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Cytoplasmic-gradient analysis of liver nuclei organization

Análisis de la organización de núcleos hepáticos mediante gradiente citoplásmico

FEDERICO LEIGHTON

International Institute of Cellular and Molecular Pathology, Brussels, and Departamento de Biología Celular, Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

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The beaviour of nuclei in undiluted liver tissue homogenates not submitted to ultracentrifugation and during cytoplasmic-gradient subcellular fractionation, allows, to recognize two nuclear states which are defined in function of the homogenate density: low density nuclei and high density nuclei. Low density nuclei are characterized by their ability to retain ATP:NMN-adenylytransferase, by a higher protein content when compared to high density nuclei, and by a transient low density state in sucrose gradients. High density nuclei do not bind ATP:NMN-adenylytransferase, contain approximately one half of the protein bound by low density nuclei and, in sucrose gradients, behave as organelles with a density higher than 1.28 g/ml.

The mechanism by which nuclei retain ATP:NMN-adenylytransferase in concentrated homogenates, could be the same by which many other nuclear proteins are retained. The characterization of such a mechanism would contribute to the understanding of the regulation of nuclear activity. In dilute homogenates the binding of the enzyme to nuclei occurs through a different mechanism, apparently an artefactual electrostatic interaction.

Low density nuclei are transformed into high density nuclei by controlled mechanical damage, by prolonged centrifugation in concentrated homogenates, or by centrifugation in concentrated sucrose gradients containing 1 mM $MgCl_2$. These observations indicate that the normal nuclear organization is better reflected in the organization of low density nuclei. The high density state of nuclei corresponds to an irreversible condition which apparently results from nuclear damage. Thus, the usual procedures to isolate nuclei in concentrated sucrose solutions, based on the assumption that normal nuclei equilibrate at densities of 1.30 g/ml or more, would be biased in favor of damaged organelles.

ATP:NMN-ADENYLYLTRANSFERASE CYTOPLASMIC GRADIENT FRACTIONATION
NUCLEAR DENSITY NONHISTONE CHROMATIN PROTEINS

A subcellular organelle has to be isolated and studied *in vitro* in order to understand its function, organization and control mechanisms. The activity of an organelle results from the interactions

among its constituent molecules, and it can not be understood only on the basis of the properties of its isolated constituents. Our present knowledge about oxidative phosphorylation and photophosphorylation, would not have been achieved without the procedures that allow to prepare functionally preserved mitochondria and chloroplasts and to study their function in the absence of other subcellular elements (1).

So far, there is no procedure available to isolate well preserved nuclei in large amounts and to keep them in a functionally active state *in vitro*. Consequently, our knowledge about the function of the nuclear envelope (2, 3), or the nuclear matrix (4, 5), or the bulk of non-histone proteins (6, 7), is incomplete, particularly when considering their regulatory properties.

The search for methods to obtain well preserved isolated nuclei has led to the development of numerous fractionation procedures (8, 9, 10), in which the state of the isolated organelles has been most frequently assesed from morphologic studies or biochemical composition analysis. Only exceptionally the viability of nuclei isolated in bulk has been controlled, and then the conclusion has been reached that, unless special precautions are taken, nuclei do not resume functional activity after transplantation (11). The organelle is very sensitive to damage during fractionation and does not resists the various steps involved in the procedures most frequently applied.

The inadequacy of the present aqueous procedures for nuclei isolation, possibly results from the indiscriminate application of the general principles of subcellular tissue fractionation. In general, these principles can be applied to the isolation of particles surrounded by a stable semipermeable membrane, or to the isolation of particles constituted by stable macromolecular aggregates like viruses or ribosomes. Nuclei do not correspond to any of these models.

The nuclear envelope, because of the presence of nuclear pore complexes permeable to macromolecules, is not a semipermeable membrane. Nor are nuclear constituents organized as a stable macromolecular aggregate, as shown by the translocation of nuclear polymerases detected during fractionation (12, 13, 14).

Based on the previous considerations, the cytoplasmic-gradient procedure for subcellular fractionation was applied to the study of liver nuclei organization. This novel method (15), is carried out on undiluted tissue homogenates. The nuclei remain in a medium formed by cytoplasmic contents, plus interstitial and intravascular components, a medium widely different from those routinely applied to nuclei isolation. Previous observations, made with the cytoplasmic-gradient procedure, have shown that nuclei behave as a low density component of the homogenate whose density gradually increases, during subcellular fractionation (15).

