STUDIES ON ISOLATED CELL COMPONENTS

I. NUCLEAR ISOLATION by DIFFERENTIAL CENTRIFUGATION

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Received May 17, 1950

THE problem of the isolation of cell components in quantity has been approached in several ways. For the nucleus the methods have included acid-pepsin digestion of the cytoplasm (22), hemolysis of nucleated erythrocytes (1, 33, 19), and recently the differential centrifugation of mechanically disrupted cells. In preparing tissues for differential centrifugation the following procedures have been employed: *1.* dessication (5); *2.* ultrasonic disintegration (16); *3.* freezing and thawing (11); *4.* heating to 51° C (38); and *5.* treatment with citric acid (30, 11, 21, 4). In most of the above methods the tissue is killed before nuclear isolation. Other workers have mechanically broken up living tissue in saline (17, 12) or sucrose solutions (28) and have then isolated the nuclei by differential centrifugation in these solutions. For further references on methods the recent review of Dounce (11) may be consulted.

The method of choice for the isolation of nuclei will be dictated by the purposes of the experiment, as emphasized by Dounce (11). For the investigation of the physiological and colloid chemical potentialities of the isolated nucleus, which is the aim of the present studies, it becomes necessary to employ procedures which will furnish quantities of pure nuclei in a state resembling the intracellular condition as closely as possible. This means giving attention to such factors as electrolyte composition, tonicity, the maintenance of pH near neutrality during and following extraction, etc. Since technics previously devised for other purposes were not suitable in these respects, modifications have been introduced. A simple method is presented which permits the isolation of rat liver nuclei in a state of purity satisfactory for various colloidal and biochemical studies and in

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a condition optically similar to nuclei within the living cell. The isolation procedure constitutes a starting point for an examination of the requisite conditions for the maintenance of nuclei *in vitro*.

METHODS

Before presenting the detailed method for nuclear isolation, mention may be made of certain general procedures which we have found useful in the preparation and centrifugal fractionation of homogenates of living tissues. An excellent general discussion has previously been given by Hoerr (17).

1. *General considerations*

Temperature.

The maintenance of low temperature during all operations is essential. At room temperatures mitochondria in liver brei fragment rapidly (2), and nuclei become granular and irregular (35). Also loss of enzymes from nuclei occurs on warming and is illustrated by the finding that nuclei prepared by the earlier method of Dounce contained no catalase, whereas nuclei prepared with a refrigerated centrifuge had a high catalase content (11). The use of a refrigerated room is only a partial answer to the problem since suspensions are warmed during centrifuging. It has been observed, for example, that the temperature of distilled water in 15 ml centrifuge tubes rose from 5.8° to 10.5° during 20 minutes centrifuging at 1,170 × g in an International Centrifuge Size 2 operated in a cold room at 5.2° C. For work involving centrifugation for long periods at high speeds a refrigerated centrifuge is almost indispensable.

Preparation of homogenates.

Rats of the Osborne-Mendel strain or hamsters (*Cricetus auratus*) were stunned by a blow on the head and rapidly decapitated. Possible effects of anesthetics are thus eliminated. The liver was flooded with cold 0.15 M NaCl and perfused with the same solution. The organ was then excised and homogenized in cold medium (usually 5 ml of medium per gram of fresh liver). The entire procedure was carried out in a refrigerated room at 5° C.

The disruption of cells has usually been accomplished by pressing the tissue through bolting silk after preliminary grinding (6); by the use of the Waring Blendor l.c. or similar device (10); or by the use of tight fitting grinders (14, 31, 25, 36). Kneading through bolting silk is not satisfactory when a high percentage of cells must be ruptured; and the Waring Blendor has been found too drastic for use with unfrozen tissues and may denature certain proteins (29). Temperature control has also been found somewhat difficult with the latter technique (10).

Simple glass, plastic, or rubber grinders operated by hand (25, 36) will give no rapid and complete homogenization of brain, liver, and kidney. For most work pestles with a diameter of approximately 0.8 inches fitted in test tubes ten inches in length have been used. A study of the grinding characteristics of eleven pestles

TABLE I

The pH of Rat Tissue Homogenates.

All homogenates consisted of 1 gram of tissue (fresh weight) per 5 ml. of medium. The entire procedure was carried out in a refrigerated room at 5° C.

Preparation	pH of medium	pH of homogenate immediately after preparation	pH of homogenate after one hour at 5° C
Perfused rat liver in:			
Distilled water	5.78	7.1	7.02
Sucrose-phosphate[1]	7.01	7.02	6.98
Isotonic KCl	5.98	6.92	6.82
Unperfused rat kidney in:			
Distilled water	5.78	6.92	6.84
Sucrose-phosphate[1]	7.01	7.05	6.92

indicates that the pestle diameter should be about 0.2 mm less than the tube diameter. About seventy slow up and down strokes of the pestle will disrupt nearly all the cells in 8 to 10 grams of rat liver as the tissue passes between the pestle and the tube wall. Since the clearance is many times the thickness of the liver cell, contact with both grinding surfaces is probably not necessary. With less clearance gases are likely to be withdrawn from the solution by the upward movement of the pestle. All homogenates are strained through bolting silk or cheesecloth before centrifugation to remove connective tissue.

Hydrogen ion concentration.

