Studies on Acute Methionine Toxicity

I. Nucleolar Disaggregation in Guinea Pig Hepatic Cells with Methionine or Ethionine and Its Reversal with Adenine.

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The effects of methionine and ethionine on the fine structure of hepatic cell nucleoli of guinea pigs and rats were investigated. A single intraperitoneal injection of methionine into guinea pigs results in the disruption of nucleolonema as early as 2 hours after the injection. By 4 hours, nucleoli show complete fragmentation consisting of many small fragments and small remnants of nucleoli. Large aggregates of interchromatinic granules and condensation of chromatin appear in the nucleoplasm. These changes are remarkably similar to the lesions induced by ethionine in the liver of the rat or the guinea pig. The methionine-induced nuclear and nucleolar lesions persist up to 10 hours after the injection. The administration of adenine 4 hours after the methionine injection reverses the nucleolar lesions by 8 hours. The appearance of incompletely reconstructed nucleoli with twisted rope-like structures suggests a pattern of recovery very similar to the adenine-induced nucleolar reformation in ethionine-treated rats. Injecting methionine into rats induced no nucleolar abnormalities. It is suggested that the mechanism of nucleolar fragmentation induced by methionine or ethionine is related to the accumulation of S-adenosyl compounds with concomitant ATP deficiency in the liver. (Amer J Path 64:241–256, 1971)

ETHIONINE, the ethyl analogue of methionine, induces severe disorganization of hepatic cell nucleoli shortly after it is administered to rats.[1-3] The nucleolar changes are either prevented or reversed by the administration of ATP precursors, such as adenine.[1] It

was suggested that the nucleolar lesions are probably related to the rapid and persistent fall of hepatic ATP concentration, one of the early metabolic effects of ethionine in the rat liver.[4,5] The ethionine-induced ATP deficiency is due to an imbalance between the rates of (1) trapping of adenosine as S-adenosylethinonine; (2) regeneration of adenosine through transethylation; (3) synthesis of adenine nucleotides *de novo*.[6]

Before methionine can be utilized for the purpose of transmethylation, it must first be activated by the enzyme, ATP-S-adenosyltransferase, to form S-adenosylmethionine.[7,8] Although the accumulation of S-adenosylmethionine in liver after the administration of excess methionine does occur, the magnitude is small and the effect is transient in the rat.[6,9] However, in the guinea pig, as reported by Hardwick et al,[10] the administration of excess methionine does induce a prolonged drop of hepatic ATP concentration, which appears to be related to the relatively large accumulation of S-adenosylmethionine and S-adenosylhomocysteine.

In order to assess the role of ATP deficiency in the induction of nucleolar changes, we have investigated the comparative effects of excess methionine and of ethionine on the nucleolar fine structure of guinea pig hepatic cells.

Materials and Methods

Albino guinea pigs of both sexes (Hilltop Laboratory Animals, Inc, Scottsdale, Pa) weighing 200–300 g each and maintained on Rockland guinea pig diet (Teklad, Inc, Monmoth, Ill), and female Wistar rats (Carworth Farms, New City, NY) weighing 160–180 g each and maintained on Wayne Lab Blox (Allied Mills Inc, Chicago, Ill) were used. In all experiments, animals were fasted overnight before the experiments began and then during the entire experimental period. All injections were given intraperitoneally. The nuclei of hepatic cells were examined in the following groups of animals.

Group I. Guinea pigs were given a single injection of L-methionine (Eastman Organic Co, Rochester, NY) in a dose of 0.5 or 1.0 mg/g of body weight. Groups of animals consisting of 4 or 5 guinea pigs were sacrificed every 2 hours up to 10 hours. Four control guinea pigs were injected with physiologic saline in an amount equivalent to the methionine, and were sacrificed 4 and 8 hours after injection.

Group II. Four guinea pigs were injected with 0.16 millimoles of adenine sulfate (Nutritional Biochemical Corp, Cleveland, Ohio) 4 hours after the L-methionine was injected and were sacrificed 2 or 4 hours later.

Group III. Four guinea pigs and 4 rats were each injected with DL-ethionine (Calbiochem, Los Angeles, Calif) or L-methionine, respectively, in a dose of 1 mg/g of body weight. They were all sacrificed 8 hours after the injection.

Tissue was removed from the left lateral lobe of the liver while the animals were under ether anesthesia. Small pieces of tissue were fixed in 1% osmium tetroxide in phosphate buffer for 1 hour at 4 C and for 30 minutes at room temperature. The tissue was dehydrated at room temperature in a graded series of ethanol solutions and was embedded in a mixture of Epon 812 and Araldite.[11] Sections were cut with glass knives on Porter Blum MT2 ultramicrotome. Sections were stained with lead hydroxide alone [12] or with uranyl acetate followed by lead citrate.[13] They were examined by Phillip EM 300 or Siemens 101 electron microscopes.

Results

Control Guinea Pigs

The fine structure of nuclei and nucleoli of guinea pig liver cells was essentially the same as that of rat liver cells previously described.[1,14] Nuclei were usually round with a distinct nuclear membrane (Fig 1). With the fixative used in the present study, the nucleoplasm appeared homogeneous and the chromatin was visualized only faintly. Occasional small aggregates of interchromatinic granules and perichromatin granules were noted. The nucleolus appeared compact and contained three distinct components, granular, fibrillar and light fibrillar, which were intimately intermingled and formed a skein-like arrangement called nucleolonema. Nucleolus-associated chromatin and intranucleolar chromatin was not distinct.

Methionine-Treated Guinea Pigs

Two levels of dosage (0.5 mg or 1 mg/g) induced similar nucleolar lesions at various time intervals after injection. However, with the higher dosage animals appeared sick by 10 hours and they usually died within 24 hours. Changes in the nuclear and nucleolar structure in both female and male guinea pigs were similar.

Two hours after the methionine was injected, the nucleolar changes were obvious in the majority of the hepatic parenchymal cells. They began to lose their compact structure and showed disruption of nucleolonema (Fig 2). In such nucleolonema, granular and fibrillar components were still recognized. By 4 hours, almost all the hepatic parenchymal cells, regardless of their zonal distribution, showed severe nucleolar changes. In contrast, the nucleoli of biliary epithelial cells and Kufpper cells showed no abnormalities.

