REFORMATION OF NUCLEOLI AFTER ETHIONE-INDUCED FRAGMENTATION IN THE ABSENCE OF SIGNIFICANT PROTEIN SYNTHESIS

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ABSTRACT

The rat liver nucleolus, after fragmentation induced by ethionine treatment, has been found to undergo complete reformation by adenine in the presence of a dose of cycloheximide sufficient to cause inhibition of protein synthesis by 90–95%. In contrast, actinomycin D given along with adenine was followed by the appearance of a small compact mass containing only the fibrillar component with no evident granules. This structure resembled pseudonucleoli seen in the anucleolate mutant of *Xenopus laevis* or in certain early stages of amphibian oocytes. Actinomycin D administered 2 hr after adenine induced a segregation of the fibrillar and granular components of nucleoli similar to that induced in the normal nucleolus. The implications of these findings in relation to nucleolar organization are briefly discussed.

INTRODUCTION

The hepatic cell nucleoli of rats undergo a progressive disorganization shortly after the administration of ethionine, leading to an eventual fragmentation and scattering of components by 12 hr (8, 12). This nucleolar fragmentation is readily reversible when the basic ATP deficiency induced by ethionine is counteracted by the administration of ATP precursors, such as adenine, or methionine, the natural metabolite of ethionine (12). An important question is whether the reformation of the nucleolar structure after adenine administration is the result of reaggregation of dispersed nucleolar components or *de novo* synthesis of an entirely new organelle. In the present study, the effect of cycloheximide and actinomycin D on the processes of nucleolar reformation was examined. The data suggest that synthesis of new protein is not essential for the restoration of nucleolar structure, and that the reconstruction of nucleoli probably represents reaggregation of nucleolar fragments. However, some DNA-related nuclear function, possibly RNA synthesis, appears to be required for the complete restoration of the nucleolus.

MATERIALS AND METHODS

White female rats of the Wistar strain (Carworth Farms, New City, N.Y.)weighing 160–190 g were used. Rats were given a single intraperitoneal injection of ethionine in a dose of 1 mg per g of body weight after 15–16 hr of fasting. 8 hr after the injection of ethionine, one group of rats was sacrificed without subsequent treatment. The remainder of the rats were given one of the following treatments: (*a*) adenine and saline; (*b*) adenine and cycloheximide; (*c*) adenine and actinomycin D; (*d*) adenine followed 2 hr later by actinomycin D; (*e*) saline; (*f*) no treatment, and were sacrificed 4 hr later. In one group, rats were sacrificed 2 hr after the

injection of adenine and saline. Control animals received an initial injection of saline followed 8 hr later by an injection of adenine alone. Adenine was given as adenine sulfate in an aqueous solution in a dose of 0.16 mmoles. Cycloheximide (Actidione, Upjohn Co., Kalamazoo, Mich.) was given in a dose of 2.5 mg per kg of body weight, and actinomycin D (Lyovac, Merck, Sharp & Dohme, West Point, Penna.) in a dose of 1 μg per g of body weight.

For electron microscopy, pieces of liver were fixed in osmium tetroxide in phosphate buffer (p^{H} 7.3) and embedded in Epon (7). The sections were stained with lead hydroxide (5) and examined with a Philips EM 100B or EM. 200 microscope. Protein synthesis, as measured by incorporation of L-leucine-1-^{14}C into total and nuclear liver protein, was determined as previously described (18). The nuclei were prepared as previously reported (21).

RESULTS

Table I shows the results of the incorporation studies. It is evident that the administration of cycloheximide continued or intensified the inhibition of protein synthesis seen with ethionine alone. The degree of inhibition in the nucleus was approximately the same as that found for total liver protein.

