AN IMPROVED METHOD FOR THE ISOLATION OF RAT LIVER NUCLEI BY DENSITY CENTRIFUGATION

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INTRODUCTION

It is generally agreed that the method of Chauveau *et al.* (1) gives the purest preparation of rat liver nuclei of any method employing sucrose solution as the suspending medium. This method involves extensive centrifugation of an homogenate of a fasted rat's liver in 2.2 M sucrose, in which nuclei migrate to the bottom of the tube and all other cell components migrate upwards. It has been used by several other workers either unmodified (2, 3) or for purification of crude nuclear fractions isolated in isotonic sucrose (4–9) and has apparently given satisfactory results. Unfortunately, at least with the animals available in this laboratory, this procedure often yields extensively agglutinated and contaminated nuclei, especially without prior starvation of the rat. Other authors have reported similar difficulties (6, 10). The addition of calcium improves the nuclei only in some cases, but slight acidity is much more effective in preventing gelation. This effect has been investigated in some detail to determine the minimum deviations necessary for the repeatable preparation of intact nuclei.

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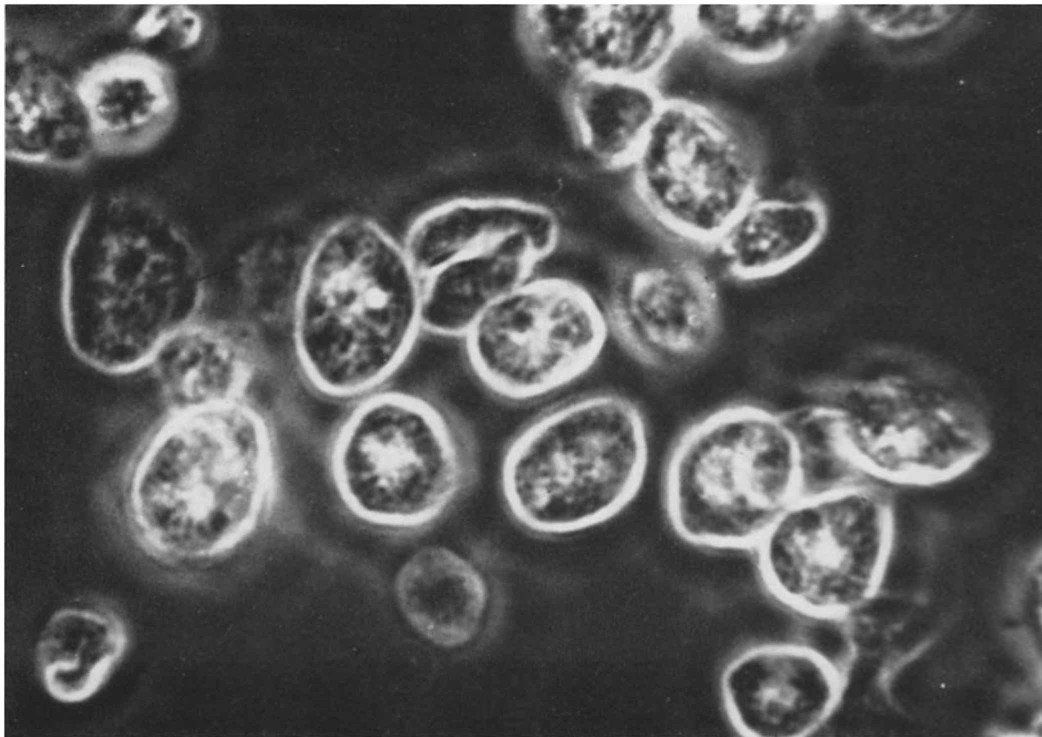

FIGURE 1 Phase contrast of an unfixed suspension of nuclei isolated as described in the text. × 1950.