The present results show that the organization of low density nuclei corresponds to a metastable equilibrium state, and is apparently similar to that of the organelle *in situ*. An expanded version of these findings will be published elsewhere (16). Low density nuclei retain a large fraction of their protein by a labile mechanism which, in concentrated homogenates, is responsible for the nuclear localization of ATP:NMN-adenylyltransferase. Upon dilution, a secondary strong binding of the enzyme to nuclear components is observed. That is why in diluted homogenates or nuclear suspensions, ATP:NMN-adenylyltransferase can not be used as an indicator of the nuclear system responsible for retaining a large fraction of the nuclear proteins. High density nuclei have a low protein content and no longer bind ATP:NMN-adenylyltransferase, suggesting that both protein loss and increase in density result from the same alteration. It is also shown, by sucrose gradient fractionation of concentrated homogenates containing either high or low density nuclei, that prior damage seems to be required in order to isolate nuclei by the usual sucrose gradient procedures.

METHODS

Male Wistar rats, weighing 170 to 230 g, were used throughout. The animals were kept under artificial illumination, with alternating 12-hour periods of light and darkness; food was supplied ad libitum. Killing was made by decapitation, during the third hour of the illuminated period.

Established procedures were used for the determinations of DNA (17), RNA (18, 19), protein. (20), alkaline phosphodiesterase I, (21), phosphoglucomutase (22) and cytochrome oxidase (23). NAD production was measured for ATP:NMN-adenylyltransferase, in a cycling reaction (24) involving ethanol and cytochrome-c

dismutation; the procedure, to be published elsewhere (16), gives a hundred-fold increase in sensitivity over that of Kornberg (25).

Homogenization and fractionation, carried on without addition of diluent, were performed as previously reported (15), with the modifications stated in the text. Briefly, the procedure involves filtration of the livers through a stainless steel sieve (Harvard tissue press) and homogenization in a standard teflon-glass homogenizer (A.H. Thomas, size C). Previous liver perfusion plus the rotational speed, clearance, and the number of strokes involved in the homogenization have been shown to be critical (16). Homogenization, performed by once displacing the rotating pestle to the bottom of the glass vessel, and then stopping and removing the pestle by manual traction, is defined as homogenization by half a stroke.

The undiluted homogenate is fractionated by centrifugation induced stratification of its components in an SW 65 Beckman rotor at 39,000 rpm. At the end of the centrifugation period, the tube is sliced into eight fractions of similar volume. After measuring its density, by isopycnic equilibration of a small fragment in a petroleum ether and o-dichlorobenzene organic gradient (26), the paste-like content of the fractions plus an aliquot of the homogenate are diluted for assays.

Additional descriptions of the methods employed are given in the legends to figures.

RESULTS

Changes in nuclei during prolonged centrifugation of undiluted homogenates

During the first hour of centrifugation, the apparent density of nuclei is lower than that of the homogenate, then it gradually increases to values higher than that of the homogenate. This phenomenon is reflected first in flotation, later in sedimentation of nuclei (Fig. 1). The sedimentation observed could also be accounted for by the progressive decrease in the density of the top fractions, subsequent to centrifugal migration of high specific gravity components like glycogen and mitochondria. From density measurements it has been established that both factors —decrease in medium density and increase in nuclear density— are involved in nuclear sedimentation. In all cytoplasmic gradients where nuclear sedimentation has been observed, the density at the peak of DNA is higher than that of the original homogenate. In addition, during prolonged centrifugation nuclei lose their ability to retain the nuclear enzyme ATP:NMN-adenylyltransferase (Fig. 2).

