The isolation of nuclear envelopes

Effects of thiol-group oxidation and of calcium ions

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The effects of (a) oxidative cross-linking of protein thiol groups and (b) the presence or absence of Ca^{2+} ions on rat liver nuclear-envelope isolation were studied. Two envelope-isolation procedures were compared: a well characterized low-ionic-strength method and a recently developed high-ionic-strength method. The latter method seems preferable to the former in respect of lower intranuclear contamination of the envelopes, suppression of endogenous serine proteinase, and maintenance of high specific activities of envelope-associated enzymes. In both procedures, however, the presence of Ca^{2+} gave rise to a rapid, apparently irreversible, contamination of the envelopes by intranuclear material. This effect was half-maximal at $20\ \mu\text{M}\text{-Ca}^{2+}$. In addition, the envelopes became contaminated with intranuclear material by a Ca^{2+} -independent mechanism, apparently resulting from *N*-ethylmaleimide-sensitive intermolecular disulphide-bond formation. This oxidative process seemed to have two major kinetic components (half-life, $t_{1/2}$, approx. 2 min and 10 min). In view of these findings, it is recommended that (i) for most purposes, nuclear envelopes be isolated by the newly developed high-ionic-strength procedure, (ii) irrespective of the method used, Ca^{2+} -chelators be included in all the buffers, (iii) thiol-group oxidation be prevented or reversed during the procedure.

Methods for isolating nuclear envelopes in bulk from liver and from other tissues fall into two broad classes: (1) those that rely on low-ionic-strength media to extract the nuclear contents, after fragmentation of the chromatin by exogenous deoxyribonuclease-I treatment or by endogenous hydrolytic enzymes; (2) those that rely on extraction of the chromatin with buffers of high ionic strength, with or without preliminary cleavage of the DNA [see Franke (1974) and Harris & Agutter (1976) for reviews].

Low-ionic-strength procedures are generally rapid and yield morphologically intact material with high specific activities of envelope-associated enzymes. One of them, the method of Harris & Milne (1974), has been used extensively in our laboratory [see, e.g., Agutter *et al.* (1977, 1979a)]. However, there are difficulties concerning the use

of this procedure. (a) Yield and composition of the envelopes appear to be functions of the concentration of nuclei during the lysis stage, and this effect has not been adequately explained (Agutter & Gleed, 1980). (b) The envelopes contain substantial amounts of the nuclear DNA, RNA and histones (Hubert *et al.*, 1981; Clawson & Smuckler, 1980; Richardson & Maddy, 1980). (c) Finally, this procedure, in common with all low-ionic-strength methods, seems to depend in part on endogenous proteolysis. Nuclear envelopes cannot be obtained by the Harris & Milne (1974) or Kay *et al.* (1972) procedures if serine proteinase inhibitors such as PMSF are included in the buffers used (Comerford *et al.*, 1984; S. H. Kaufmann, personal communication).

In general, high-ionic-strength isolation procedures involve long operation times, during which the degradation of envelope components causes (i) substantial inactivation of the endogenous NTPase (Porteous *et al.*, 1978), (ii) morphological damage to the material (Harris & Agutter, 1976)

Abbreviations used: NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; NTPase, nucleoside triphosphatase (EC 3.6.1.15).

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and (iii) irreproducibilities in the amounts of most associated enzyme activities (Richardson & Agutter, 1980). However, the most recently developed high-ionic-strength procedure, that of Kaufmann *et al.* (1983), seems more attractive than its predecessors because it is much more rapidly completed and yields envelopes largely in the form of hollow nucleus-sized spheres, containing well-preserved pore-complexes. The envelopes isolated by this method, which involves the suppression of serine proteinases, have not yet been characterized in terms of the activities of their associated enzymes, including the NTPase. One of the objectives of the work described in the present paper was to compare the enzyme activities of envelopes isolated by the Kaufmann *et al.* (1983) and Harris & Milne (1974) procedures, the latter being already well characterized (Milne *et al.*, 1978).