ISOLATION PROCEDURE

It is convenient for practical reasons to use rats weighing between 130 and 150 gm, but rats of any weight may be used providing the homogenate volumes are kept in the right proportions. The liver is perfused *in situ*, excised, freed from obvious fibrous material, rinsed in isotonic saline, blotted, and weighed. All further operations are performed at 4°C. 6 gm of liver are broken up in a glass homogeniser and then homogenised in about 20 ml of dense sucrose medium[1] containing 3 mm $CaCl_2$ to which has been added 1 mm acetic acid.[2] Fifty up-and-down strokes of a loose stainless steel pestle (200-μ radial gap) followed by fifty strokes with a tighter pestle (125-μ radial gap) is sufficient to break most of the cells without damaging many nuclei. The homogenate is filtered through nylon organdie and diluted with sucrose medium to 15 ml/gm wet liver. The pH is adjusted to 6.0, if necessary, with 0.05 N HCl or NaOH, and the homogenate is centrifuged at 40,000 *g* (av) for 25 minutes ("preparative spin"). Pellicles and supernatants are discarded, the tube walls wiped clean, and the nuclear pellet very gently resuspended in 20 ml of sucrose medium (without added acid) and centrifuged again at 40,000 *g* for 15 minutes ("washing spin"). The preparation takes about 75 minutes from the death of the animal.

Representative fields of nuclei prepared in this way are shown in Figs. 1 and 2. Fibrous contamination is usually of a very low level. There is less than one whole cell per thousand nuclei, and only an occasional mitochondrion is seen in the electron microscope. This is confirmed by estimation of succinate-INT reductase activity (Table I). Undoubtedly the most serious remaining contamination is microsomal, representing the outer nuclear membrane and pieces of the endoplasmic reticulum attached to it. This is indicated by the high glucose-6-phosphatase content (Table I), as found by other workers (11), although, since the enzyme cannot be brought into true solution, the validity of this assay must be considered dubious. About 65 per cent of the DNA in the filtered homogenate is recovered,

[1] Specific gravity 1.28 to 1.29, prepared by dissolving two pounds of Tate and Lyle granulated sugar in 700 ml of distilled water.

[2] This is merely to adjust the final homogenate pH to about 6.0.

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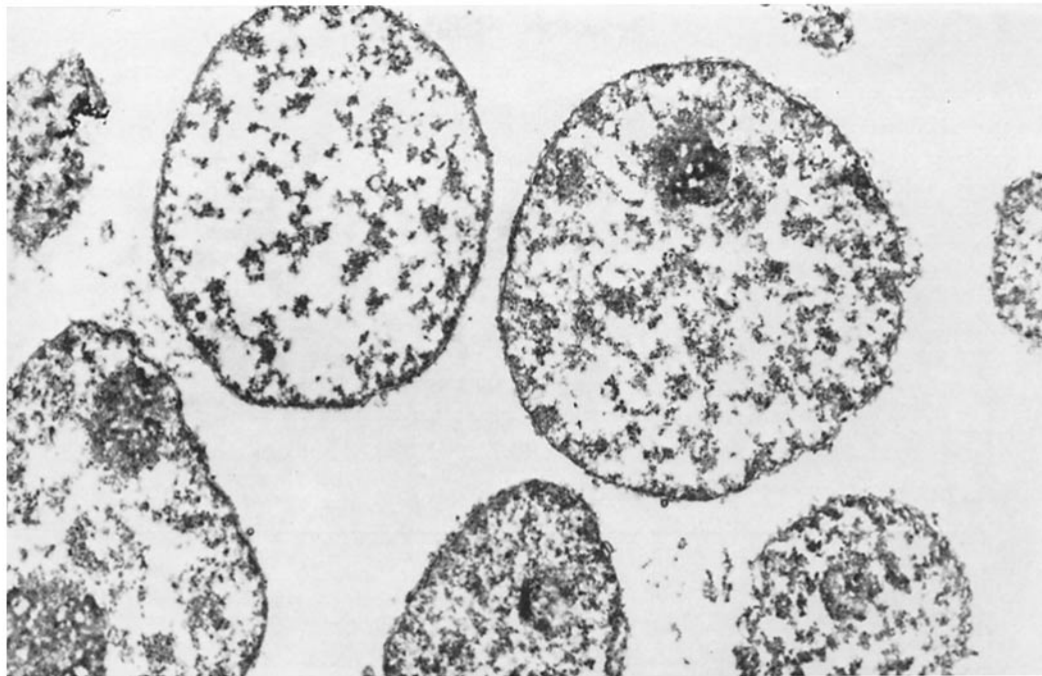

FIGURE 2 Same nuclei as in Fig. 1. Fixed in formaldehyde and osmium tetroxide, dehydrated, and embedded by Luft's method (16), and stained with uranyl acetate. × 8500.

with 0.175 mg RNA/mg DNA in the final preparation.