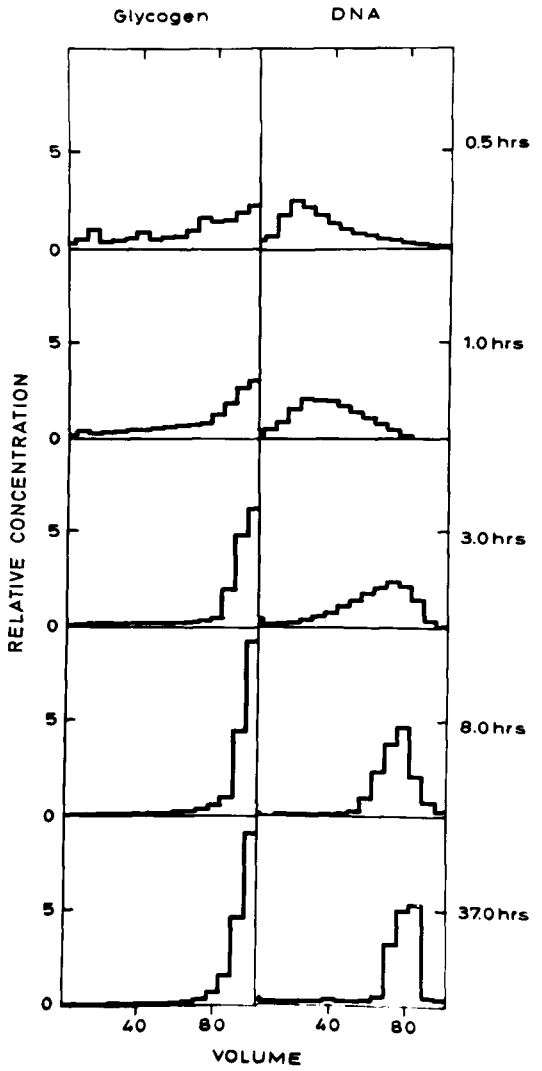

Fig. 1. Time course of centrifugation-induced nuclear stratification during cytoplasmic-gradient fractionation. Undiluted homogenates from unperfused livers centrifuged at 39,000 rpm for the time intervals indicated. The graphs show relative purification versus cumulative volume from the meniscus. Glycogen behaves as a dense component which gradually sediments into the bottom fraction. Nuclei, as shown by the DNA profiles, behave initially as low density components floating towards the meniscus but, after 1 hour, their relative density apparently increases since the organelles start sedimenting. Equilibrium is finally reached at a density higher than that of the homogenate, indicating that a change in the physical properties of the organelle has occurred. Data taken from Leighton *et al.*, (15).

Nature of the ATP:NMN-adenylyltransferase binding to nuclei

The release of ATP:NMN-adenylyltransferase observed during centrifugation also occurs

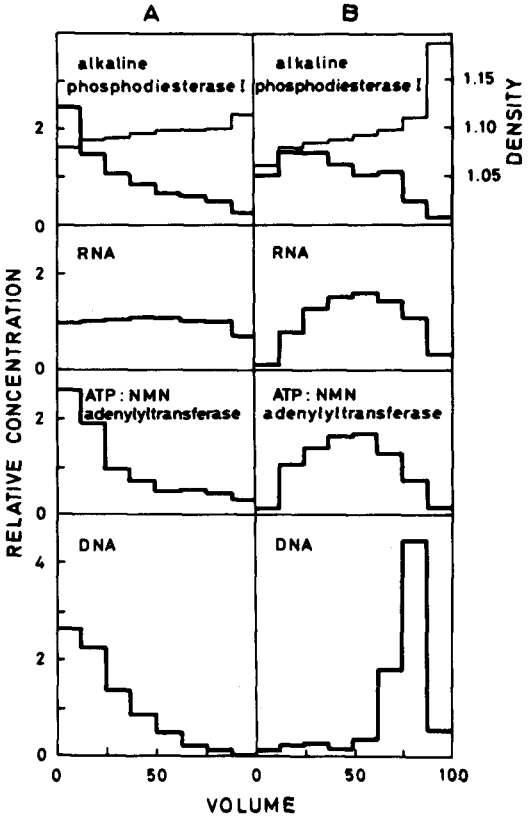

Fig. 2. Release of ATP:NMN-adenylyltransferase from nuclei in homogenates submitted to prolonged centrifugation. Unperfused liver homogenized by half a stroke of a normal clearance homogenizer at 1400 rpm. The undiluted homogenate was centrifuged at 2°C. 39000 rpm, for 10 min (colum A), or 13 hours (column B). The density of the fractions is shown in thin line graphs. The distributions of DNA and ATP:NMN-adenylyltransferase show that high density nuclei no longer bind the enzyme which now follows an RNA-like distribution. Plasma membrane fragments also behave as a low density component; the change in distribution observed after 13 hours centrifugation would simply reflect the change in density profiles.