Apart from some proteolytic degradation, at least two other factors can affect the reproducibilities of subnuclear-fractionation procedures, and these might account for some of the differences between nuclear-envelope preparations described by different laboratories [see Richardson & Agutter (1980) for review]. One of these factors is the presence of Ca^{2+} ions in the media used: Ca^{2+} causes both irreversible aggregation of intranuclear constituents and degradation of these constituents by Ca^{2+} -activated proteinases (Laemmli *et al.*, 1981; Lebkowski & Laemmli, 1982a,b). The other factor is the oxidative cross-linking of endogenous protein thiol groups. This seems to be crucial in stabilizing the rat liver intranuclear matrix and in effecting the attachment of this structure, and probably of other nuclear components, to the envelope (Kaufmann *et al.*, 1981). Such cross-linking also determines the state of the lamina (the Triton-X-100-resistant perinuclear fibrillar system contiguous with the inner nuclear membrane) and hence, presumably, the behaviour of the envelope as a whole (Kaufmann *et al.*, 1983). The second main objective of the studies described in the present paper was to determine the effects of Ca^{2+} and of endogenous thiol-group oxidation on the characteristics of nuclear envelopes isolated by the Harris & Milne (1974) and Kaufmann *et al.* (1983) procedures.

Materials and methods

ATP (disodium salt, grade II), NEM, EGTA, NADH and 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate) were obtained from Sigma Chemical Co., Poole, Dorset, U.K., PMSF and *p*-nitrophenyl phosphate from BDH, Poole, Dorset, U.K., deoxyribonuclease I and ribonuclease A from Boehringer, Mannheim, West Germany, and sodium

tetrathionate was from Fluka, Buchs, Switzerland. All other reagents were of AnalaR grade from BDH.

Isolation of nuclei and nuclear envelopes

Liver nuclei were isolated from male rats (100–150 g body wt.) by the method of Blobel & Potter (1966), except that in some experiments 1.0 mM-PMSF, 1.0 mM-EGTA or 5.0 mM-NEM was added to the homogenization and dense-sucrose buffers. Nuclear envelopes were isolated from the nuclei by modifications of one of two procedures. (1) For the method of Harris & Milne (1974), nuclei were isolated in the presence or absence of EGTA or NEM and in the absence of PMSF. In some experiments 1 mM-EGTA was added to the bicarbonate buffer used for nuclear lysis and washing of the envelopes. In others, various concentrations of CaCl_2 were added at the lysis stage. In all experiments, the nuclei were resuspended to a concentration of $4.5 \times 10^7/\text{ml}$ (1 mg of protein/ml) at the lysis stage (Agutter & Gleed, 1980). (2) For the method of Kaufmann *et al.* (1983), nuclei isolated in the presence of PMSF were used in most experiments. In experiments where NEM had been added to the homogenizing buffer, the 2-mercaptoethanol was omitted in the 1.6 M-NaCl extraction step. In experiments where EGTA had been present throughout the nuclear-isolation procedure, either 1 mM-EGTA was added to all the buffers used in the later steps, or the nuclei were washed once with 250 mM-sucrose/50 mM-Tris/HCl (pH 7.5)/5.0 mM- MgCl_2 /1.0 mM-PMSF containing 0–100 μM - CaCl_2 , immediately before the combined nuclease-digestion stage. In this case, later steps were performed as described by Kaufmann *et al.* (1983) without modification.

Chemical determinations

Protein was measured by a modification (Maddy & Spooner, 1970) of the method of Lowry *et al.* (1951), DNA by the method of Burton (1956), RNA by the method of Ashwell (1957) and phospholipid as described by Agutter & Gleed (1980).

Enzyme assays

NTPase was measured as described by Agutter *et al.* (1979a), peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) by the spectrophotometric method of Stubbs & Harris (1978), glucose-6-phosphatase (*D*-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) as described by Kadlowski & Agutter (1977), succinate dehydrogenase (succinate:acceptor oxidoreductase, EC 1.3.99.1) by the method of King (1963), NADH dehydrogenase (NADH:acceptor oxidoreductase, EC 1.6.99.3) by the method of Zamudio *et al.* (1969),

with ferricyanide as receptor, monoamine oxidase (amine:oxygen oxidoreductase, EC 1.4.3.4) by the method of Erwin & Hellerman (1967), with tyramine as substrate, 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) by the method of Segal & Brenner (1960), with AMP as substrate, and alkaline and acid phosphatases (orthophosphoric-monoester phosphohydrolases, EC 3.1.3.1 and 3.1.3.2) as described by Kadlowski & Agutter (1977), with *p*-nitrophenyl phosphate as substrate.