DISCUSSION OF THE ISOLATION METHOD

The isolation conditions were chosen after investigating the changes produced by varying pH and metal ion content during the "preparative spin." The medium composition is critical only during this first centrifugation, and is relatively unimportant during homogenisation or during the "washing spin." For this investigation, identical portions of filtered homogenate from female albino rats were divided into separate centrifuge tubes containing various amounts of acid and metal ion, mixed well, and spun simultaneously. The pH of each tube was recorded, and the nuclear pellet, supernatant, and pellicle were examined.

Variation of pH[3]

1. The amount of microscopically visible material remaining in the supernatant becomes progressively less with pH, down to about pH 5.4. There is a sharp boundary between about pH 5.8 and 6.0, above which the supernatant contains very much material, including a few unsedimented nuclei, with many free nuclei in the pellicle.

2. If the liver has not been perfused, the nuclear pellet is pink at neutrality, but extremely red below pH 6.5 because of the sedimentation of a larger proportion of the erythrocytes. Prior perfusion is, therefore, necessary.

3. Contamination of the pellet and agglutination of the nuclei are strongly correlated, both being minimal between pH's of about 5.4 and 6.0, outside of which range it seems impossible to obtain morphologically unaltered nuclei. This "acceptable" range is, unfortunately, extremely variable among rats, but it is possible in every case to prepare unagglutinated nuclei at pH 5.8. At pH's outside this range, DNA seems to escape from the nuclei and "glue" them together, making it impossible to resuspend them as discrete bodies. It has not been found possible to reverse this agglutination by moderate chemical method.

[3] The pH of an homogenate in dense sucrose alone is 6.8.

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Variation of pH in the Presence of Calcium Chloride

Calcium chloride is itself acidic, and its addition to an homogenate reduces the pH according to the amount of tissue present. This fact caused some confusion in interpreting experimental results before the importance of pH was realised.

By adding 3 mM $CaCl_2$ and readjusting the pH to that of the control, a small reduction in the degree of agglutination and contamination is effected without any marked difference in the clarity of the supernatant. Lowering the pH in the presence of calcium has an effect qualitatively the same as in its absence, but calcium does slightly improve the appearance of the nuclei at all pH's, and, more important, slightly widens the pH range, in which unagglutinated nuclei can be obtained, to 5.2 to 6.1 or 5.0 to 6.5 in the best cases. At these lower pH's calcium does help to clarify the supernatant, probably by agglutination of microsomes (12), but this is not so marked as clarification by reduction of pH. One mM $CaCl_2$ has very little effect.

The removal of calcium by 3 mM EDTA produces changes opposite to those of adding calcium. Nuclei are more agglutinated at all pH's, and a pH cannot be found at which there is no agglutination. At pH 5.6 (at which agglutination is the least), the nuclei are extremely deformed, readily form attachments to cytoplasmic debris, and have poorly defined nucleoli which stain abnormally with methylene blue.

Magnesium does not duplicate these actions of calcium, except that it produces a slight clarification of the supernatant. Moreover, the nuclei tend to break up into small fragments.

Other Animals

Despite considerable variations among individual rats, much the same pattern of behaviour at different pH's, with and without calcium, is obtained using livers from both males and females, of 50- to 250-gm body weight, for two other strains of rats (Norwegian black and white hooded, and August strains), for regenerating liver, and also from mice, although in this case the nuclei tend to be contaminated with erythrocytes because perfusion is more difficult. Chauveau's observation (1) has been confirmed that at pH's near neutrality much better nuclei can be isolated from rats starved for 24 hours before sacrifice. At pH 6.8, such nuclei are distinctly less strongly agglutinated and rather less contaminated than are nuclei from normally fed rats. They are, however, only completely discrete when prepared at a pH below about 6.