While many nucleoli in this group showed no trace of the normal configuration of nucleolonema, in occasional nucleoli in 1 animal nucleolonema persisted and small dense spherules appeared adjacent to the partially unwound nucleolus, forming into small electron-opaque masses (Fig 3 and 4). Small spherules measured about 0.2–

0.5 μ in diameter and consisted of a central dense core, associated
with small granules of about 300–450 Å diameter at their periphery
(Fig 5). Frequently, irregularly shaped masses composed of granular
and/or fibrillar components of normal nucleoli were observed in the
midst of dispersed fragments (Fig 5). In the nucleoplasm, the chro-
matin pattern became distinct around the nuclear membranes and
the areas of distorted nucleoli (Fig 4). In addition, large aggregates
of interchromatinic granules appeared in the nucleoplasm. These
granules were smaller than granules of normal nucleoli and appeared
in clusters (Fig 4).

Nucleolar lesions and the accompanying nuclear changes per-
sisted in the hepatic cells of guinea pigs sacrificed 6, 8 or 10 hours
after the methionine injection (Fig 6 and 7). The changes were es-
sentially similar to those described above, although variation in forms
from one cell to another was apparent. Even in these groups, the
nucleoli of neither the Kupffer cells nor biliary epithelial cells were
altered.

Effects of Adenine on the Methionine-Treated Guinea Pigs.

When guinea pigs were given adenine sulfate 4 hours after meth-
ionine was injected and were sacrificed 2 hours later, the majority
of hepatic cell nucleoli were fragmented. However, 4 hours after ad-
enine was injected, many nucleoli no longer had any signs of frag-
mentation. Instead, various forms of simplified nucleoli were ob-
served. The nuclei of many hepatic cells contained multiple small
nucleoli consisting of granular and fibrillar components of normal
nucleoli (Fig 8). Some of these simple nucleoli showed a twisted
rope-like structure with constant width of 0.5 μ and with varying
lengths. These rope-like structures formed loops encircling large nu-
cleolar vacuoles (Fig 9 and 10). These structures resemble the nu-
cleoli of rat hepatic cells during adenine-induced or spontaneous
recovery after ethionine administration.[1,3] Some nucleoli showed an
apparent fusion of simplified forms (Fig 11) and some showed al-
most complete recovery (Fig 12). It was also apparent that the in-
tensity of chromatin patterns and aggregates of interchromatinic gran-
ules became less prominent (Fig 8).

Ethionine-Treated Guinea Pigs

In hepatic cell nucleoli of guinea pigs 8 hours after ethionine was
injected, nucleolonema were disrupted and fragmented into small
spherules (Fig 13). The changes were similar to those of ethionine-

treated rats. Changes in the chromatin pattern and aggregates of interchromatinic granules were also present (Fig 13).

Methionine-Treated Rats

No nucleolar abnormalities were noted in the hepatic cells of rats sacrificed 8 hours after L-methionine of a higher dosage was injected.

Discussion

It is evident from this study that the nuclear and nucleolar alterations induced in guinea pig liver by methionine are essentially the same as the lesions induced in rat and guinea pig liver by ethionine. It is also clear that the methionine-induced nucleolar lesions in guinea pigs can be reversed with adenine and that the pattern of reversal appears to be the same as the adenine-induced nucleolar reformation in the ethionine-treated rats. These observations indicate an essential similarity in the nucleolar response induced by ethionine and by methionine.

A most intriguing aspect of this study is the striking difference in the acute response of the guinea pig and the rat to excess methionine in the face of an essentially similar response of each species to ethionine. This is best explained by the differences in the quantitative balance in the two species between rates of formation and utilization of the sulfonium compounds of ethionine and methionine. With the artificial analogue, the rate of formation of S-adenosylethionine (SAE) far exceeds the rate of utilization [6] and, therefore, SAE rapidly accumulates in each species.[6,9,15–17] With methionine, in the rat, the rate of generation of S-adenosylmethionine (SAM) is so balanced by the rate of utilization that only a slight transient elevation of SAM occurs,[9,15] without any measurable decrease in ATP concentration.[6] However, in sharp contrast, the guinea pig apparently generates SAM (and S-adenosylhomocysteine, SAH) at a rate considerably in excess of the rate of utilization, thus causing a rapid and large accumulation of the adenosyl derivatives with a concomitant decrease in ATP concentration lasting many hours.[10]

The major difference in the response of the two species to methionine seems then to reside in the ability of the guinea pig, but not the rat, to accumulate sufficiently large amounts of SAM (and SAH) to produce significant trapping of adenosine and as a consequence the induction of an acute deficiency of ATP.

Ethionine has been shown to have at least four major metabolic effects:[4,5] (1) incorporation into protein in place of methionine; (2)

formation of S-adenosylethionine with subsequent transethylation; (3) competitive inhibition of metabolic reaction of methionine and (4) decrease in cellular ATP concentration.

The known basic biochemical changes in the liver common to ethionine and excess methionine are the accumulation of the corresponding adenosyl sulfonium compounds and the induction of an acute deficiency of ATP. SAM and SAE are highly charged compounds that can bind avidly with DNA.[18] Conceivably, such reactions could modify the functional interaction of the DNA in its relationship to the nucleolus, and thus result in nucleolar disturbance. However, the administration of adenine does not decrease the concentration of SAE or SAM in the liver [6] under conditions in which it effectively prevents or reverses the nucleolar alterations.[1] Thus, it appears that the only known biochemical consequence common to ethionine and methionine is the induction of an ATP deficiency. This conclusion supports that previously proposed for the nucleolar effects of ethionine alone in the rat.[1]

The mechanism whereby low ATP induces nucleolar aberations is not established. In the whole liver, the low ATP concentration induces an inhibition of both protein [19] and RNA [20] synthesis. Since complete nucleolar reformation can occur in the ethionine-treated rat in the presence of almost complete inhibition of protein synthesis,[21] it must be tentatively concluded that the nucleolar fragmentation with ethionine and with methionine are not due to inhibition of protein synthesis but rather are the consequence of inhibition of RNA synthesis resulting from the induction of an acute ATP deficiency. Recent unpublished results in this laboratory [17] have demonstrated inhibition of hepatic RNA synthesis in the guinea pig with methionine.