Results

Modifications of the isolation procedure of Kaufmann et al. (1983)

The overall composition of envelopes isolated by this procedure corresponded closely to that described by Kaufmann *et al.* (1983), irrespective of the addition of NEM at the homogenization stage (Table 1, columns 1 and 2). When Ca^{2+} was removed by addition of EGTA to the buffers at all stages, the recovery of phospholipid was slightly increased and the recoveries of protein and RNA were decreased (Table 1, column 3). The increased phospholipid recovery may indicate suppression of residual Ca^{2+} -activated proteinases. In any case, it suggests that the decreased protein and RNA recoveries reflect decreased intranuclear contamination rather than fragmentation and loss of a more fragile nuclear envelope. When (a) nuclei were isolated in the absence of EGTA and then 1 mM-EGTA was added to all the buffers used subsequently, or (b) envelopes were isolated by the unmodified procedure and then washed in the sucrose/Tris/ Mg^{2+} buffer supplemented with 1 mM-EGTA, the compositions of the envelopes were not significantly altered (Table 1, columns 4 and 5). This suggests that the presumed effects of

trace Ca^{2+} concentrations on envelope composition are not reversed by the usual Ca^{2+} chelators (cf. Lebkowski & Laemmli, 1982a,b). This view is supported by the results of experiments in which nuclei were isolated in the presence of EGTA and were then washed with various concentrations of Ca^{2+} before the nuclease treatment. The recovery of protein and RNA in the envelopes increased with increasing Ca^{2+} concentration, and the effect was half-maximal when the Ca^{2+} concentration was about 20 μM (Fig. 1). Finally, omission of PMSF from the media decreased the recovery of protein and phospholipid in the envelopes (Table 1, column 6). This indicates that, even in a rapid

Fig. 1. Effects of Ca^{2+} on composition of envelopes isolated by the Kaufmann *et al.* (1983) procedure

Nuclei were isolated in the presence of 1 mM-EGTA, and CaCl_2 (0–100 μM) was added and the nuclei were washed once before nuclease treatment. Envelopes were then isolated as described by Kaufmann *et al.* (1983), and the percentage recoveries of protein (○) and RNA (●) were measured. Results are means $\pm$ S.E.M. for three experiments, each done in triplicate.

Table 1. Compositions of envelopes isolated by modifications of the Kaufmann *et al.* (1983) procedure

Envelopes were isolated by the modifications of the procedure described in the text. Nuclei were isolated by the unmodified procedure (columns 1, 4 and 5), in the presence of EGTA (column 3), after addition of 5 mM-NEM to the homogenizing buffer (column 2) or in the absence of PMSF (column 6). Column 4: 1 mM-EGTA was added to the nuclei before nuclease treatment. Column 5: the envelopes were washed in 1 mM-EGTA before analysis. The percentage recovery of phospholipid from nuclei was determined directly. The percentage recoveries of the other components were corrected for phospholipid recovery, assuming that 100% of the nuclear phospholipid is located in the envelopes. Results are means $\pm$ S.E.M. of three or four experiments, each done in triplicate. Abbreviation: ND, not detectable.

	Recovery from nuclei (%)					
	1	2	3	4	5	6
Protein	11.6 $\pm$ 0.6	11.0 $\pm$ 0.4	6.4 $\pm$ 0.4	14.1 $\pm$ 0.3	10.7 $\pm$ 0.8	7.5 $\pm$ 0.6
DNA	0.6 $\pm$ 0.2	1.0 $\pm$ 0.2	ND	0.5 $\pm$ 0	0.8 $\pm$ 0.2	1.2 $\pm$ 0
RNA	3.1 $\pm$ 0.4	3.5 $\pm$ 0.6	1.4 $\pm$ 0	2.6 $\pm$ 0.3	3.4 $\pm$ 0.4	3.0 $\pm$ 0.6
Phospholipid	45 $\pm$ 2	48 $\pm$ 4	51 $\pm$ 3	42 $\pm$ 3	47 $\pm$ 3	33 $\pm$ 5

envelope-isolation procedure carried out entirely at 4°C, overall effects of endogenous serine proteinases are marked.