The nucleolar alterations 8 hr after the ethionine injection were characterized by fragmentation of nucleolar components (Fig. 1). Fragments varied in size and were frequently surrounded by small satellite granules. In accord with previous observations (12), virtually all the hepatic cells were affected. The accentuation of the chromatin pattern and its margination, and the aggregation of interchromatinic granules were prominent changes in the nucleoplasm. The nucleolar lesions 12 hr after the ethionine injection were very much the same as the lesion at 8 hr. Despite the fragmentation, the administration of adenine at 8 hr induced a rapid restoration of nucleolar structure, such that the nucleoli were normal or almost so when viewed 4 hr later. Rats sacrificed 2 hr after the administration of adenine showed many nuclei which contained abortive forms of nucleoli characterized by thin twisted rodlike structures (Figs. 2, 3).

The simultaneous administration of cycloheximide with adenine 8 hr after ethionine did not interfere with the process of nucleolar reformation. 4 hr after the injection of adenine plus cycloheximide, all nucleoli showed a structure close to

TABLE I

Effect of Ethionine, Adenine, and Cycloheximide on the Incorporation of Radioactive Leucine into Total and Nuclear Protein of Liver

Exp. No.	Treatment* Initial	Treatment* Subsequent	Cell fraction	Incorporation‡ into protein	Radioactivity of acid soluble fraction	Corrected values	Inhibition
				cpm/mg	*cpm/0.5 ml*		%
1	Saline (2)	Adenine +	Total	987±380	1556±283	0.62±.13	—
		Saline	nuclear	813±409		.54±.16	—
	Ethionine (2)	Saline +	Total	36±3	1384±375	0.025±0.002	96
		Saline	nuclear	39±1		0.028±0.008	95
	Ethionine (2)	Adenine +	Total	57±3	6175±422	0.045±0.005	93
		Cycloheximide	nuclear	27±8		0.004±0.000	99
2	Saline (3)	Adenine +	Total	1346±197	1693±163	0.805±0.18	—
		Saline	nuclear	1103±142		0.680±0.14	—
	Ethionine (3)	Saline +	Total	117±24	2489±990	0.049±.013	94
		Saline	nuclear	99±29		0.041±0.01	94
	Ethionine (3)	Adenine +	Total	106±8	5092±93	0.020±.001	98
		Cycloheximide	nuclear	86±5		0.016±0.0007	98

* The *initial* treatment refers to that given at zero time. The dose of ethionine was 1 mg/g body weight given intraperitoneally. The *subsequent* treatment refers to that given at 8 hr. The animals were killed 4 hr later at 12 hr. The dose of adenine was 0.16 mmoles and that of cycloheximide 2.5 mg/kg body weight. The number of animals is in parentheses.

‡ 5 μc L-leucine-1-^{14}C (20 mc/mmole), 0.2 ml was injected intraperitoneally 20 min before the animals were killed.

