Instability of Rat Liver Chromatin and other Nuclear Non-Histone Proteins in Alkaline Solution

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Non-histone proteins from rat liver nuclei and chromatin were shown to be hydrolysed in 0.1 M or 1 M-NaOH solutions both at 4° and 18°C; 24 h in 1 M-NaOH at 18°C is sufficient to break down approx. 77% of these proteins to low-molecular-weight peptides. Loss of protein material banding in the region of pH 5.5-8.0 has been demonstrated by isoelectric focusing in polyacrylamide gels, and fine high-molecular-weight bands are no longer visible on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The results indicate that care must be taken when analysing non-histone-protein fractions to avoid exposure to alkaline pH conditions.

After the rediscovery of the non-histone or nuclear 'acidic' proteins by the Stedmans in the 1940s there was little progress in the fractionation of this important group of cellular proteins until the early 1970s (see Elgin & Bonner, 1970; Gronow & Griffiths, 1971; MacGillivray *et al.*, 1972), difficulties being mainly due to solubility problems. Although these problems are now being resolved, in the past often the only means of solubilizing the non-histone proteins tightly bound to the DNA, the so-called residual portion, characterized by a rapid turnover, was to dissolve it in dilute NaOH (e.g. see Busch, 1965). We have noted that, although NaOH in high concentration (2-4 M) at 100°C is known to hydrolyse proteins to amino acids, the effect of lower molarities at lower temperatures does not seem to have been published.

Some workers have reported on the dissociation of histones from DNA at alkaline pH (Matsuyama *et al.*, 1971; Ramm *et al.*, 1972; Ohba & Hayashi, 1972; Russev *et al.*, 1974). Murphy & Bonner (1975) and Russev *et al.* (1975) have used alkaline conditions for the selective dissociation of non-histone proteins from chromatin. During our investigations on the chemical and physical properties of the nuclear proteins of rat liver (and other tissues) we observed considerable hydrolysis in dilute NaOH even with fairly short exposure times and at 4°C. This effect was first noticed during sodium dodecyl sulphate-electrophoresis studies on whole nuclear and chromatin non-histone-protein fractions.

Materials and Methods

Rat liver nuclei were isolated by a modification of the Chauveau procedure (Chauveau *et al.*, 1956), and from these chromatin was prepared by extraction

with 0.14 M-NaCl/0.05 M-Tris/[HCl/0.05 M-EDTA, pH 7.5. Non-histone proteins were extracted by using the 8 M-urea/0.05 M-sodium phosphate, pH 7.6, technique (Gronow & Griffiths, 1971), usually also including a thiol-blocking reagent such as *N*-ethyl-maleimide. The protein mixture obtained was either used directly or passed through a Bio-Gel P4 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column to remove low-molecular-weight material together with some RNA.

In an initial simple experiment rat liver whole nuclear non-histone proteins were precipitated with 15% (w/v) trichloroacetic acid, washed with ethanol/diethyl ether (1:1, v/v) and then with diethyl ether, and dried. Samples were dissolved in NaOH (5 mg of protein/ml) under different conditions and then the trichloroacetic acid precipitation step was repeated. The percentage difference by weight after various treatments, including a ribonuclease digest (pancreatic ribonuclease, EC 2.7.7.16; Worthington Biochemical Corp., Freehold, NJ, U.S.A.), was calculated and the results are shown in Table 1. Control samples treated with sodium phosphate buffer, pH 7.0 or 7.6, did not show any decrease in trichloroacetic acid-precipitable material.

For Sephadex chromatography the whole nuclear or chromatin non-histone protein was dissolved in NaOH (5 mg of protein/ml). Samples were kept for 24 h at 4° or 18°C, neutralized with HCl and freeze-dried. They were dissolved in 8 M-urea/0.05 M-sodium phosphate, pH 7.6 (with warming when necessary) (6.6 mg of protein/ml), applied to a column (25 cm × 2 cm) of Sephadex G-50 and the column was eluted with the same buffer. Fractions (3 ml; 88 drops) were collected and the protein contents of the fractions containing material in the exclusion volume of the column were determined by the Folin-Lowry method (Lowry *et al.*, 1951).

Table 1. *Effect of various NaOH treatments on rat liver non-histone protein precipitated with 15% (w/v) trichloroacetic acid*

Rat liver non-histone proteins were extracted from isolated nuclei by the technique of Gronow & Griffiths (1971). The protein material was precipitated with 15% (w/v) trichloroacetic acid at 4°C washed with ethanol/diethyl ether followed by diethyl ether, and then dried. Samples (5 mg/ml) were treated as shown, and at the end of the incubation procedure they were reprecipitated with trichloroacetic acid and weighed.

Treatment		Percentage loss of trichloroacetic acid-precipitable material
(1) 0.1 M-NaOH	18°C for 24 h	30.5
(2) 0.25 M-NaOH	18°C for 24 h	37.6
(3) 0.5 M-NaOH	18°C for 24 h	58.0
(4) 1 M-NaOH	18°C for 24 h	77.3
(5) 0.5 M-NaOH	100°C for 1 min	61.5
(6) Ribonuclease (0.5 mg/ml)	37°C for 2 h	3.7

Table 2. *Sephadex G-50 chromatography of NaOH-treated rat liver non-histone proteins*

Non-histone proteins, extracted as described in the legend of Table 1, were treated with NaOH as shown, neutralized with HCl and freeze-dried. After dissolving in 8 M-urea/0.05 M-sodium phosphate, pH 7.6, the mixture was applied to a column (25 cm × 2 cm) of Sephadex G-50 and eluted with the same solution. The protein material eluted in the void volume was measured by the Folin-Lowry method (Lowry *et al.*, 1951).