Modifications of the isolation procedure of Harris & Milne (1974)

The composition of the envelopes isolated by the unmodified procedure (Table 2, column 1) was similar to that previously described (Milne *et al.*, 1978; Agutter & Gleed, 1980). With this preparation, however, the addition of NEM to the homogenizing buffer affected the composition of the envelopes markedly, both in the presence and in the absence of EGTA (Table 2, columns 2-4).

When nuclei were isolated in the presence of EGTA and then various concentrations of CaCl_2 were added during lysis in 1 mM- NaHCO_3 , the recovery of protein in the envelopes increased with increasing Ca^{2+} concentration, and again the effect of Ca^{2+} was half-maximal at around 20 μM (Fig. 2a). The effect on the specific activity of the NTPase, which is apparently located exclusively in the nuclear envelope (Agutter *et al.*, 1977, 1979a), is consistent with the view that Ca^{2+} causes contamination of the isolated envelopes with intranuclear material (Fig. 2b). In these experiments, NEM again seemed to decrease the extent of contamination of the envelopes by intranuclear protein (Figs. 2a and 2b).

Stubbs & Harris (1978) reported that a characteristic nuclear peroxidase was envelope-associated and might be used as a marker enzyme for nuclear envelopes. To examine this possibility further, nuclei were isolated in the presence of 1 mM-EGTA and were lysed in the presence of 0-100 μM - CaCl_2 . The peroxidase activities in the purified envelopes and in the supernatant from the lysis step were measured as described by Stubbs & Harris (1978), except that 0.5 mM-KCN was added to the lysis supernatant to suppress an endogenous catalase activity. The results (Fig. 3a) indicate that the peroxidase is present in both the envelope and

Fig. 2. Effects of Ca^{2+} on envelopes isolated by the Harris & Milne (1974) procedure.

Nuclei were isolated in the presence of 1 mM-EGTA. In some experiments (●), 5 mM-NEM was added to the homogenizing buffer, and in others (○) it was omitted. The nuclei were then lysed in 1 mM- NaHCO_3 containing 0-100 μM - CaCl_2 , and envelopes were isolated as described by Harris & Milne (1974). (a) The percentage recovery of protein from the nuclei was measured; (b) the specific activity of the envelope NTPase was measured. Results are means $\pm$ S.E.M. for three experiments, each done in triplicate.

Table 2. Compositions of envelopes isolated by modifications of the Harris & Milne (1974) procedure. Envelopes were isolated by the following modifications of the procedure: column 1, no modification; columns 2 and 4, liver homogenized in the presence of 5 mM-NEM; columns 3 and 4, 1 mM-EGTA added to all the buffers used. Results are means $\pm$ S.E.M. of three or four experiments, each done in triplicate. The recoveries of the components were calculated as described in the caption for Table 1.

	Recovery from nuclei (%)			
	1	2	3	4
Protein	35.3 $\pm$ 1.6	26.6 $\pm$ 1.3	15.4 $\pm$ 1.3	10.8 $\pm$ 0.9
DNA	15.2 $\pm$ 0.5	8.3 $\pm$ 1.4	7.2 $\pm$ 0.5	4.2 $\pm$ 0.8
RNA	41.2 $\pm$ 2.2	24.0 $\pm$ 0.7	15.4 $\pm$ 1.5	8.9 $\pm$ 1.1
Phospholipid	37.4 $\pm$ 1.4	43.3 $\pm$ 0.9	44.1 $\pm$ 2.8	45.2 $\pm$ 2.7

the intranuclear material, and becomes more completely envelope-associated with increasing Ca^{2+} concentration during the isolation procedure.

When this experiment was repeated on nuclei isolated from livers homogenized in the presence of 5 mM-NEM, the effect of Ca^{2+} was more marked

and the total measurable peroxidase activity was about 5-fold greater (Fig. 3b). These results suggest that the peroxidase is (i) partially inactivated and (ii) more readily associated with the nuclear envelope, by the spontaneous formation of intermolecular disulphide bonds during manipulations of the nuclei.