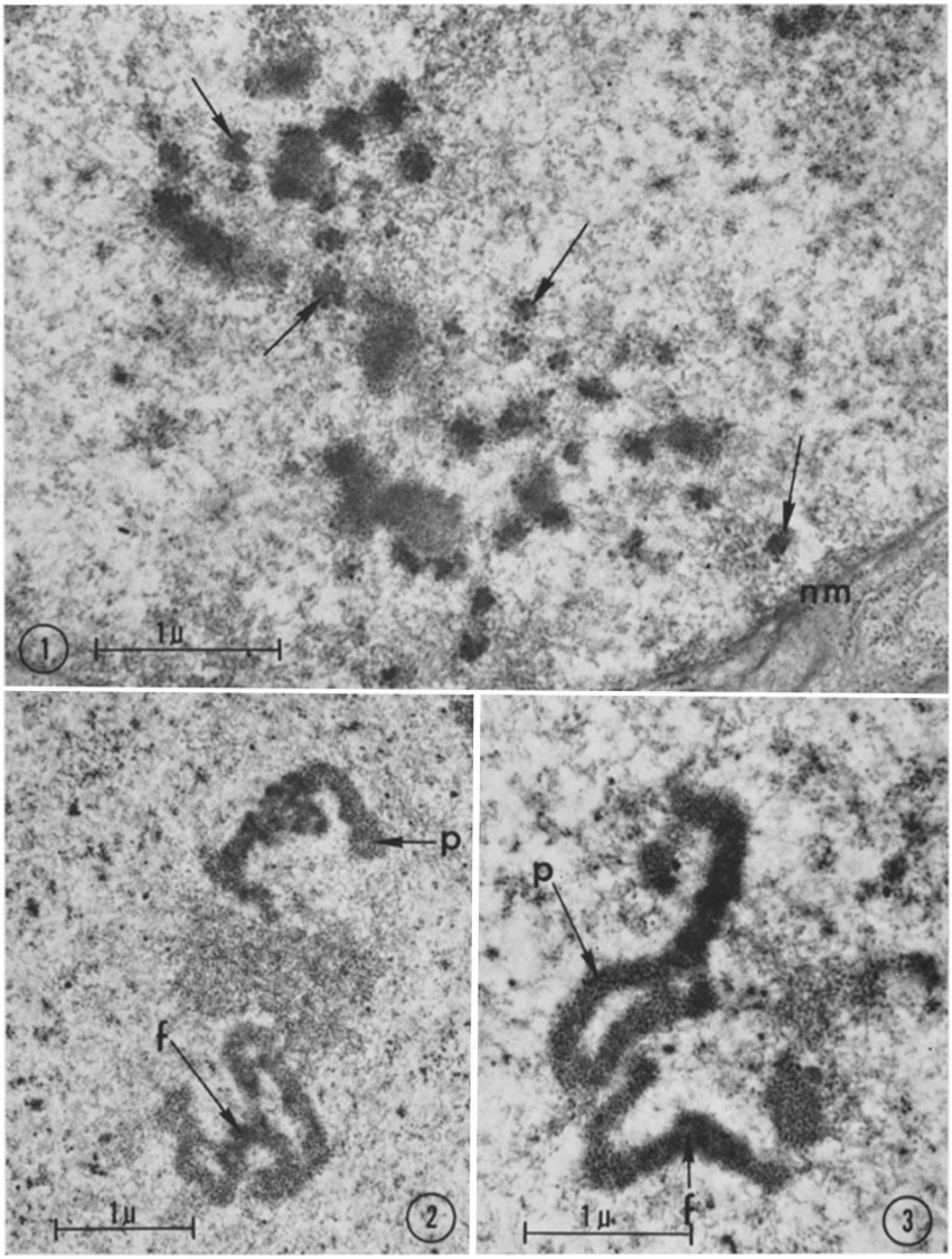
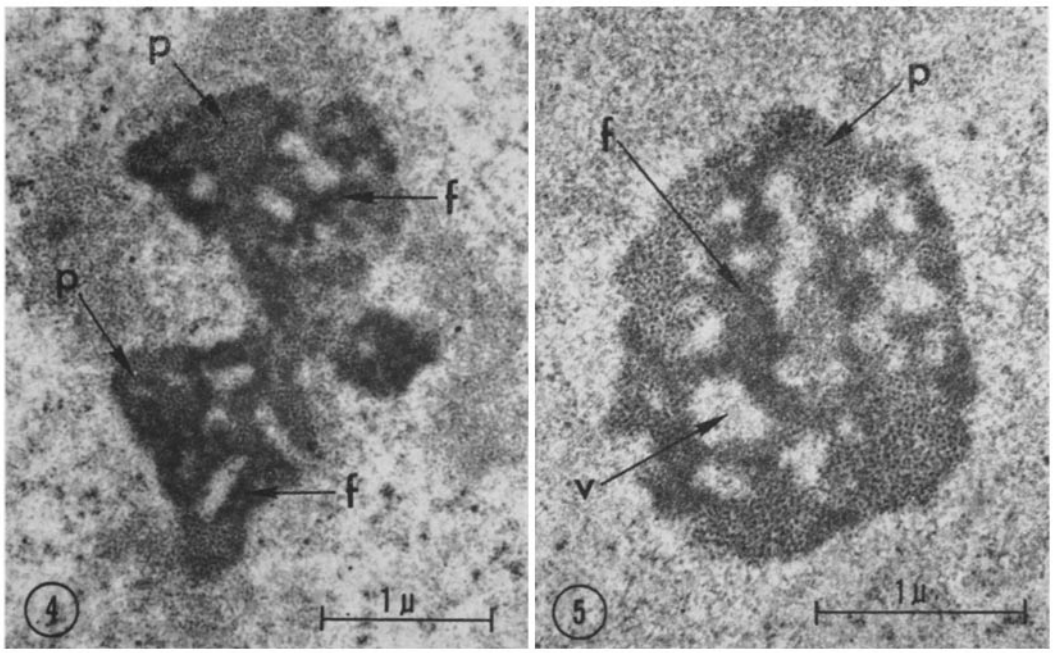