Maggio *et al.* (10) were able to prepare unagglutinated nuclei from the livers of fasted guinea pigs with only 1.5 mM calcium without any further pH adjustment. This is slightly different from the behaviour of rat liver nuclei, and so guinea pig homogenates were tested in the same way as rat liver homogenates. Without added calcium, the pH must be lowered to 5.6 before clean, unagglutinated nuclei sediment. With 3 mM $CaCl_2$ the best nuclei are obtained below pH 6, but,

TABLE I

Contamination of Nuclei

	Succinate-INT reductase*	Calculated per cent of whole cells, or their equivalent‡	Whole cell count§	Glucose-6-phosphatase‖
Filtered homogenate	2.06			2.89
Nuclei	0.04 (1.9%)	0.12%	<0.1%	0.73 (25%)

* μMoles formazan produced/mg protein/hour (8.5 mM succinate, 0.05 mgINT/ml, pH 7.0 (14).

‡ Calculated from column 1 on the basis of an average protein distribution between cytoplasm and nucleus of 16:1.

§ The difference between the second and third columns represents contamination by free mitochondria.

‖ μMoles phosphate released from glucose-6-phosphate at pH 6.5/mg protein/hour (15).

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unlike rat liver nuclei, the nuclei sedimenting at pH 6.5 are entire, largely discrete, and only slightly contaminated, whether or not the animal has been starved before death. It must be concluded that a slight difference between the animals exists, in that guinea pig liver nuclei are the rather more sensitive to calcium.

In general, it is clear that free, unagglutinated nuclei with a minimum of cytoplasmic tags are best prepared in the presence of 3 mM $CaCl_2$ at slightly acid pH's. Contamination by cytoplasmic debris left in the supernatant decreases as the pH is lowered, but most of this contamination can be removed by a further "washing spin" providing the nuclei can be completely redispersed. A pH of 6.0 seems to be the best compromise between contamination and any potentially deleterious effects of departing too far from the physiological value, and at this pH nuclei can be reproducibly prepared without any need to use fasted animals. An incidental advantage of working at pH 6 is that this gives the minimum intracellular protease activity in the liver (13).

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REFERENCES

1. Chauveau, J., Moulé, Y., and Rouiller, C., *Exp. Cell Research*, 1956, **11**, 317; *Bull. Soc. Chim. Biol., Paris*, 1957, **39**, 1521.
2. Rendi, R., *Exp. Cell Research*, 1960, **19**, 489.
3. Miller, L. A., and Goldfeder, A., *Exp. Cell Research*, 1961. **23**, 311.
4. Fisher, R. F., Holbrook, D. J., Jr., and Irvin, J. L., *J. Cell Biol.*, 1963, **17**, 231.
5. Penniall, R., Hall, A. H., Griffin, J. B., and Irvin, J. L., *Arch. Biochem. and Biophysics*, 1962, **98**, 128.
6. Sporn, M. B., Wanko, T., and Dingman, W., *J. Cell Biol.*, 1962, **15**, 109.
7. Widnell, C. C., *Biochem. J.*, 1963. **89**, 11P.
8. Wilczok, T., and Chorazy, K., *Nature*, 1960, **188**, 516.
9. Zbarskii, I. B., and Georgiev, G. P., *Biokhimiya*, 1959, **24**, 192.
10. Maggio, R., Siekevitz, P., and Palade, G. E., *J. Cell Biol.*, 1963, **18**, 267.
11. Siebert, G., *Exp. Cell Research*, 1963, suppl. **9**, 389.
12. Abood, L. G., Koyama, I., and Kimizuka, H., *Nature*, 1963, **197**, 367.
13. Dounce, A. L., *Exp. Cell Research*, 1963, suppl. **9**, 126.
14. Shelton, E., and Rice, M. E., *J. Nat. Cancer Inst.*, 1957, **18**, 117.
15. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60**, 604.
16. Luft, J. H., *Exp. Cell. Research*, 1961, **9**, 409.

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