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A Quantitative Analysis of Histone H1 in Rabbit Thymus Nuclei

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The relative quantity of histone H1 in rabbit thymus whole histone was determined to be 17.2% (w/w). This implies that there is, on average, one histone H1 molecule per nucleosome.

Models of the structure of chromatin suggest that it is composed of repeating units, each unit or nucleosome consisting of about 200 base-pairs of DNA associated with an octamer of two each of the histones H2A, H2B, H3 and H4 (Kornberg, 1974; Thomas & Kornberg, 1975; Noll, 1974). No evidence has been found for an association between histone H1 and the histones of this octamer, and it is likely that histone H1 is bound to the DNA on the outside of the nucleosomes or in the portion of the DNA linking the nucleosomes (Baldwin *et al.*, 1975; Varshavsky *et al.*, 1976; Whitlock & Simpson, 1976; Noll & Kornberg, 1977).

One vital piece of information required to fit histone H1 into the existing chromatin models is the quantity of histone H1 relative to the other four histones. Although it is thought that there is probably one histone H1 molecule per nucleosome, a reliable value for the quantity of histone H1 in chromatin is lacking; values quoted in the literature vary widely (Johns, 1967; Panyim & Chalkley, 1969; Panyim *et al.*, 1971; Garrard *et al.*, 1974; Sonnenbichler & Zetl, 1975; Berdnikov & Gorel, 1975; Holmgren *et al.*, 1976), and this is probably due to the notorious susceptibility of histone H1 to proteolytic degradation. The fact that histone H1 could be covering a fairly large piece of internucleosomal DNA (up to 60 base-pairs) (Whitlock & Simpson, 1976; Noll & Kornberg, 1977) suggests that there could be more than one histone H1 molecule per nucleosome, and it is possible that, because of degradation, the quantity of histone H1 in chromatin has been underestimated. We have therefore carried out a re-examination of the quantity of histone H1 relative to the other four histones in nuclei.

Materials and Methods

Preparation of histone H1 and whole histone standards

Chromatin was prepared at 4°C from fresh rabbit thymus (approx. 15g) by blending the tissue with 500ml of 0.075M-NaCl/0.025M-EDTA (pH7.5)/1mM-phenylmethanesulphonyl fluoride/0.5% dimethyl sulphoxide in an MSE blender, centrifuging at 2000*g* for 20min, and then washing the chromatin twice more in a similar manner. Non-histone proteins were removed by three extractions with 0.35M-NaCl/1mM-phenylmethanesulphonyl fluoride/0.5% dimethyl sulphoxide, pH7. The chromatin was divided into two portions. Histone H1 was obtained from one portion by 5% (w/v) $HClO_4$ extraction (Sanders & Johns, 1974); whole histone was extracted from the other portion with 0.25M-HCl. After acetone precipitation and drying, the proteins were redissolved in water to give stock solutions, and the protein concentration in each was determined by amino acid analysis (see below).

Preparation of standard DNA

DNA was prepared by the method of Kay *et al.* (1952). A stock solution of the DNA (approx. 200μg/ml) in water was prepared and the concentration of DNA determined by phosphorus analysis (Jaenicke, 1974; Chen *et al.*, 1956) [the DNA had less than 1% RNA as determined by the orcinol method (Lin & Schjeide, 1969)].

Protein determination of standard solutions

Samples were hydrolysed for 24h with 6M-HCl at 110°C. After evaporation to dryness the hydrolysates were redissolved in loading buffer [0.04M-citric acid/0.16M-NaCl/0.35% (v/v) Brij/0.25% (v/v) thiodiglycol (2,2′-thiodiethanol)] containing norleucine internal standard (0.1μmol/ml) and analysed with a Rank–Hilger Chromospek analyser. A standard amino acid mixture (0.1μmol of each amino acid/ml, including norleucine) was analysed also and was used to calculate the quantity of each amino acid in the hydrolysate and hence the total weight of protein in the original solution. Each analysis was carried out in triplicate. The values thus obtained agreed to

within 10% with results obtained by nitrogen determination (Jaenicke, 1974).

DNA determination

A time-course study showed that to completely solubilize the DNA in nuclei (see below) a 1h hydrolysis with 5% (w/v) trichloroacetic acid at 70°C was required. The hydrolysates of the nuclear DNA and DNA standards prepared from the stock solution described above were then analysed by the indole method as described by Hubbard *et al.* (1970).

Quantitative analysis of histone H1 and whole histone in acid extracts of nuclei

Nuclei were prepared at 4°C from fresh rabbit thymus as follows. All solutions apart from the 0.25M-HCl extracting solution contained 0.5mM-phenylmethanesulphonyl fluoride, which was added fresh after first dissolving it at a concentration of 0.1M in dimethyl sulphoxide.