Many agents that interfere with cellular metabolism of RNA are known to induce nucleolar alterations.[22-24] It is generally considered that the reaction pattern of the nucleolus is related more to the mechanism of inhibition of RNA synthesis than to the inhibition per se.[24-26] One group of agents, such as actinomycin D, 4-nitroquinoline-N-oxide, aflatoxin, proflavin and nagalomycin, all bind to DNA and thereby interfere with the template function of DNA in RNA synthesis.[22-24] A common feature of these compounds is the induction of distinct nucleolar alterations called "nucleolar segregation." [22,27-29] Nucleolar segregation is not a prominent feature of the lesions induced by methionine or ethionine. These agents, as well as α-amanitin [30] and toyocamycin,[31] induce a second pattern, nucleolar fragmentation, which is considered to be related to an inhibition of RNA synthesis by mech-

anisms other than through interference with the template function of DNA.[26] α-Amanitin has been shown to inhibit RNA polymerase II [32,33] and not to bind to DNA. Liver RNA polymerase has been reported to be less active in nuclear preparations from ethionine-treated rats.[34] However, the exact relationship, if any, between inhibition of RNA synthesis and of RNA polymerase and nucleolar fragmentation remains to be clarified.

The finding of methionine-induced nucleolar changes in guinea pigs may have broader implications in regard to its biologic significance. Ethionine is known to induce fatty liver and pancreatitis in varieties of experimental animals after short-term administration and to induce hepatic carcinoma after long-term administration.[4] Although many studies were done to evaluate the effects of excess methionine on experimental animals, the information other than that on rats is relatively restricted.[35-41] The necessity of extending these studies to other species of animals is obvious, in view of the clear differences in reaction to excess methionine between rats and guinea pigs that was demonstrated in this study. The comparative study of the effects of methionine and ethionine in these two species may lead to the elucidation of common denominator(s) for the molecular mechanism of their actions.

References

1. Shinozuka H, Goldblatt PJ, Farber E: The disorganization of hepatic cell nuceoli induced by ethionine and its reversal by adenine. J Cell Biol 36:313–328, 1968
2. Miyai K, Steiner JW: Fine structure of interphase liver cell nuclei in acute ethionine intoxication. Lab Invest 16:677–692, 1967
3. Shinozuka H, Reid IM, Shull KH, Liang H, Farber E: Dynamics of liver cell injury and repair. I. Spontaneous reformation of the nucleolus and polyribosomes in the presence of extensive cytoplasmic damage induced by ethionine. Lab Invest 23:253–267, 1970
4. Farber E: Ethionine carcinogenesis. Advances Cancer Res 7:383–474, 1963
5. Stekol J: Biochemical basis for ethionine effects on tissue. Advances Enzym 25:369–393, 1963
6. Shull KH, McConomy J, Vogt M, Castillo A, Farber E: On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine. J Biol Chem 241:5060–5070, 1966
7. Cantoni GL: Activation of methionine for transmethylation. J Biol Chem 189:745–754, 1951
8. Shapiro SI, Schlenk F: The biochemistry of sulfonium compounds. Advances Enzym 22:237–280, 1960
9. Stekol JA: Formation and metabolism of S-adenosyl derivatives of S alkyl-homocysteins in the rat and mouse, Transmethylation and Biosynthesis of

Methionine. Edited by SK Shapiro, F Schlenk. Chicago, University of Chicago Press, 1965, pp 235–252

10. Hardwick DF, Applegarth DA, Cockcraft DM, Ross PM, Calder RJ: Pathogenesis of methionine-induced toxicity. Metabolism 19:381–391, 1970

11. Mollenhauer HH: Plastic embedding mixtures for use in electron microscopy. Stain Techn 39:111–114, 1964

12. Karnovsky MJ: Simple methods for "staining with lead" at high pH in electron microscopy. J Biophys Biochem Cytol 11:729–732, 1961

13. Reynolds ES: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212, 1963

14. Shinozuka H: Intranucleolar dense particles in rat hepatic cell nucleoli. J Ultrastruct Res 32:430–442, 1970

15. Farber E, Shull KH, Villa-Trevino S, Lombardi B, Thomas M: Biochemical pathology of acute hepatic adenosine-triphosphate deficiency. Nature 203: 34–40, 1964

16. Smith RC, Salmon WD: Formation of S-adenosylethionine by ethionine-treated rats. Arch Biochem Biophys 111:191–196, 1965.

17. Cox, Shinozuka H: Unpublished data

18. Gnatt R, Montes de Oca F, Bump E: Binding of S-adenosylmethionine to DNA as a model for S-adenosylethionine–DNA interaction, Tenth International Cancer Congress. Vol 6. Houston, Texas, 1970, p 6, abstr

19. Villa-Trevino S, Shull KH, Farber E: The role of adenosine triphosphate deficiency in ethionine-induced inhibition of protein synthesis. J Biol Chem 238:1757–1763, 1963

20. Idem: The inhibition of liver ribonucleic acid synthesis by ethionine. J Biol Chem 241:4670–4674, 1966

21. Shinozuka H, Farber E: Reformation of nucleoli after ethionine-induced fragmentation in the absence of significant protein synthesis. J Cell Biol 41:280–286, 1969

22. Bernhard W: Ultrastructural aspects of the normal and pathological nucleolus in mammalian cells. Nat Cancer Inst Monogr 23:13–38, 1966

23. Busch H, Smetana K: The Nucleolus. New York, Academic Press, Inc, pp 472–510, 1970

24. Simard R: The nucleus: action of chemical and physical agents. Int Rev Cytol 28:169–211, 1970

25. Simard R, Bernhard W: Le phénomène de la ségrégation nucléolaire: Spécificité d'action de certains antimétabolites. Int J Cancer 1:463–479, 1966