FIGURE 1 Typical fragmentation of hepatic cell nucleolus 8 hr after the ethionine injection. Small fragments are indicated by arrows. Some of them show satellite granules. *nm*, nuclear membrane. × 27,000.
FIGURES 2 and 3 Rodlike structure found in nuclei of hepatic cells in rats given adenine 8 hr after the ethionine injection and sacrificed 2 hr later. The rodlike structures consist of particulate (*p*) and fibrillar (*f*) elements of nucleolus. Fig. 2, × 20,000; Fig. 3, × 24,500.



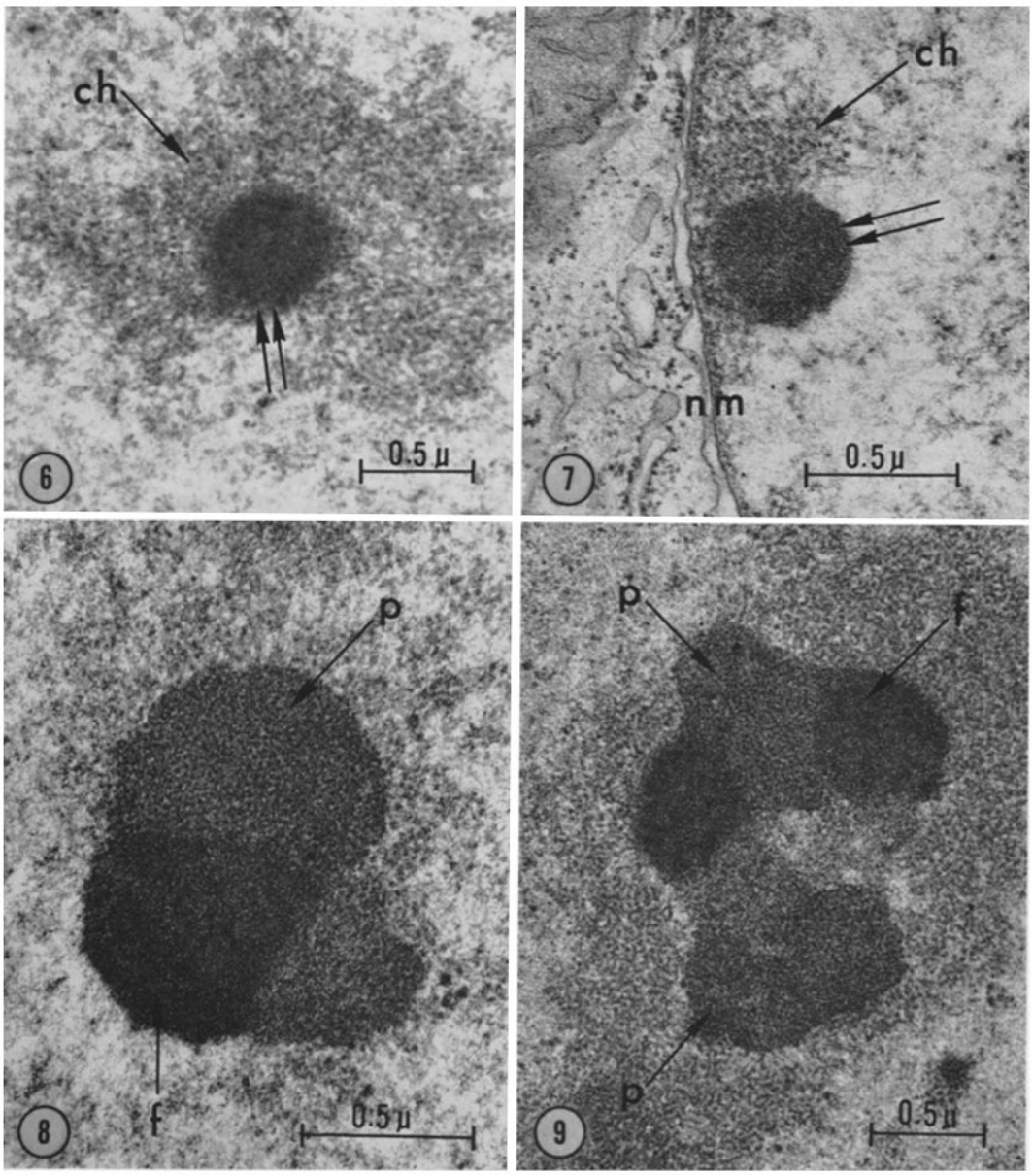
FIGURES 4 and 5 Hepatic cell nucleoli of rats given adenine and cycloheximide 8 hr after the ethionine injection and sacrificed 4 hr later. Nearly complete restoration of nucleolar structure is evident. Nucleoli consist of fibrillar (*f*) and particulate (*p*) components and form distinct nucleolar vacuoles (*v*). Fig. 4, × 22,500; Fig. 5, × 28,000.