The tissue (4g) was blended in 600ml of 0.25M-sucrose/10mM-$MgCl_2$ and centrifuged at 1000*g* for 10min. The pellet was resuspended in 30ml of 0.25M-sucrose/10mM-$MgCl_2$ and mixed thoroughly with 6vol. of 2.4M-sucrose/10mM-$MgCl_2$. The nuclear pellet, obtained by centrifuging at 30000*g* for 1h, was washed three times with 10mM-$MgCl_2$. Samples of the nuclei were taken for DNA determination and the rest extracted three times with 15ml of 0.25M-HCl. The first extraction was overnight, the following two for 2h each.

The combined acid extract thus prepared was diluted with an equal volume of 9M-urea and samples were loaded in triplicate on 20% polyacrylamide gels (0.6cm×10cm) containing 0.9M-acetic acid (Johns, 1967). At the same time, various quantities of the standard histone H1 (2–10μg) and whole histone (5–30μg) from the stock solutions diluted with 9M-urea/0.1M-HCl were loaded on gels in duplicate. Electrophoresis was carried out at 180V for 3h. The gels were stained overnight with 0.2% (w/v) Procion Navy (I.C.I, Ltd. Organics Division, Manchester, U.K.) in 7% (v/v) acetic acid and destained with 40% (v/v) ethanol at 55°C.

The gels were scanned at 580nm with a Gilford 240 spectrophotometer equipped with a linear-transport device. The areas under the histone H1 and total whole histone peaks were obtained by weighing cut-outs of photocopies of the chart-recorder traces.

Results

One of the problems of trying to measure chromosomal proteins is the possibility of losses due to proteolytic degradation during the isolation of nuclei or chromatin. Histone H1 appears to be particularly susceptible to this, presumably because it is in a more exposed location in the chromatin. In calf thymus this degradation results in the appearance of extra bands of faster electrophoretic mobility than histone H1 in acid extracts (Kinkade & Cole, 1966), and also in the appearance of a basic protein, high-mobility-group protein 8, in 0.35M-NaCl extracts (G. H. Goodwin, J. M. Walker & E. W. Johns, unpublished work). Similarly, degradation of non-histone high-mobility-group protein 1 occurs in calf thymus. However, we have found that rabbit thymus does not appear to have these high concentrations of proteolytic enzymes (as shown by the lack of these extra bands in acid and 0.35M-NaCl extracts) (Goodwin *et al.*, 1977) and it was for this reason that rabbit thymus was used in the present study. In addition to using fresh tissue for each experiment, the proteolytic inhibitor phenylmethanesulphonyl fluoride was used throughout.

Nuclei were isolated and the histones extracted with acid. Instead of recovering the histones by acetone precipitation (which may result in differential losses of histones), the acid extracts were loaded directly on the polyacrylamide gels for the quantitative analysis of histone H1 and whole histone. After the electrophoresis of the acid extracts together with known amounts of the standard histone H1 and whole histone, the gels were stained with Procion Navy, a dye that binds to and fixes protein very strongly in polyacrylamide gels. Fig. 1 shows that the relationship between the amount of standard histone H1 and whole histone loaded and dye bound is linear in the range used. From these two graphs the amount of histone H1 and whole histone in the acid nuclear extracts could be calculated. The results obtained for three different nuclear preparations are given in Table 1. The values agree fairly well with one another and the overall average value is 17.2% for the percentage by weight of histone H1 in whole histone.

A histone H1:DNA ratio was also obtained for one nuclear preparation, and from this it was calculated that there are, on average, 1.3 histone H1 molecules per 200 base-pairs of DNA.

Apart from proteolytic losses it is possible that histone H1 could be lost from the chromatin as a result of endogenous nuclease digestion during the nuclear isolation. To check this, chromatin was isolated in the presence of EDTA. Fresh rabbit thymus was blended six times with 0.075M-NaCl/0.025M-EDTA, pH7.5, containing 0.8mM-phenylmethanesulphonyl fluoride, the chromatin extracted with 0.25M-HCl, and the histones were analysed by polyacrylamide-gel electrophoresis. The histone H1:whole histone ratio for this preparation was identical with that of histones prepared from nuclei as described in the Materials and Methods section.