Rapidity of the effects of Ca^{2+} and of thiol-group oxidation

The following experiment was designed to investigate the time course of the Ca^{2+} effect. Nuclei were isolated in the presence of 1 mM-EGTA and were resuspended in 2.5 ml of 50 mM-Tris/HCl (pH 7.5)/5.0 mM- MgCl_2 containing 15% (w/v) sucrose. The suspension was layered over 2.5 ml steps of 20, 25, 30, 35 and 40% (w/v) sucrose in the same buffer. The 25%-sucrose layer contained 100 μM - CaCl_2 and the 35%-sucrose layer contained 1 mM-EGTA. The gradient was centrifuged at 15000g for 15 min, the nuclear pellet was divided into two portions, and envelopes were isolated from these by the two methods under investigation. As a control, the CaCl_2 was omitted from the 25%-sucrose layer. The results (Table 3) indicate that the irreversible effect of Ca^{2+} was complete within the duration of the experiment (compare with Figs. 1 and 2a and Tables 1 and 2). A series of brief centrifugations of such gradients showed that at least 90% of the nuclei had passed through the 25%-sucrose layer in less than 1 min under the conditions used.

To determine the rate of oxidative cross-linking of endogenous thiol groups, the following experiments were performed. First, liver was homogenized, and nuclear envelopes were isolated by the procedure of Harris & Milne (1974), with 1 mM-EGTA added to all the media used. NEM (5 mM) was added at various stages between homogenization of the tissue and lysis of the nuclei. Peroxidase activity was measured in the envelopes and in the lysis supernatant; this seems to be a sensitive indicator of thiol-group oxidation (Figs. 3a and

Fig. 3. Effects of CaCl_2 and of NEM on distribution of nuclear peroxidase activity

Nuclei were isolated and the CaCl_2 concentration during lysis was altered as described in the legend to Fig. 2. Peroxidase activity was measured in the envelopes (○) and in the supernatant after lysis (●) (see the text). (a) No NEM added to homogenizing buffer; (b) 5 mM-NEM added to homogenizing buffer. Results are means $\pm$ S.E.M. for three determinations, each done in triplicate.

Table 3. Rapidity of Ca^{2+} effect

Columns 1 and 3: nuclei were treated briefly with 100 μM - CaCl_2 and the excess Ca^{2+} was quickly removed (see the text). Envelopes were then isolated by the Harris & Milne (1974) or Kaufmann *et al.* (1983) procedures (columns 1 and 2, and columns 3 and 4, respectively). Columns 2 and 4: nuclei were treated in the same way, except that the 100 μM - CaCl_2 was omitted. Results are means of two experiments, each done in triplicate.

	1	2	3	4
Percentage recovery of:				
Protein	12.8	7.5	5.9	3.6
DNA	5.4	3.7	0.4	0.2
RNA	14.7	8.1	1.5	1.0
NTPase activity (μmol of P_i /h per mg of protein)	5.5	12.4	4.3	7.4

3b). The results (Table 4) suggest that about 40–50% of the thiol-group oxidation that alters the activity and distribution of the peroxidase occurs very rapidly; the remainder occurs more slowly. Secondly, liver was homogenized and nuclei were isolated in media supplemented with 1 mM-EGTA and 5 mM-2-mercaptoethanol. The nuclei were washed in sucrose/Tris/Mg²⁺ buffer supplemented with 1 mM-EGTA, and were then resuspended to a concentration of 5×10^8 nuclei/ml in the same buffer containing 0.5 mM-sodium tetrathionate (Kaufmann *et al.*, 1981). After incubation at 4°C for 0–20 min, 5 mM-NEM was added to the suspension to prevent further cross-linking, and the nuclei were then subjected to the envelope-isolation procedure of Kaufmann *et al.* (1983), but without the addition of 2-mercaptoethanol. After prolonged incubation with tetrathionate, nuclei yield chromatin-depleted residues with extensive matrices and substantial quantities of intranuclear protein as well as envelopes (Kaufmann *et al.*, 1981). The results (Fig. 4) showed that protein recovery from the nuclei increased markedly with incubation time over the interval 0–4 min, and afterwards increased more slowly. Thus both sets of data indicate that those endogenous nuclear protein thiol groups that are capable of forming intermolecular cross-links comprise rapidly oxidized and more slowly oxidized components.