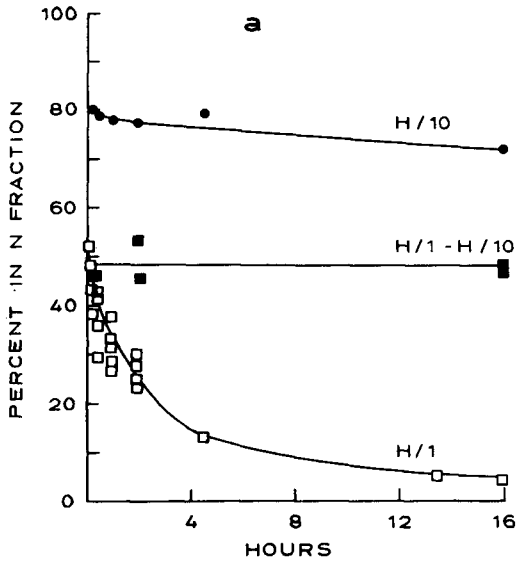

Fig. 3a. Release of ATP:NMN-adenylyltransferase as a function of time and concentration of the homogenate. H/1: Unidiluted homogenate prepared by half a stroke at 1400 rpm, kept at 0° and diluted to 1/10 w/v with 0.25 M sucrose at the time intervals shown. H/1-H/10: Homogenate prepared as for H/1, adjusted to 1/10 w/v inmediately after preparation. H/10: homogenate prepared 1/4 w/v in 0.25 M sucrose with two strokes of the same homogenizer, then diluted to 1/10. The amount of enzyme retained by nuclei is estimated from the proportion bound to an unwashed N fraction prepared without additional dilution (43).

ATP:NMN-adenylyltransferase remains bound to the nuclear fraction in diluted homogenates, conforming a well established fact (27); however in concentrated homogenates, the enzyme is released from nuclei.

upon storage of concentrated homogenates at 0° (Fig. 3a). It ceases after dilution with 0.25 M sucrose, and is not observed in homogenates prepared directly in 0.25 M sucrose at concentrations of 1/3 g liver/ml or lower. Addition of 0.2 M KCl to the sucrose homogenization medium results in a complete release of the enzyme with no nuclear damage detectable by phase contrast microscopy. This, and similar observations, allow to postulate that in homogenates diluted with 0.25 M sucrose, the fixation of the enzyme to nuclei, corresponds to a secondary electrostatic interaction. This type of interaction is not possible *in situ* because of the high ionic content of nuclei. As a consequence, another mechanism must be proposed for the nuclear localization of ATP:NMN-adenylyltransferase in the cell.

In liver tissue, kept for several hours at 0° before homogenization in 0.25 M sucrose, the enzyme remains bound to nuclei (Fig. 3b) The same behaviour is observed at 37° (results not shown). Then, a mechanism must exist capable of retaining the enzime in the abscense of normal energy supply and under normal, tissue composition.

Such a mechanism should be mechanically labile since release occurs as soon as the tissue is homogenized, provided the homogenate remains concentrated.

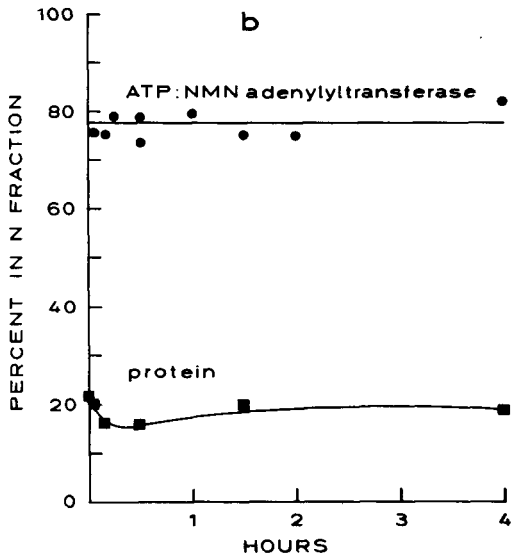

Fig. 3b. Stability of the nuclear binding of ATP:NMN-adenylyltransferase in undisrupted liver tissue. At the intervals shown, pieces of liver tissue kept at 0° were homogenized in 0.25 M sucrose at a 1/4 w/v concentration, diluted to 1/10 and fractionated into a crude nuclear fraction and a postnuclear supernatant. Protein was measured as a control.

This observation indicates that the binding of ATP:NMN-adenylyltransferase to nuclei does not require metabolic activity and further, that it is not destroyed by the tissue ionic environment which is similar to that of undiluted homogenates. Together with the observation from Fig. 3a, these evidences indicate that the homogenization step is responsible for the release observed in undiluted homogenates.