26. Farber E: Biochemical pathology. Ann Rev Pharmacol 11:71–96, 1971

27. Journey LJ, Goldstein MN: Electron microscope studies on HeLa cell line sensitive and resistant to actinomycin D. Cancer Res 21:929–932, 1961

28. Reynolds RC, Montgomery PO'B, Hughes B: Nucleolar "caps" produced by actinomycin D. Cancer Res 24:1269–1277, 1964

29. Schoefl GI: The effect of actinomycin D on the fine structure of the nucleolus. J Ultrastruct Res 10:224–243, 1964

30. Fiume L, Marrinozzi V, Nardi F: The effects of amanitin poisoning on mouse kidney. Brit J Exp Path 50:270–276, 1969

31. Bonar RA, Chabot JF, Longlois AJ, Sverak L, Vepreck L, Beard JB:

Influence of tovocamycin on ovian leukemia myeloblasts: cell growth, ultrastructure, RNA synthesis and elaboration of BAI strain A virus. Cancer Res 30:753–762, 1970

32. Stirpe F, Fiume L: Effect of α-amanitin on ribonucleic acid synthesis and on ribonucleic acid polymerase in mouse liver nuclei. Biochem J 105:779–782, 1967

33. Lindell TJ, Weinberg F, Morris PW, Roeder RG, Rutter WJ: Specific inhibition of nuclear RNA polymerase II by α-amanitin. Science 170:447–449, 1970

34. Smuckler EA, Koplitz M: The effects of carbon tetrachloride and ethionine on RNA synthesis *in vivo* and in isolated rat liver nuclei. Arch Biochem Biophys 132:62–79, 1969

35. Russell WC, Taylor MW, Hogan JM: Effect of excess essential amino acids on growth of the white rat. Arch Biochem Biophys 39:249–253, 1952

36. Cohen HP, Choitz HC, Berg CP: Response of rats to diets high in methionine and related compounds. J Nutrition, 64:555–569, 1958

37. Stekol JA, Szaran J: Pathological effects of excessive methionine in the diet of growing rats. J Nutr 77:81–90, 1962

38. Klavins JV, Kinney TD, Kaufman N: Histopathologic changes in methionine excess. Arch Path 75:661–673, 1963

39. Klavins JV, Johansen PV: Pathology of amino acid excess. IV. Effects and interactions of excessive amounts of dietary methionine, homocystine and serine. Arch Path 79:600–614, 1965

40. Jeanjean M, Japer H: Toxicité de la méthionine chez le lapin. Path Europ 2:93–104, 1967

41. Boquist L: The effect of excess methionine on the pancreas: a light and electron microscopic study in the Chinese hamster with particular reference to degenerative changes. Lab Invest 21:96–104, 1969