normal (Figs. 4, 5). Occasional nucleoli showed a slight distortion of their shape (Fig. 4), although the arrangement of the nucleolar components was similar to that seen in normal nucleoli. Accentuation of chromatin pattern and its margination, and the aggregation of interchromatinic granules became less prominent after treatment with adenine plus cycloheximide. However, the reversal of these alterations was less complete and somewhat variable in extent, as compared to those of the nucleolus.

The results with actinomycin D were in sharp contrast. This antibiotic, when given together with adenine 8 hr after the ethionine injection, completely inhibited normal nucleolar reformation. In this group, no organized nucleoli were found in any hepatic cell nuclei. Many nuclei contained small, round masses which consisted mainly of dense fibrillar material without any evident granular component (Figs. 6, 7). These small masses were frequently embedded in the condensed chromatin material. Some of them were located close to the nuclear membrane (Fig. 7). The change in the nucleoplasm was the same as observed with ethionine treatment alone. When actinomycin D was given 2 hr after the administration of adenine, the appearance of the nucleoli was entirely different. They now contained both fibrillar and granular components, but these components were segregated into distinct zones (Figs. 8, 9). This appearance was more or less the same as that of liver nucleoli in animals given actinomycin D alone without pretreatment with ethionine (10, 11, 13–15).

DISCUSSION

Although the mechanism(s) involved in the ethionine-induced nucleolar fragmentation and its reversal by adenine is not established, an attractive working hypothesis is that the processes are probably related to the biochemical changes in hepatic cells initiated by the fall of hepatic ATP concentration (12). ATP deficiency induced by ethionine rapidly leads to the inhibition of RNA and protein synthesis (19–21). It is possible, therefore, that nucleolar alterations induced by ethionine may be a reflection of the disturbance of RNA metabolism and/or protein metabolism, and that reformation



FIGURES 6 and 7 Small, round fibrillar masses (double arrows) found in nuclei of hepatic cells in rats given adenine and actinomycin D 8 hr after the ethionine injection and sacrificed 4 hr later. *ch*, chromatin; *nm*, nuclear membrane. Fig. 6, × 30,000; Fig. 7, × 41,000.