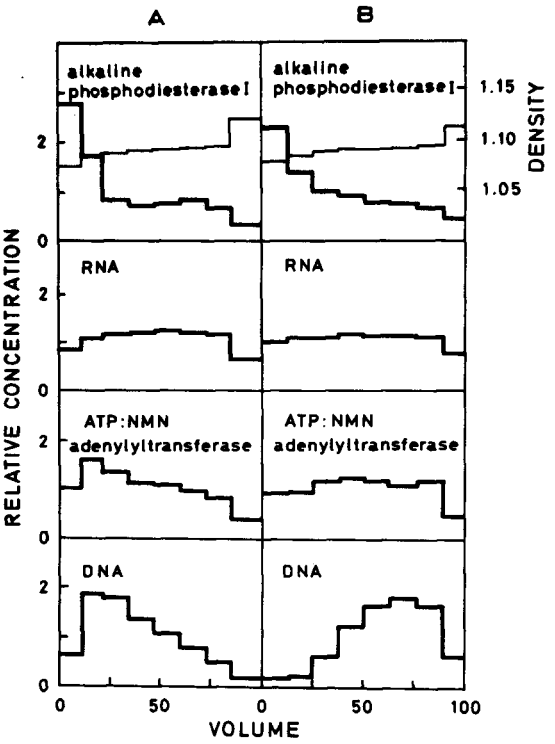

Fig. 4. Release of ATP:NMN-adenylyltransferase from nuclei induced by the application of shearing forces of different intensities for the homogenization. Unperfused livers homogenized at 1400 rpm by half a stroke (colum A) or two strokes (colum B) of a normal clearance homogenizer. Fractionation at 2°C, 30 000 rpm, for 30 min.

Increased shearing forces lead to nuclei which behave as dense components with decreased retention of ATP:NMN-adenylyltransferase. Observations by phase contrast microscopy and on Azur B stained smears of fractions and homogenates, allow to discard nuclear fragmentation as the explanation for the release of the enzyme.

Short-term cytoplasmic-gradient fractionation of homogenates prepared with increased shearing forces (Fig. 4), confirms the previous conclusion, i.e., mechanical damage favors ATP:NMN-adenylyltransferase release. These results also illustrate that nuclei which have lost the ability to retain the enzyme have a higher density. Further support for the correlation among mechanical damage and enzyme release is given by the kinetics of release observed in concentrated homogenates prepared by shearing forces of different magnitudes. (Fig. 5).

Sucrose gradient fractionation of concentrated homogenates

A maximal nuclear retention of ATP:NMN-adenylytransferase is observed in concentrated homogenates prepared from livers perfused with 0.15 M sucrose. The hypotonicity of the perfusion fluid leads to tissue hydration, approximately 10-15% by weight. Presumably because of this hydration, which greatly increases the fluidity of the concentrated homogenates, there is less nuclear damage (Fig. 5) and also a sharper band of low density nuclei after cytoplasmic gradient fractionation of these homogenates (results not shown).

To attempt sucrose gradient fractionation of concentrated homogenates, the homogenate from hypotonically perfused livers was chosen because of its low apparent viscosity and better nuclei preservation. In addition, homogenates

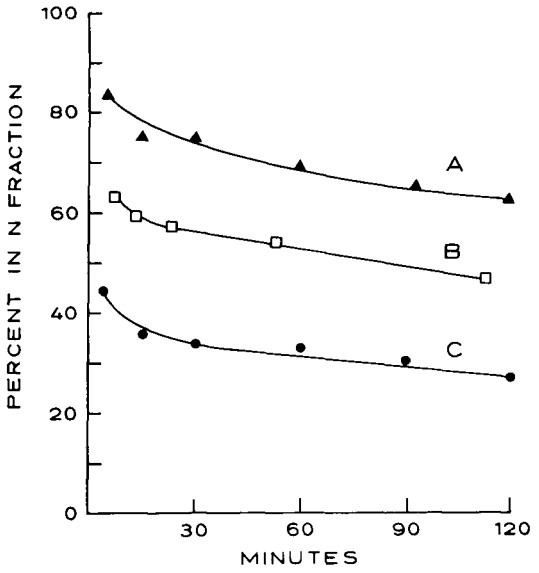

Fig. 5. Influence of the homogenization conditions on the binding of ATP:NMN-adenylyltransferase to nuclear fractions prepared from undiluted liver homogenates.