Comparative enzymologies of the preparations

Table 5 summarizes the specific activities of a number of enzymes measured in nuclear envelopes isolated by (a) the Harris & Milne (1974) procedure, either (i) unmodified or (ii) modified by addition of 5 mM-NEM to the homogenizing buffer and by inclusion of 1 mM-EGTA in all the buffers, or (b) the Kaufmann *et al.* (1983) procedure, either (i) unmodified or (ii) modified by the addition of

1 mM-EGTA to all the buffers used. The values in column 1 of Table 5 are in close agreement with those previously reported for this preparation (Milne *et al.*, 1978). Generally, EGTA seems to decrease contamination by apparently extra-nuclear enzymes. The endogenous enzyme activities in the Kaufmann *et al.* (1983) envelope preparations appear to be well preserved, and contaminant activities are low.

The high NTPase activity in the Harris & Milne (1974) preparation has been described previously (Agutter *et al.*, 1977, 1979a,b), and seems surprising because this enzyme is readily inactivated by extremes of pH and ionic strength, urea, detergents and some proteinases (Porteous *et al.*, 1978). One

Fig. 4. Effects of tetrathionate-induced cross-linking on protein recovery from nuclei

Nuclei were isolated under reducing conditions and in the presence of 1 mM-EGTA. After incubation for 0–20 min at 4°C in the presence of tetrathionate, they were subjected to the procedure of Kaufmann *et al.* (1983), with the reduction step omitted. The percentage recovery of protein in the residual material was measured. Results are means $\pm$ S.E.M. for five experiments, each done in triplicate.

Table 4. Peroxidase distributions after various times of thiol-group oxidation

During the isolation of nuclear envelopes by the method of Harris & Milne (1974) modified by the addition of 1 mM-EGTA to all the buffers, 5 mM-NEM was added at one of the following stages: (A) before homogenization; (B) after homogenization and before centrifugation; (C) to the crude nuclear pellet; (D) to the purified nuclei after centrifugation through dense sucrose; (E) to the nuclei during lysis in 1 mM-NaHCO₃. (F) = control (equivalent volume of buffer added instead of NEM). Peroxidase activity was measured in the lysis supernatant and in the envelopes. Results are means $\pm$ S.E.M. of three experiments, each done in triplicate.

Addition of NEM	Peroxidase (μ mol of dye/h per mg of protein) in			Activity ratio (supernatant/envelopes)
	Supernatant	Envelopes	Total	
A	1032 $\pm$ 52	97 $\pm$ 9	1129 $\pm$ 61	10.8 $\pm$ 1.5
B	903 $\pm$ 26	86 $\pm$ 8	989 $\pm$ 34	10.6 $\pm$ 1.3
C	681 $\pm$ 43	92 $\pm$ 6	773 $\pm$ 49	7.5 $\pm$ 1.0
D	306 $\pm$ 18	117 $\pm$ 12	423 $\pm$ 30	2.7 $\pm$ 0.4
E	251 $\pm$ 13	106 $\pm$ 4	357 $\pm$ 17	2.4 $\pm$ 0.2
F	224 $\pm$ 18	113 $\pm$ 7	337 $\pm$ 25	2.0 $\pm$ 0.3

Table 5. *Nuclear-envelope enzyme activities*

Envelopes were isolated (a) by the method of Harris & Milne (1974), unmodified (column 1) or modified by the addition of 5 mM-NEM to the homogenizing buffer and of 1 mM-EGTA to all the buffers used (column 2), or (b) by the method of Kaufmann *et al.* (1983), unmodified (column 3) or modified by the inclusion of 1 mM-EGTA in all the buffers (column 4). All activities are expressed in $\mu\text{mol/h}$ per mg of protein and are means $\pm$ S.E.M. for three to five determinations. Abbreviation: ND, not detectable.