[*Illustrations follow*]
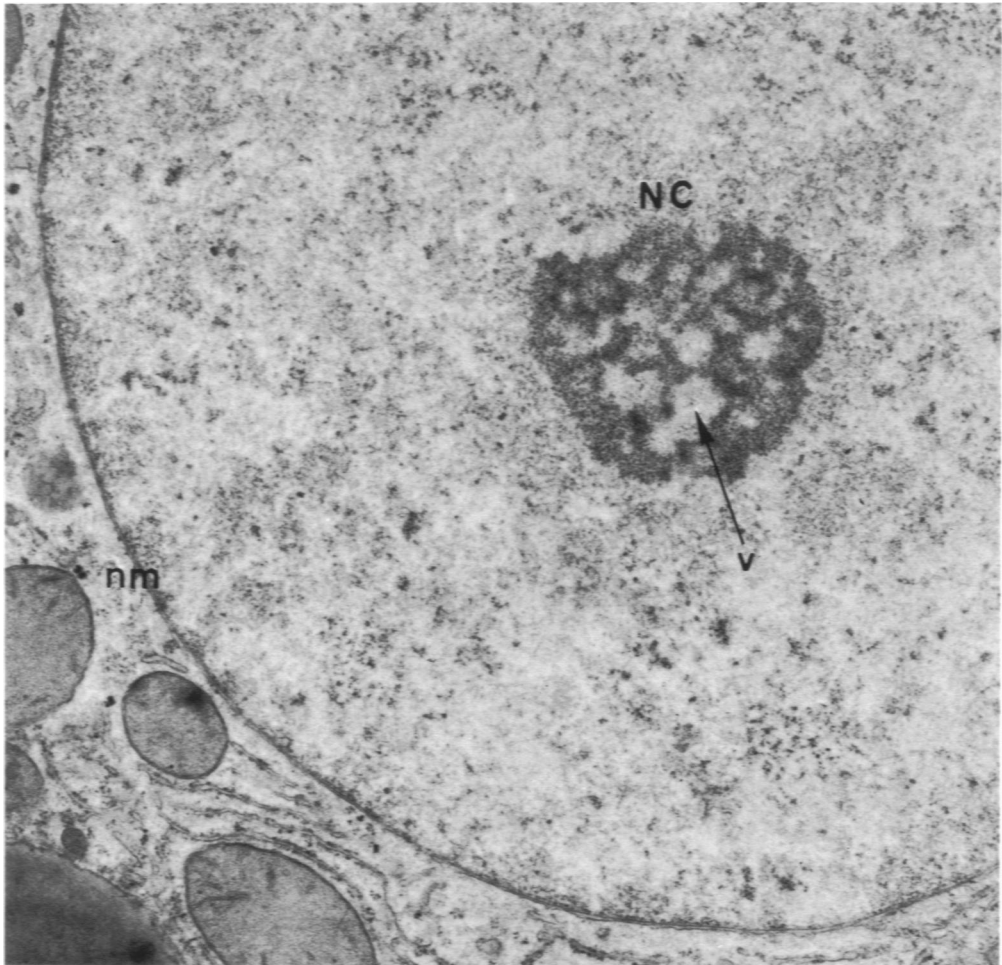
Fig 1—A portion of hepatic cell nucleus of control guinea pig. Nucleus shows a distinct nuclear membrane (*nm*) and a relatively homogeneous nucleoplasm with indistinct chromatin. Nucleolus (*NC*) is compact and shows skein-like arrangement of nucleolomena encircling nucleolar vacuoles (*v*). Granular and fibrillar components are intermingled (× 18,000).
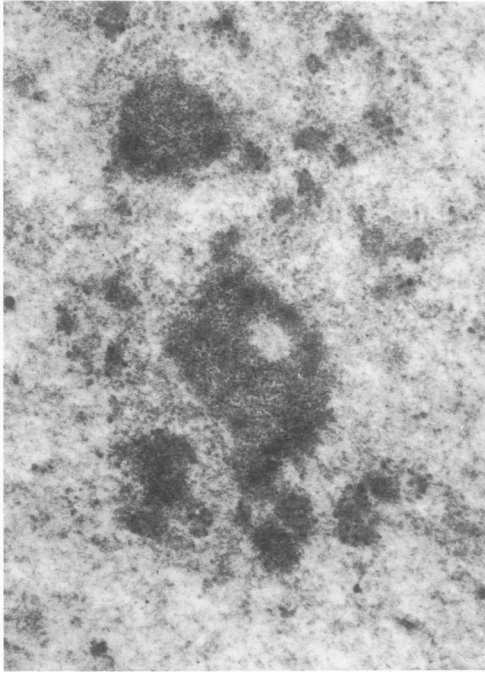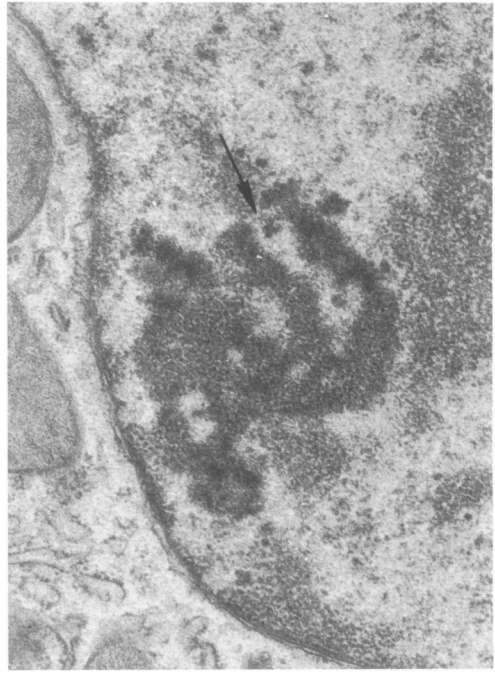
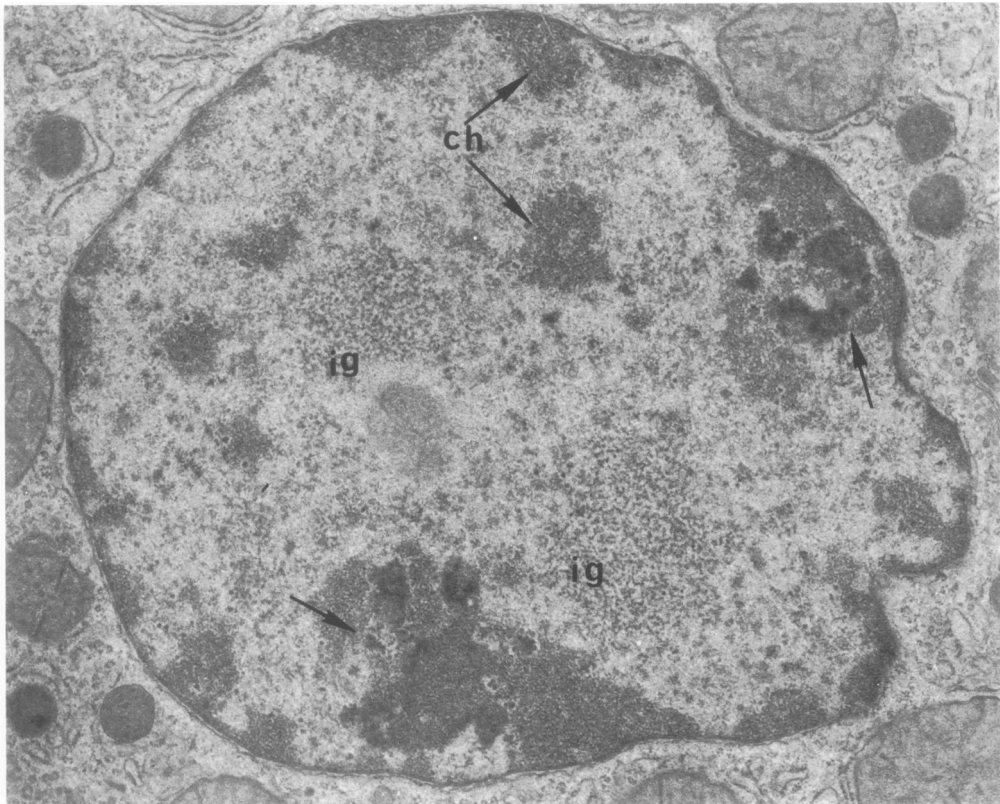Fig 2—Hepatic cell nucleolus of guinea pig sacrificed 2 hours after methionine injection. Disruption of nucleolomena is apparent. Fragments vary in size and show granular and fibrillar components of normal nucleolus (× 23,000).　　