	Protein recovered in exclusion volume (μg)	Percentage change
(a) Chromatin non-histone protein		
(1) Control, pH 7.6, 4°C or 18°C	3280	—
(2) 0.1 M-NaOH at 4°C	2780	-15
(3) 1 M-NaOH at 4°C	1630	-50.3
(4) 1 M-NaOH at 18°C	1020	-68.8
(b) Whole nuclear non-histone protein		
(1) Control	4250	—
(2) 0.1 M-NaOH at 4°C	3350	-21
(3) 1 M-NaOH at 4°C	2490	-41.5

For analytical examination of the effects of NaOH treatment urea was removed by exhaustive dialysis against double-distilled water at 4°C and the mixture freeze-dried.

Samples (usually 3–5 mg/ml) were taken for treatment with NaOH, and controls were made up in pH 7.0 buffer. At the end of the allotted time the samples were brought to pH 7.0 by the addition of HCl, divided into two halves and freeze-dried.

One-half was dissolved in 5 M-urea/5 mM-sodium phosphate, pH 7.6 (1 mg of protein/ml), heated briefly to 100°C and incorporated into a 5% (w/v) polyacrylamide-gel mixture in 8 M-urea with 2% (w/v) Ampholine carrier ampholytes (pH range 3.5–10; LKB-Producter A.B., Stockholm, Sweden). Isoelectric focusing was basically by the method of Gronow & Griffiths (1971), but larger gels were run (11.5 cm) and the initial voltage was set at 400 V for 16 h. This produces a plateau effect that helps to spread out the protein species, the bulk of which have pI values between 5 and 8.

The other one-half was dissolved in 5 M-urea/2% (w/v) sodium dodecyl sulphate/0.01 M-sodium phosphate/10% (w/v) sucrose/1% (v/v) 2-mercapto-ethanol, pH 7.0 (1 mg of protein/ml), and heated briefly to 100°C. Samples were analysed electrophoretically in 10% (w/v) polyacrylamide gels by using the method of Weber & Osborn (1969).

Results and Discussion

From Tables 1 and 2 it is evident that the NaOH is hydrolysing the protein; even 0.1 M-NaOH at 4°C. The hydrolysis is probably proportional to the concentration of NaOH. Certainly breakdown in these experiments is not due to the presence of an internal proteinase, as the control experiments demonstrate. Ribonuclease treatment has little effect on the amount of trichloroacetic acid-precipitable material (sodium dodecyl sulphate gels were identical with control samples).

The polyacrylamide gels clearly show a loss of

EXPLANATION OF PLATE I

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels (Weber & Osborn, 1969)

Chromatin non-histone protein was treated with different concentrations of NaOH; samples (100 μ g) were applied to a 10cm $\times$ 0.5cm gel. (a) Control; (b) 0.1M, 5h at 4°C; (c) 0.1M, 24h at 4°C; (d) 0.5M, 24h at 4°C; (e) 1M, 2h at 4°C; (f) 0.1M, 3min at 100°C.

EXPLANATION OF PLATE 2

Isoelectric focusing in 5% (w/v) polyacrylamide gels (Gronow & Griffiths, 1971)

Chromatin non-histone protein was treated with different concentrations of NaOH; samples (220 μ g) were incorporated into a 11.5 $\times$ 0.5 cm gel. (a) Control; (b) 0.1 M, 2 h; (c) 0.1 M, 12 h; (d) 1 M, 1 h; (e) 1 M, 24 h.

stainable protein bands even after 2 h exposure to 0.1 M-NaOH at 4°C (Plates 1 and 2). The pH of this solution was found to be 12.7 (by pH-meter). Since no dialysis step was used in the preparation of samples this means that in the case of the isoelectric-focusing gels the peptide breakdown products are either soluble in the initial 12% (w/v) trichloroacetic acid wash used or that the dye complexes diffuse out of gels during staining-de-staining. The latter probably happens in the case of sodium dodecyl sulphate gels.

The destruction of proteins with pI values above 5.5 is very noticeable, correlating with the rapid loss of fine high-molecular-weight bands in sodium dodecyl sulphate electrophoresis. Similar results have been obtained with non-histone proteins isolated from other rat tissues and tumour cells. By using these analytical systems we are now investigating the molecular-weight range of the polypeptides released at low NaOH concentrations. In addition, we are looking at the rate of hydrolysis and attempting end-group analysis to ascertain if any particular peptide link is preferentially attacked at low temperatures during treatment with dilute NaOH. These findings might be useful for sequencing studies.

Obviously the maximum concentration of NaOH and the highest possible buffer pH that can be used in analysing non-histone proteins has yet to be ascertained. However, it is quite clear that under some conditions used for the analysis of materials closely associated with DNA, e.g. alkaline sucrose gradients and the classical Schmidt-Thannhauser procedure, there will be considerable hydrolysis of protein material. The possibility exists that some proteins present in trace amounts, such as repressor molecules,

could be preferentially destroyed under even mild alkaline conditions.

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