Curve A: Homogenization with a large clearance glass-teflon homogenizer (A.H. Thomas, size C, pestle diameter reduced by 0.1 mm), half a stroke at 1400 rpm. The liver was previously perfused with cold 0.15 M sucrose. Curve B: Homogenization as for curve A, but perfusion made with 0.25 M sucrose. Curve C: Homogenization with 2 strokes of a normal clearance homogenizer, at 1400 rpm, without prior perfusion. The undiluted homogenates were kept at 0°; dilution to 1/10 w/v in 0.25 M sucrose and fractionation were performed at the time intervals shown.

On the basis of this and other evidences (16), it is concluded that the small degree of tissue hydration which follows hypotonic perfusion, results in decreased shearing during homogenization. The conditions for curve C correspond approximately to the maximal shearing forces that nuclei will sustain in undiluted homogenates before signs of generalized disruption appear in optical microscopy observations.

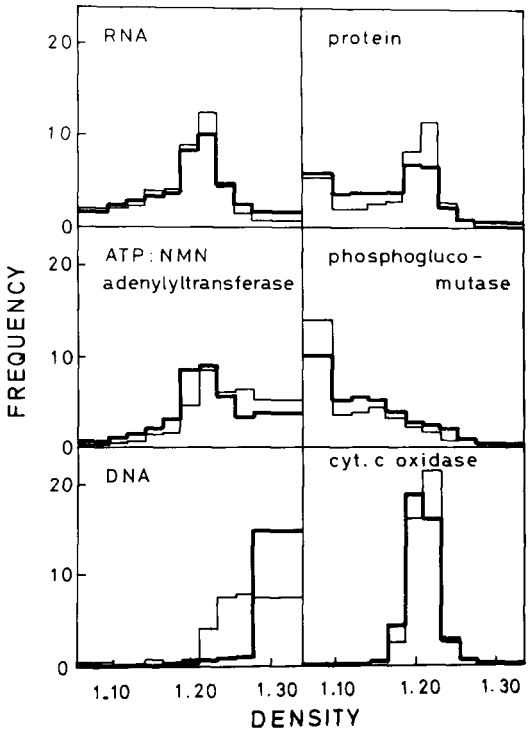

Fig. 6. Sucrose density gradient fractionation of liver concentratated homogenates prepared under low or high shearing forces. Thin-line graphs: Homogenization at 1400 rpm, half a stroke of the large clearance homogenizer after perfusion with 0.15 M sucrose. Thick-line graphs: Homogenization with 2 strokes of a normal clearance homogenizer at 1400 rpm; this homogenate, from an unperfused liver, was slightly diluted with 15% by weight of 0.15 M sucrose in order to match the density and fluidity of the preceding one.

The fractionation was performed layering 0.5 g of each homogenate above 4.6 ml of a continuous sucrose gradient, extending from 1.13 to 1.29 g/ml, plus 0.2 ml of 1.34 g/ml sucrose as a cushion. 1 mM Mg Cl_2 was added to all gradient solutions. Centrifugation was performed in an SW 65 rotor, 30 min at 39000 rpm and 2°C. The results are presented as standardized density distributions (26).

Practically all the nuclei from the homogenate prepared under high shearing forces sediment into the high density bottom fraction, whereas only about 50% of the nuclei from the homogenates prepared with the gentler procedures sediment into the high density bottom fraction. The extranuclear ATP:NMN-adenylyltransferase follows the RNA distribution and not that of phosphoglucomutase, a cell sap marker. The mitochondrial distribution, as shown by the activity of cytochrome c oxidase, is the same for both homogenization conditions.

containing high density nuclei, prepared by mechanical damage, were used to determine if the loss of the nuclear properties of low relative density and high degree of ATP:NMN-adenylyltransferase retention could be detected in sucrose gradients. In order to preserve nuclear organization 1 mM Mg Cl_2 was added to the sucrose solutions. The viscosity of the homogenate containing damaged nuclei was reduced by adding 0.25 M sucrose in a proportion similar to that observed in hypotonic perfusion. As shown in Fig. 6, after 30 min of centrifugation, the nuclei showing high density in short term cytoplasmic gradients have already sedimented beyond the 1.28 g/ml density level. In contrast, about one half of the nuclei displaying low density in cytoplasmic gradients remain at density levels lower than

1.28 g/ml. Of these, one half sediment into the high density bottom fraction after 2.5 hours centrifugation (results not shown).