Fig 3—Hepatic cell nucleolus of guinea pig sacrificed 4 hours after methionine injection. Overall structure of nucleolonema still remains, with an area (*arrow*) suggestive of partial disruption. Small electron-dense spherules are seen in area of disrupted nucleolomena (× 29,000).　　Fig 4—Hepatic cell nucleus of guinea pig 4 hours after methionine injection. Two disorganized nucleoli are seen (*arrows*). Chromatin (*ch*) is condensed around nuclear membrane and in nucleoplasm (compare with Fig 1). Two areas of aggegated interchromatinic granules (*ig*) are also seen (× 16,000).
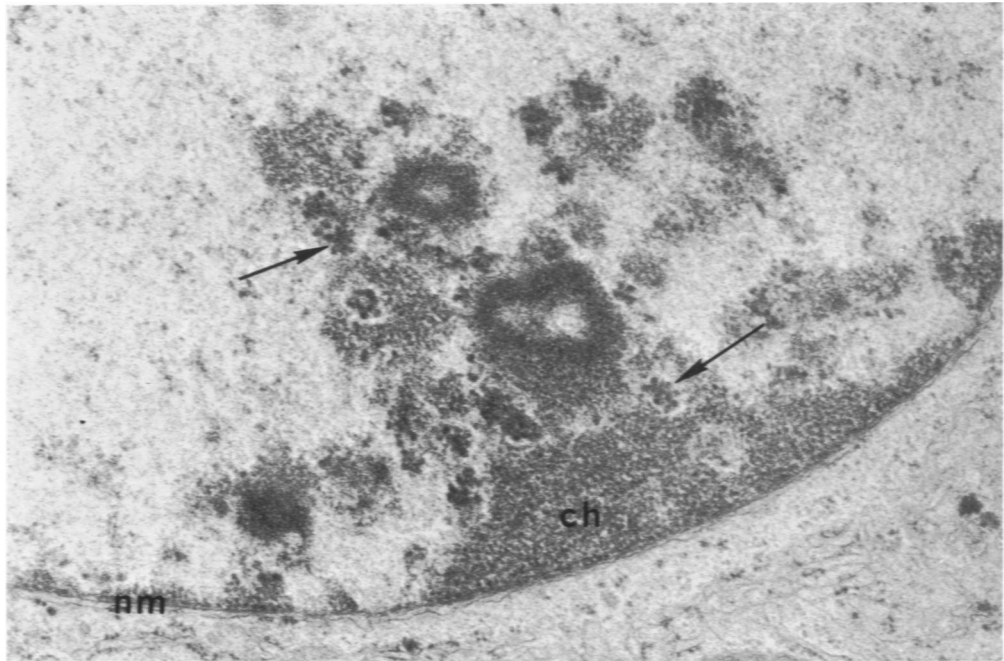
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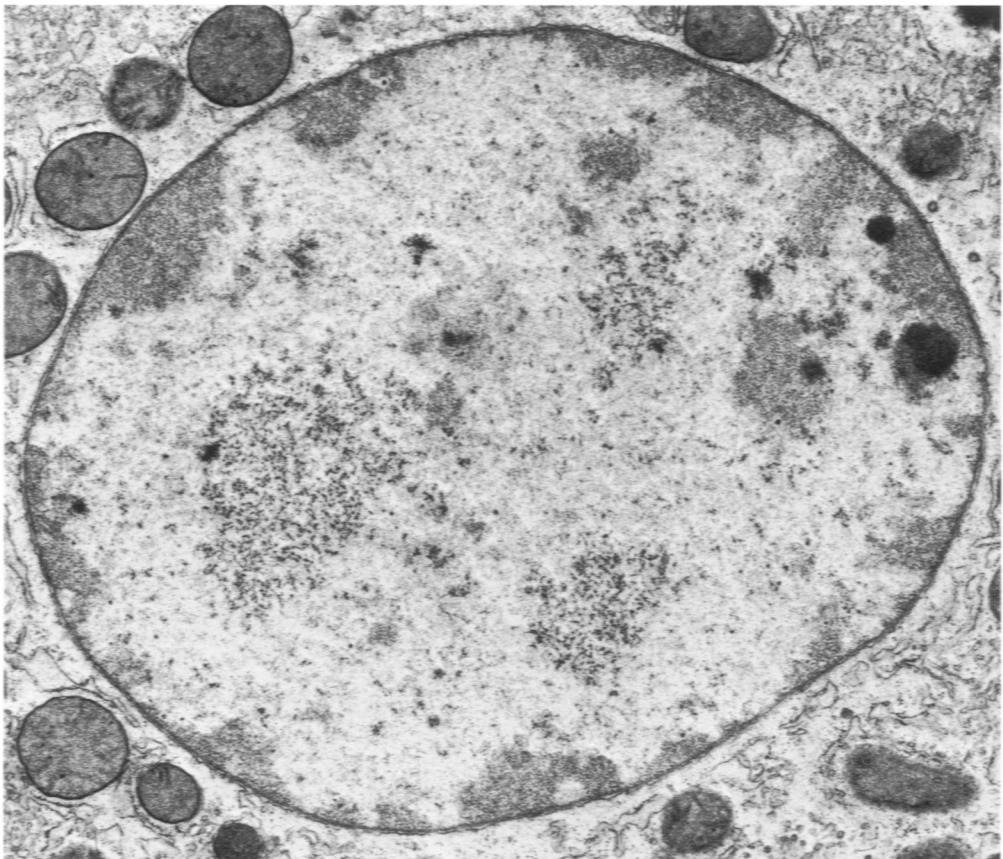
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Fig 5—Fragmentation of nucleolus 4 hours after methionine injection. Small fragments are dispersed around the cores of remnant nucleolus. Fragments (*arrow*) show small granules in the periphery. Nuclear membrane indicated by *nm*, chromatin by *ch* (× 26,000).