FIGURES 8 and 9 Hepatic cell nucleoli of rats given adenine 8 hr after the ethionine injection, then 2 hr later actinomycin D, and sacrificed 2 hr later. A sorting out of nucleolar components is evident. *p*, particulate component; *f*, fibrillar component. Fig. 8, × 47,000; Fig. 9, × 31,000.

of nucleoli may follow the recovery of these metabolic processes. It is evident from the present studies that the synthesis of new protein is not essential for the complete restoration of liver cell nucleoli from the fragmentation induced by ethionine. Since protein is one of the major constituents of normal nucleoli (1, 22), it is not likely that this reformation of nucleolar structure constitutes

synthesis of a new organelle. The fact that reformation occurs with nearly complete absence of protein synthesis suggests the possibility that the recovery of nucleolar structure is due to the reaggregation of dispersed nucleolar fragments. However, it should be pointed out that a pool of ribosomal proteins is known to occur in cells and that, at least in some cells, this pool may last for several hours after almost complete inhibition of protein synthesis (9, 23). Conceivably, a comparable pool of nucleolar protein may exist in the liver, and such a pool may actively participate in the reformation of the nucleolus after disorganization by ethionine. Under such circumstances, the reformation may be more complex than the reunification of the broken-up fragments.

These results suggest that the inhibition of protein synthesis induced by ethionine is not the major factor in the fragmentation of the nucleolus seen with this methionine analogue. Consistent with this conclusion is the finding that cycloheximide administered by itself at a dose sufficient to inhibit protein synthesis about 95% had no evident effect upon the ultrastructure of the liver cell nucleolus within a 12 hr time period (17). Thus, it is tempting to consider that the ATP deficiency *per se* induced by ethionine, rather than the inhibition of protein synthesis resulting from the low ATP concentration, is the major biochemical basis for the nucleolar fragmentation. Hopefully, the further analysis of this nucleolar reformation, especially in vitro, may give new insight into the nature of the smaller fragments and how they are held together to form a nucleolus.

The role of synthesis of new RNA during the process of nucleolar reformation is more difficult to analyze, since many agents which selectively inhibit RNA synthesis induce nucleolar alteration (13) and at least some of them have biochemical effects in addition to the inhibition of RNA synthesis (6, 16). In the present study, the administration of actinomycin D together with adenine 8 hr after the ethionine injection inhibited the nucleolar reformation and resulted in the appearance of small dense fibrillar masses in the nuclei. It is especially noteworthy that these dense fibrillar masses resemble the pseudo-nucleoli of the anucleolate mutant of *Xenopus laevis* described by Hay (2) and by Jones (3) and the primitive nucleoli found in the amphibian oocytes described by Karasaki (4). Since the anucleolate mutant is deficient in ribosome formation and since actinomycin D is known to have an inhibitory effect on the synthesis of ribosomal RNA (9), it is possible that the nucleolus seen in each of these conditions represents an abortive form of organization of this organelle in a state of functional frustration vis-a-vis the ribosome. Possibly, the synthesis of one or more essential components of the ribosome is required for the proper reformation of the nucleolar structure from its fragmented precursor. If this proves to be so, the major essential component of the ribosome for this reformation is RNA rather than protein, since the granular component of nucleoli appears in normal amount in the animals treated with cycloheximide. This agrees well with the findings of Warner et al. (23) who reported that ribosome formation continues for some time in HeLa cells in the presence of sufficient cycloheximide to inhibit protein synthesis by over 90%. Thus, both studies suggest that a pool of ribosomal proteins exists preformed at any one time in either the liver or HeLa cells and that this pool is readily available for ribosome formation.

The observation that actinomycin D, when given 2 hr after adenine, induced typical segregation of the nucleolar components offers further evidence that these ropelike structures probably represent simplified precursors of normal nucleolonema. The further study of each of these different types of liver cell nucleoli or precursors with a combined biochemical-structural approach may help to throw new light upon how the nucleolus is organized to perform its major function in RNA and protein synthesis.

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