Enzyme	1	2	3	4
Nucleoside triphosphatase	5.2 $\pm$ 0.5	17.1 $\pm$ 0.8	4.3 $\pm$ 0.2	7.4 $\pm$ 0.5
Glucose-6-phosphatase	14.3 $\pm$ 3.1	21.8 $\pm$ 2.2	26.2 $\pm$ 1.1	31.5 $\pm$ 3.0
Alkaline phosphatase	1.4 $\pm$ 0.2	5.1 $\pm$ 0.8	11.4 $\pm$ 0.7	14.2 $\pm$ 1.0
Acid phosphatase	6.3 $\pm$ 0.5	2.1 $\pm$ 0.3	0.3 $\pm$ 0	0.15 $\pm$ 0
NADH-ferricyanide reductase	11.4 $\pm$ 1.2	15.6 $\pm$ 0.3	20.0 $\pm$ 1.7	23.5 $\pm$ 1.2
Monoamine oxidase	1.5 $\pm$ 0.3	ND	ND	ND
5'-Nucleotidase	1.3 $\pm$ 0.3	0.6 $\pm$ 0	ND	ND
Succinate dehydrogenase	0.7 $\pm$ 0.1	0.3 $\pm$ 0	0.1 $\pm$ 0	ND

set of preliminary results may suggest interesting reasons for the observed high activity. Two preparations of rat liver nuclei were made, in (a) the presence and (b) the absence of PMSF. From (a), envelopes were isolated by the Kaufmann *et al.* (1983) procedure. From (b), a lysate (containing some endogenous serine proteinase activity) was prepared by nuclease treatment, 1.6M-NaCl extraction and high-speed centrifugation. When samples of this lysate (10 μg of protein) were incubated with suspensions containing 1 mg of the nuclear envelopes at 20°C, the NTPase activity in the envelopes rose from 3.9 to 8.7 μmol of P_i/h per mg of protein in 20 min, and afterwards decreased. It is therefore possible that the NTPase can be activated by limited proteolysis.

Discussion

There are several reasons for considering the method of Kaufmann *et al.* (1983) to be the most satisfactory of the available nuclear-envelope isolation procedures. These include: (a) its rapidity and (b) the well-preserved envelope morphology, in which respects the method is comparable with the low-ionic-strength methods; (c) the very low contamination with histone and DNA; (d) the suppression of endogenous proteolysis, in which respects it is markedly superior to the low-ionic-strength procedures; (e) the reproducibly good yield of envelopes, in which respect it is at least comparable with other methods. To these points two others can be added, in view of the results shown in Table 5: (f) the freedom from extra-nuclear contamination and (g) the high specific activities of endogenous enzymes. On the basis of these characteristics, it seems reasonable to conclude that the procedure of Kaufmann *et al.* (1983) is the most satisfactory envelope isolation procedure to use for most purposes (see discussion of

alternative methods in Harris & Agutter, 1976), with the possible exception of permeability studies (Kondor-Koch *et al.*, 1982).

Apart from proteolysis, (i) the presence of Ca^{2+} ions and (ii) oxidative cross-linking of intranuclear and envelope proteins can apparently interfere with nuclear-envelope isolation. Ca^{2+} ions cause contamination of the envelopes, as of other sub-nuclear fractions (Lebkowski & Laemmli, 1982a,b), with intranuclear components. Such contamination cannot be removed by subsequent washings, or by EGTA treatment. Irrespective of the type of isolation procedure used or of the parameter chosen to assess Ca^{2+} -related contamination (see, e.g., Figs. 1–3), approximately the same sensitivity to Ca^{2+} ions (half-maximal effect at around 20 μM) was observed. In view of this, it seems surprising that the effect has not been noted in any previous discussion of nuclear-envelope isolation. This concentration is likely to be attained or exceeded in most tissue homogenates, even after preliminary washing or perfusion, because of persistent traces of extracellular fluid. Because of this and of the rapidity (Table 3) and irreversibility of the effect, it seems reasonable to recommend the addition of EGTA to the homogenizing buffer before the cells are broken, and to include this chelating agent in the other buffers used during envelope isolation.