Fig 6—Hepatic cell nucleus of guinea pig 8 hours after methionine injection. Distorted nucleolus is seen in one corner close to the nuclear membrane. In the nucleoplasm, three well-circumscribed aggregates of interchromatinic granules are also apparent. Changes are similar to the ones shown in Fig 4 (× 15,000).
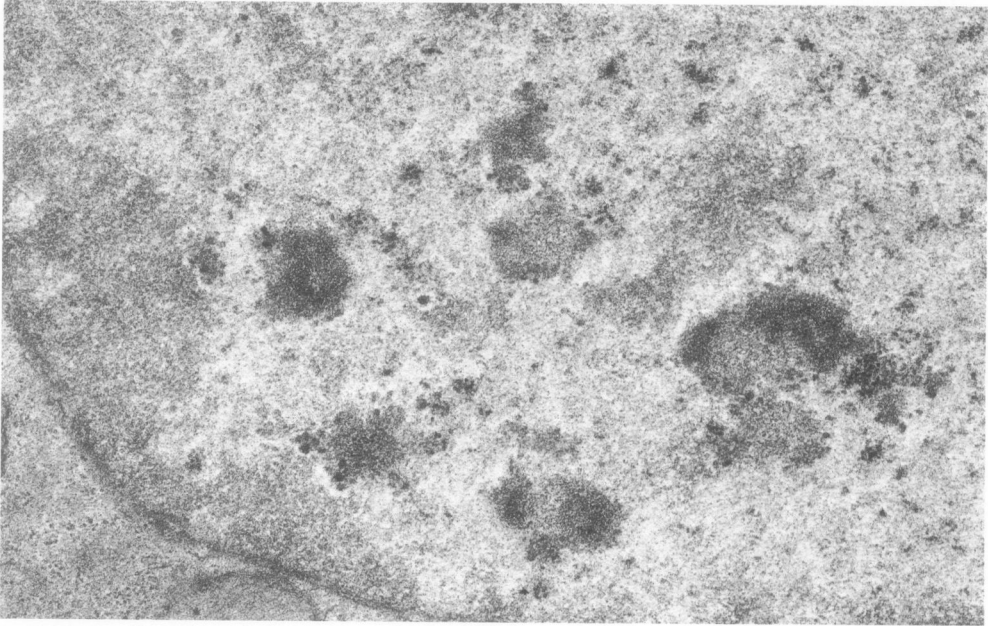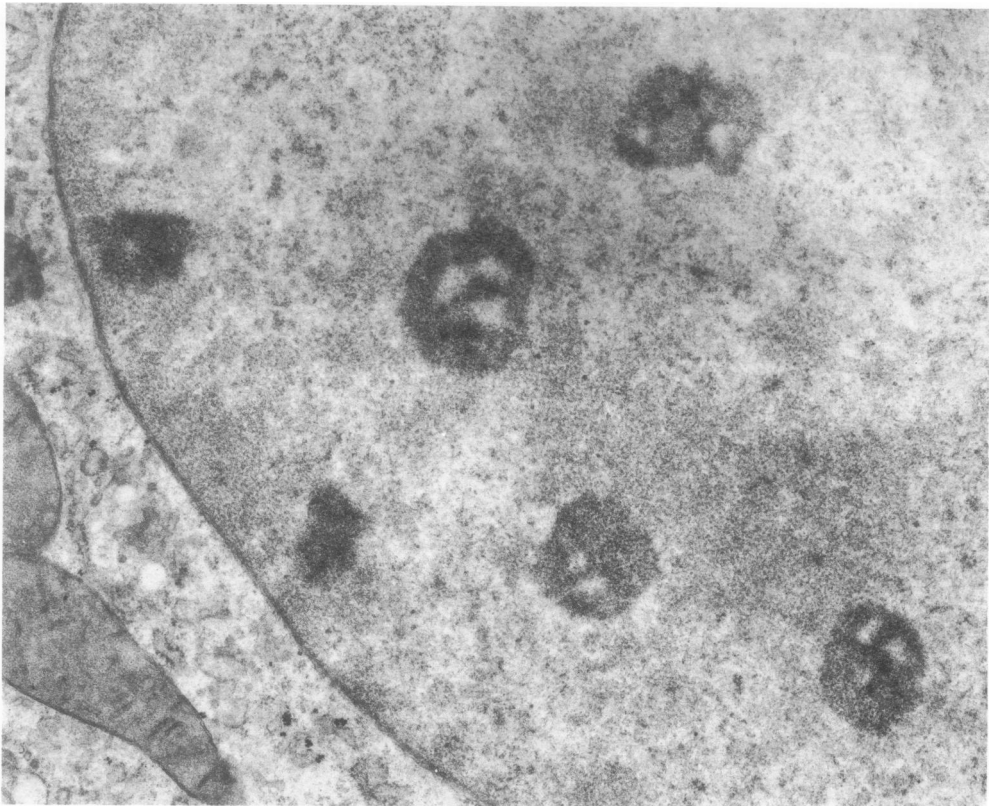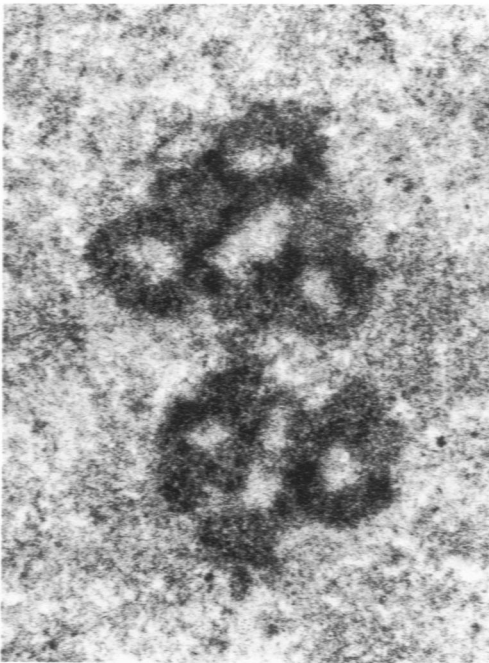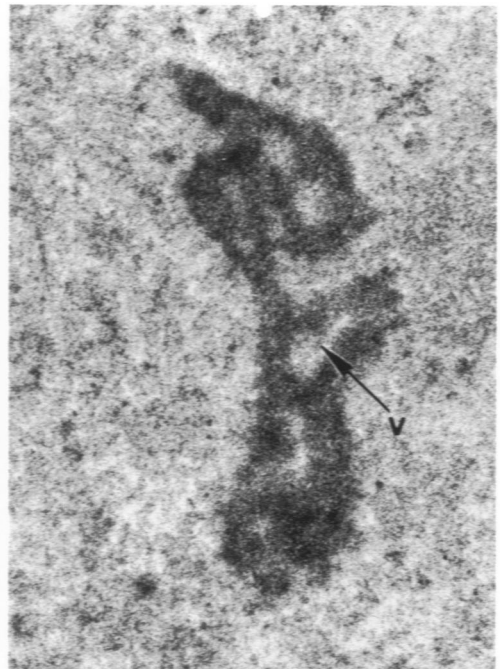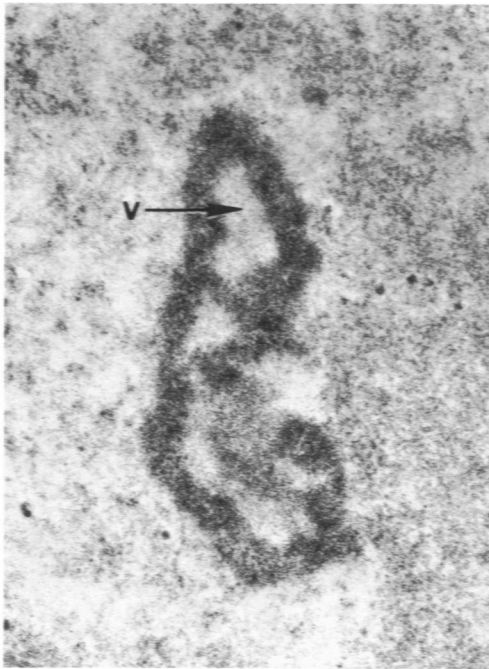
Fig 7—Varying sizes of nucleolar fragments seen 8 hours after methionine injection (× 33,800).   **Fig 8**—A portion of hepatic cell nucleus of guinea pig given adenine 4 hours after methionine injection and sacrificed 4 hours later. Six small nucleoli, which show relatively simple organization, are seen. Chromatin in the nucleoplasm is less condensed (× 19,000).
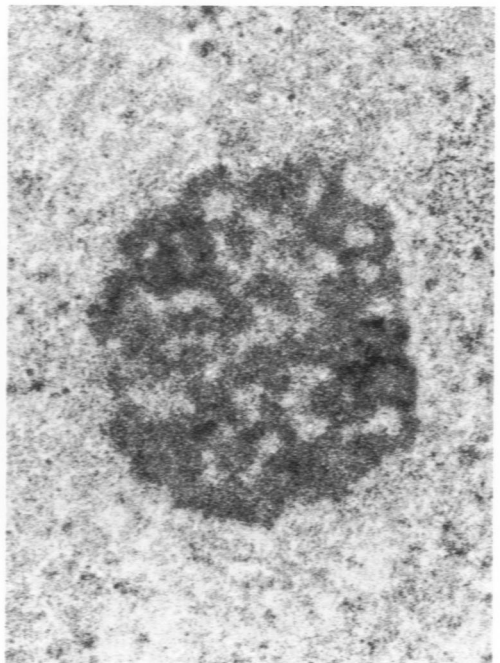