A check was also made that 0.25M-HCl does in

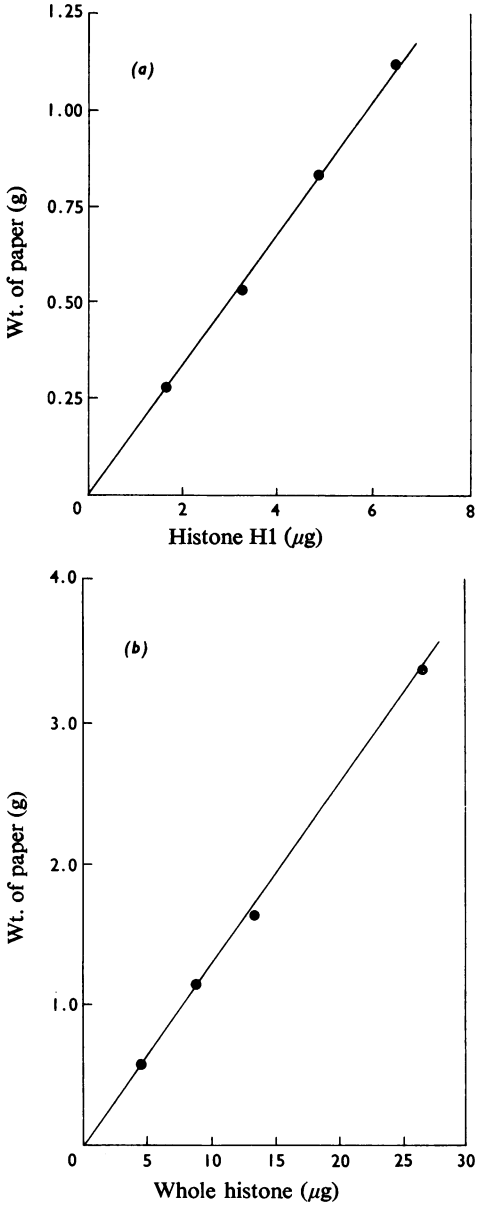

Fig. 1. *Procion Navy stain uptake by (a) histone H1 and (b) whole histone standards*

The abscissae indicate the amounts of standard histone H1 and whole histone loaded (in duplicate) on polyacrylamide gels. The ordinates give the amounts of stain absorbed as measured by weighing cut-outs from the 580nm scans of the gels. Each point is the mean of the duplicates.

fact extract total histone quantitatively. The sediment remaining after 0.25M-HCl extraction of nuclei (containing about 20% protein by weight) was dissolved in sodium dodecyl sulphate and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weintraub *et al.*, 1975). Most of the protein was high-molecular-weight non-histone protein, but two bands had the same mobility as histones H4 and H3. However, these two bands accounted for only approx. 1% of the total nuclear histone. Thus it appears that the acid extraction method used removes whole histone almost quantitatively.

Table 1. *Percentage by weight of histone H1 in whole histone*

The acid extracts of three different preparations of thymus nuclei were loaded on polyacrylamide gels, each in triplicate. After staining and scanning, the mean peak areas of histone H1 and whole histone for each preparation were obtained and by using the standard graphs (Fig. 1) the quantities of histone H1 and whole histone in each preparation were determined. From these two quantities the percentages by weight of histone H1 in whole histone were obtained. In addition, one preparation (preparation 3) was analysed three times by the method described.

Preparation no.	Percentage by weight of histone H1 in whole histone
1	17.2
2	16.8
3	16.7, 17.2, 18.3

Since globin migrates in approximately the same position as histone H1 on gels containing acetic acid (Johns, 1971), contamination of nuclear extracts with this protein could give erroneously high values for the quantity of histone H1. Globin is not soluble in 5% (w/v) $HClO_4$, and it was therefore possible to verify that this protein was not present in the acid extracts by precipitating all the protein except histone H1 with 5% (w/v) $HClO_4$, washing the precipitate thoroughly with 5% (w/v) $HClO_4$, and re-running the histones (minus histone H1) on polyacrylamide gels; no protein migrated at the histone H1 position.

Discussion

Several studies have been carried out to quantify the histone fractions, and values ranging from 10.5% to 23% for the quantity of histone H1 in whole histone have been quoted (Johns, 1967; Panyim & Chalkley, 1969; Panyim *et al.*, 1971; Garrard *et al.*, 1974; Sonnenbichler & Zetl, 1975; Berdnikov & Gorel, 1975; Holmgren *et al.*, 1976). This variation could be due to a genuine difference in the amount of histone H1 in the different tissues studied, but it could also be due to variable proteolytic degradation of histone H1. Other factors such as the failure to account for differential dye uptake by the different histones and inaccuracies in measuring protein standards could also account for this variability. In the present study, proteolytic degradation

has been minimized by using phenylmethanesulphonyl fluoride and a tissue, rabbit thymus, shows little histone degradation. Care has also been take to measure protein accurately in the standards by amino acid analysis. The value obtained in the present study for the quantity of histone H1 in rabbit thymus whole histone (17.2%) agrees with the value originally obtained by Johns (1967) for calf thymus (17%) and with the values obtained by Holmgren *et al.* (1976) for *Drosophila* histone H1 (16.3–19.7%).

The four core histones H2A, H2B, H3 and H4 are present in equimolar quantities in chromatin (Olins *et al.*, 1976; Joffe *et al.*, 1977), and each nucleosome contains two each of these histones, forming an octamer. From the molecular weights of the histones (Spelsberg, 1974) it is calculated that the mol.wt. of the octamer is 109400. If 17.2% of the total histone in chromatin is histone H1 (mol.wt. 21000) then there are 1.1 molecules of histone H1 per octamer or nucleosome, assuming that each nucleosome has the same quantity of histone H1. A somewhat higher value is obtained from our DNA measurements, 1.3 histone H1 molecules per 200 base-pairs of DNA. However, measurements of DNA in crude extracts are never very accurate for the reasons fully discussed by Munro & Fleck (1966), and therefore the latter value is less reliable and is only useful as a rough check on the former value. The present study thus confirms that there is probably only one histone H1 molecule per nucleosome.

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