It is noteworthy that the effective Ca^{2+} concentrations are substantially higher than those (up to 1–2 μM) that seem to be involved in metabolic regulation *in vivo*. Therefore it seems clear that the effect of Ca^{2+} on subnuclear fractionation is not an indication of any Ca^{2+} -mediated regulatory process affecting the envelopes or other nuclear structures. However, it seems to be established that there are binding sites on the non-histone chromatin proteins, perhaps including the nuclear-matrix proteins, that are highly specific for Ca^{2+} (Schibeci

& Martonosi, 1980). Moreover, at least some nuclear matrices seem to include actin among their major polypeptide components (Nakayasu & Ueda, 1983), and others exhibit Ca^{2+} -dependent hormone-sensitive calmodulin binding (Simmen *et al.*, 1982). Therefore, although the present results suggest that the effects of Ca^{2+} on nuclear subfractionation are artefacts, the possibility of a physiological role for intranuclear Ca^{2+} cannot be discounted.

The studies by Kaufmann *et al.* (1981, 1983) have clarified some of the issues about the importance of protein thiol-group oxidation for the isolation and characterization of subnuclear fractions. Broadly, they have shown that: (1) intranuclear proteins can become cross-linked by slow spontaneous oxidation or by more rapid oxidation induced by such reagents as tetrathionate, a conclusion which has important implications in the study of the nuclear matrix; (2) some major nuclear-envelope proteins, notably lamins A and B (see Gerace & Blobel, 1980), are probably monomeric *in vivo*, but are rapidly assembled into homopolymers by spontaneous oxidation during the isolation of nuclei. This second conclusion has probably resolved the controversy about whether the lamins are oligomeric (Lam & Kasper, 1979; Maul & Avdalovic, 1980) or monomeric (Shelton & Cochran, 1978; Gerace & Blobel, 1980) *in situ*. Both types of oxidative cross-linking process can be prevented by alkylation of the reactive protein thiol groups. Hydrophobic alkylating agents such as NEM are more effective on the lamins, and more hydrophilic ones such as iodoacetamide are more effective inhibitors of cross-linking of intranuclear proteins (Kaufmann *et al.*, 1983). High concentrations of 2-mercaptoethanol reduce the cross-links, but only in buffers of high salt concentration (Kaufmann *et al.*, 1981, 1983). The results described in the present paper extend these findings as follows. (a) The nuclear envelope can become contaminated by intranuclear components as a result of fast and slower spontaneous oxidative processes (Table 4, Fig. 4), which can be inhibited by treatment of the material by NEM. Thus spontaneous cross-linking of proteins can lead to contamination of the envelopes as well as to oligomerization of lamins. (b) At least one enzyme is partially inactivated by such oxidative cross-linking (contrast Figs. 3a and 3b), and this can result in misleading observations about the distribution of the enzyme between subnuclear fractions. Because of the complications caused by artefactual thiol-group oxidation, it seems to us that the process should be inhibited or reversed, as described by Kaufmann *et al.* (1983), during nuclear subfractionation.

The damaging effects of endogenous serine

proteinases on envelope preparations are significant even in rapid, low-temperature, methods (see, e.g., Table 1), and one of the most serious criticisms of low-ionic-strength methods may be that such activities make an integral contribution to the procedure (Kaufmann *et al.*, 1983). However, the possibility that endogenous nuclear serine proteinases have some physiological function should not be overlooked. For example, the preliminary results pertaining to the high specific activity of the NTPase in envelopes prepared by the Harris & Milne (1974) method, which suggest that the enzyme is activated by limited proteolysis (see the last paragraph of the Results section), could imply that at least one serine proteinase, if it is active in the microenvironment of the NTPase *in situ*, has a role in the regulation of nucleocytoplasmic RNA transport.

The principal conclusion to be drawn from this study is that the nuclear-envelope isolation procedure of Kaufmann *et al.* (1983) is more generally satisfactory than any alternative method so far published, in terms of the purity and of the morphological and biochemical integrity of the envelopes obtained. However, particular care should be taken to suppress or to reverse thiol-group oxidation, and there seems to be a good case for supplementing all the buffers used, particularly the homogenizing buffer, with a Ca^{2+} chelator such as EGTA. It seems likely that more widespread use of this procedure, perhaps with the modification suggested, will facilitate studies on several aspects of the structure and function of the nuclear envelope during the next few years.

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