Fig 9–12—Various forms of hepatic cell nucleoli seen in guinea pigs given adenine 4 hours after methionine injection and sacrificed 4 hours later. In **Fig 9 and 10,** an elongated rope-like structure is seen which forms loops, and encircles large nucleolar vacuoles (*v*). In **Fig 11,** coalescence of simplified nucleoli is seen. Structure of nucleolus in **Fig 12** appears to be close to normal. (Fig 9, × 27,500; Fig 10, × 29,000; Fig 11, × 32,000; Fig 12, × 22,000).
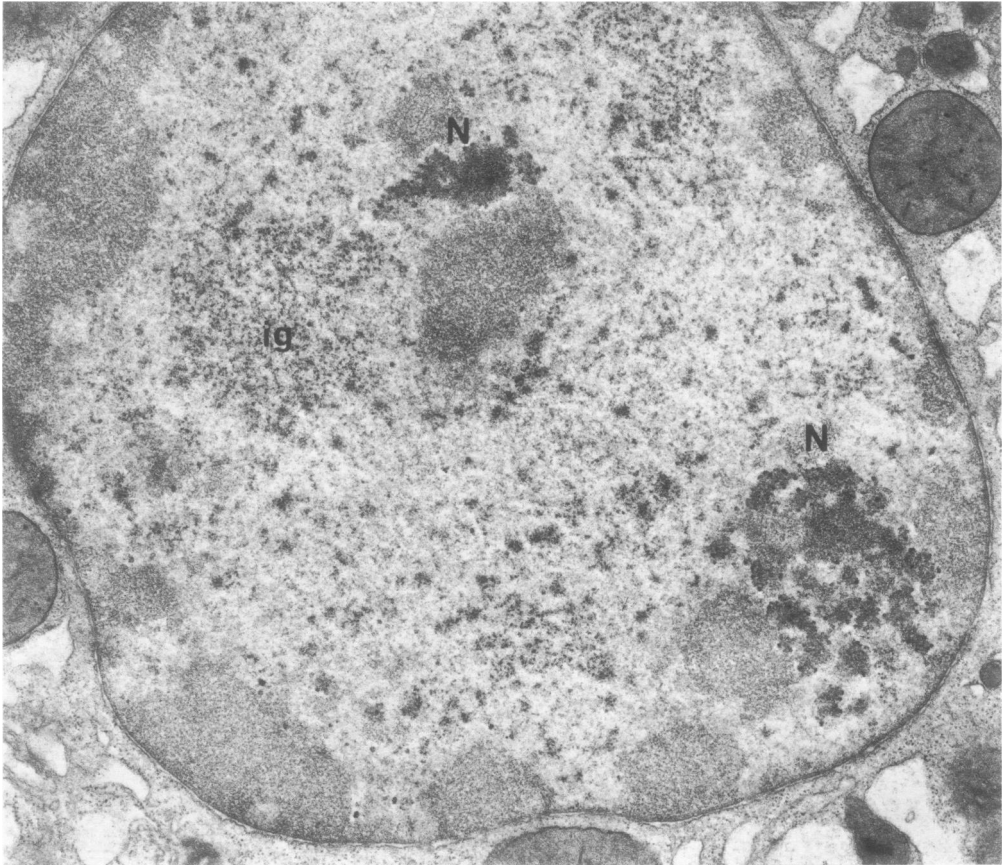
Fig 13—Hepatic cell nucleus of guinea pig sacrificed 8 hours after ethionine injection. There are two disorganized nucleoli (*N*), one of which shows obvious disruption of nucle-omena into small fragments. The pattern of condensed chromatin and the aggregates of interchromatinic granules (*ig*) are similar to those of methionine-treated animals (Fig 4 and 8) (× 18,000).