These observations suggest that the density of well preserved nuclei, in sucrose gradients containing 1 mM Mg Cl_2 , is lower than 1.28 g/ml. Higher values are observed when nuclei are damaged. This is a surprising conclusion since most nuclei isolation procedures are based on the hypothesis that the density of nuclei in sucrose gradients is 1.30 g/ml or more.

Protein content of low and high density nuclei

The subcellular fractionation observations presented in this article routinely include the determination of enzyme markers as follows: Nuclei, DNA, Mitochondria, cytochrome oxidase. Cell sap, phosphoglucomutase. Ribosomes, RNA. Endoplasmic reticulum membranes, glucose-6-phosphatase. Plasma membrane, alkaline phosphodiesterase I. Golgi apparatus, galactosyl transferase. Lysosomes, acid phosphatase. Peroxisomes, catalase.

The protein bound to nuclei was determined indirectly applying previously established procedures based on the relative specific activity of each component (15, 23). The results obtained for cytoplasmic gradient and sucrose gradient fractionation, clearly indicate that nuclei transformation from low to high density state is a process accompanied by the release of a large amount of proteins (Table I). ATP:NMN-adenylyltransferase behaves as a marker for this group of weakly bound nuclear proteins, as long as nuclei remain under ionic strength conditions similar to those of the cytoplasm.

TABLE I

Percent of the total liver protein bound to nuclei in nuclear fractions conteining "low" or "high" density nuclei*

	*Low density nuclei***	*High density nuclei****
Cytoplasmic-gradient	9.1 ± 1.7	4.6 ± 1.3
Sucrose gradient	9.1 ± 1.7	4.0 ± 1.1

*In this calculation it is assumed that the protein remaining after correction for the contribution of contaminant organelles is nuclear. The livers contained 186 ± 21 mg protein and 2.05 ± 0.33 mg DNA per g. Values in the table correspond to averages ± standard deviation.

**Correspond to the peak of nuclei floating afer cytoplasmic-gradient and to the low density nuclear fractions in sucrose gradients.

***Correspond to the peak of DNA in cytoplasmic and sucrose gradient fractionation of concentrated homogenates prepared under conditions leading to the transformation of nuclei into the high density state.

DISCUSSION

The present results establish that liver nuclei, from rats maintained and killed under controlled conditions, display two states in vitro: a metastable initial condition, defined as "low density state", and a subsequent, apparently irreversible condition, defined as "high density state".

Low density nuclei, detected by flotation in short term cytoplasmic-gradient subcellular fractionation, have the following properties: density lower than the homogenate; retention of ATP:NMN-adenylyltransferase under high ionic strength conditions; high protein content; and, a transitory low density state in concentrated sucrose gradients.

High density nuclei -which originate from low density nuclei after either prolonged centrifugation, or mechanical damage, or exposure to hypertonic sucrose— have the following characteristics: high density relative to the homogenate; loss of the ability to retain ATP:NMN-adenylyltransferase; loss of approximately one

half of the nuclear protein and, high density in sucrose gradients.

ATP:NMN-adenylyltransferase is localized in nuclei, as shown by 0.25 M sucrose fractionation (27); non aqueous media nuclei isolation procedures (28), or cytoplasmic-gradient fractionation when short term centrifugation and gentle homogenization procedures are applied. However, the mechanism responsible for the in vitro binding of ATP:NMN-adenylyltransferase to nuclei in concentrated homogenates differs from that observed in dilute homogenates or in fractions suspended in 0.25 M sucrose. In the latter condition, binding to nuclei appears as a strong electrostatic interaction which fixes or stabilizes the enzyme in its original subcellular localization. This interaction does not take place at the high ionic concentration characteristic of cell nuclei *in situ* (29) or of concentrated homogenates (16). The preservation of the mechanism by which nuclei normally retain this enzyme and other nuclear proteins can be monitored by the nuclear content of ATP:NMN-adenylytransferase only when the enzyme does not establish a secondary interaction with insoluble nuclear material.

Should the mechanism damaged during the *in vitro* transformation of nuclei from low into high density state participate *in vivo* in the regulation of nucleo-cytoplasmic transfer, it could also be involved in the physiological control of nuclear activity. If that were the case, the density states of nuclei, determined by the procedures outlined here, or their ability to retain ATP:NMN-adenylyltransferase, should correlate with their functional states. Such correlation is supported by work presently in progress (30), and by the fact that nuclei enlarge and increase their content of proteins and water (31, 32) during activation.

