure of the extent of dissociation of histone as a function of radiation dose, it has yielded analytical evidence that such dissociation does occur. Moreover, it shows that the dissociated histone does not remain free in solution but reassociates with the nucleohistone in such a way as to cause aggregation.

[This aggregation would not affect the interpretation of our earlier light-scattering results (Lloyd & Peacocke, 1966), since the aggregates would be removed by the clarification procedure (by centrifugation) before the light-scattering measurements.]

It has been suggested (Hagen, 1960) that irradiation does not actually cause dissociation of the histone from the DNH but 'weakens' the bond between the histone and the DNA, rendering the histone more easily removed by other means. Our results with salt solutions are obviously consistent with this view, but the results of fractional sedimentation definitely show loss of histone, though the separation was not sufficiently clear to enable quantitative estimation of the extent of dissociation to be made.

The results presented here may be compared

with those published by Robinson *et al.* (1966), who detected about 1.3% dissociation of histone ($\alpha=0.013$) after a dose of about 20 eV/P. Their technique, however, depended on a separation of free histone from the nucleohistone by sedimentation for 48 hr. at 70 000g, followed by a chromatographic separation and concentration of the histone from the remaining nucleohistone in the supernatants. Their results are therefore quite consistent with our failure to detect free histone in such supernatants, since aggregates containing extra dissociated histone have been removed in their experiments along with the nucleohistone by the sedimentation procedure they employed.

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