Assuming that the mechanical damage resposible for the change in density state acts directly upon the nuclei, structures like the nuclear envelope or the nuclear matrix, could be involved. The recently described nuclear matrix is supposed to control nuclear shape (33) and maybe dimension (34), but so far very little is known about its function. The nuclear envelope is a well characterized structure, but again not much is known about its function and participation in the control of nuclear activity (2, 3). Proteins and other macromolecules can enter the nuclei from the cytoplasm in a process which is limited by the size of the molecules (35, 36). An apparent patent radius of 45 A for the pores has been calculated (37). The molecular weight of ATP:NMN-adenylyltransferase is 300.000 (38) well above the limits imposed by the pores for nucleo-cytoplasmic transport. These considerations are based on data from various cell types and no evidence indicates that nuclear envelopes of different cells have the same properties. In fact, even the flow of ions seems to be restricted under certain experimental conditions (39, 40).

In the light of these facts the damage of cell nuclei induced by homogenization could be the result either of disruption of the pore organization leading to increased permeability, or of localized damage to the membrane allowing for protein translocation but not affecting the shape of nuclei in optical microscopy controls.

The methods used for the observation of nuclear organization in concentrated homogenates and in cytoplasmic-gradient, constitute simple analytical procedures which allow to define two density states of liver nuclei. In addition, the procedures employed in this work provide methodological elements to explore the functional meaning of the nuclear density states. Mirsky and Osawa (41) concluded: "considering how drastically liver nuclei are altered in the course of isolation, it is doubtful whether observations on isolated liver nuclei have any relevance to an understanding of the nuclear membrane within the cell". If definite proof is established that the functional states of nuclei correlate with the density states defined by cytoplasmic-gradient fractionation, such statement might no longer be valid.

In addition to the observations of nuclear organization in concentrated homogenates, the present work provides new insight into the general principles on which the procedures for nuclear isolation in concentrated sucrose are based. The generally accepted characteristic high density of the organelle (42) might well be the result of nuclear damage. Consequently, isolation procedures relying

on this property to separate nuclei from other subcellular components may have been optimized for the isolation of damaged nuclei, and also for a maximum degree of nuclear transformation into the damaged or high density state.

RESUMEN

Se analiza la organización nuclear en homogenizados de hígado de rata preparados sin agregar diluyente, a los que se somete a diversas condiciones experimentales, incluyendo fraccionamiento subcelular mediante estratificación de sus componentes por ultracentrifugación. Este procedimiento de fraccionamiento subcelular, denominado gradiente-citoplásmico, permite mantener los núcleos en un ambiente similar al natural. Los resultados muestran que en homogenizados concentrados los núcleos exhiben dos estados definidos en función de la densidad del homogenizado o tejido: núcleos de baja densidad, y núcleos de alta densidad. Los núcleos de baja densidad se caracterizan por retener la actividad ATP:NMN-adenililtransferasa, por un alto contenido proteico y por un estado transitorio de baja densidad en gradientes de sacarosa concentrada. Los núcleos de alta densidad se caracterizan por haber perdido la ATP:NMN-adenililtransferasa y aproximadamente la mitad de sus proteínas originales y por comportarse como organelos de alta densidad en gradientes de sacarosa concentrada.

El mecanismo responsable de la union a núcleos de la ATP:NMN-adenililtrasnferasa en homogenizados concentrados parece ser el mismo por el cual se retienen muchas otras proteínas nucleares no histonas, hecho que, potencialmente, le confiere importancia en los procesos de control de la actividad nuclear. En cambio, en homogenizados diluidos, la enzima se une firmemente a núcleos o retículos endoplásmico por una interacción secundaria, aparentemente electrostática.

Los núcleos de baja densidad pueden transformarse, de manera controlada, en núcleos de alta densidad; la situación inversa no se ha observado. El cambio puede inducirse por daño mecánico controlado, por centrifugación prolongada de homogenizados concentrados y además sometiendo homogenizados inicialmente concentrados a centrifugación en gradientes de sacarosa concentrada. Este último hecho junto a la conclusión, derivada de nuestras observaciones, que los núcleos de baja densidad preservan mejor su organización natural, señala que los procedimientos usuales de separación de núcleos en sacarosa concentrada favorecen la preparación de organelos dañados.

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