Since the pH of the cytoplasm of certain individual cells has been shown to fall from about pH 6.8 to 5.4 on injury (8, 37) homogenates prepared in unbuffered media may be expected to be decidedly acid. This is not the case with rat liver, even though a large portion of the blood buffers has been removed by perfusion. Table I shows that the pH of liver and kidney homogenates in unbuffered solutions is close to neutrality and falls no more than 0.1 pH unit in the course of an hour at low temperature. Greene et al (13) reported similar results but stated that rabbit kidney formed acid homogenates; and Hoerr (17) found that guinea pig liver homogenates in neutral water reached a pH of 5.6.

Centrifugal procedures.

The principles involved in differential centrifugation are simple and well known. However, they are sometimes disregarded, making the purification of material more laborious than necessary. A few practical applications of the principles which have proved helpful in isolating nuclei may be noted.

[1] Composition listed under section on solutions.

In centrifuging a tube filled with a homogenate the separation of components in initial centrifugings will inevitably be incomplete except for the lightest fraction, even though the sedimentation velocities of the various components are quite different. This follows from the fact that a portion of all the components is in the centrifugal end from the beginning and only the last to sediment can be obtained in pure form. Also, the particles near the centrifugal end of the tube are subjected in many centrifuges to twice the force exerted on the top of the tube—a factor of some importance in cases in which one wishes to sediment whole cells or nuclei and leave mitochondria in suspension. Repeated differential centrifugations will eventually give relatively pure preparations, however.

A much more efficient procedure consists in layering the homogenate over a denser medium in the centrifuge tube. In the sucrose method for isolating mitochondria (18) approximately 10 ml of homogenate are layered over one ml of a denser sucrose solution. Ideally, a very thin layer of homogenate would be placed at the top of a very long centrifuge tube. The components would then have ample opportunity to separate according to their individual sedimentation velocities. In practice, layers one or two centimeters in thickness layered on top of a denser medium in 50 ml conical centrifuge tubes have been used to separate nuclei from whole cells and mitochondria as described below. Care must be taken to avoid mixing of the layers during acceleration and deceleration of the centrifuge. This has been accomplished with the International refrigerated centrifuge, model PR-1, by attaching an accessory rheostat which permitted the transition between the vertical and horizontal positions of the centrifuge tubes to occur over a 30–60 second period.

Microscopic observations.

Identification of nuclei in homogenates of fresh liver tissue presents no problem with dark field or phase, but with the light microscope individual clear, hyaline nuclei may be difficult to see. For this reason we have found it convenient to use methylene blue dried on slides from an alcohol solution for routine examination of samples of the homogenate during the course of purification of the nuclei. Whole cells, mitochondria, and nuclei will all take up the dye. It should be pointed out that methylene blue and other similar basic dyes cause clumping of the mitochondria and precipitate cytoplasmic proteins of the fresh liver homogenate.

Solutions.

Our present knowledge of the constitution of cytoplasm is too fragmentary to permit us to duplicate its inorganic composition. As a beginning we used a modification of a solution containing KCl, NaCl, and sodium citrate which Chambers found maintained chromosomes of Chironomus larvae in good condition (8). This solution was unsatisfactory due to clumping of cytoplasmic constituents. It was felt advisable to eliminate the chloride ion since it is found only in very low concentration in the liver cell (32). Calcium was not included in view of the marked effects produced even in low concentrations on the cytoplasm (8) and on the isolated nucleus (34, and below). Since potassium and phosphate predominate in the cytoplasm (15) these ions are used here. Bicarbonate is included in a somewhat lower concentration than found in fresh liver (9).

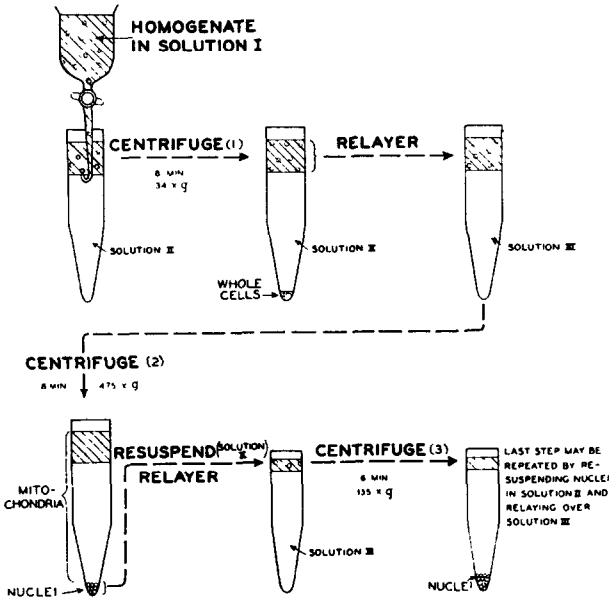

Fig. 1. Nuclear isolation procedure.

In devising solutions for the present purposes the following factors were taken into account: tonic composition, freezing point depression, density, and pH. Sucrose solutions, though satisfactory for the preservation of mitochondria (18) are not entirely suitable for the isolation and maintenance of nuclei.

The following solutions have been used in the present work:

1. *Solution I.* 0.0094 M KH_2PO_4; 0.0125 M K_2HPO_4; 0.0015 M $NaHCO_3$; 0.145 M sucrose. Sp. gr. 1.023 at 25° C; 0.7 × isosmotic; pH 7.1.
2. *Solution II.* Same salt concentration as solution I, sucrose 0.218 M. Sp. gr. 1.031 at 25° C; 0.9 × isosmotic.
3. *Solution III.* Same salt concentration as solution I, sucrose 0.272 M. Sp. gr. 1.040 at 25° C; 1.2 × isosmotic.

The salt concentration and pH are constant in all three solutions. The density requirement for differential centrifugation has been met by variations in sucrose concentration, which in turn alters the tonicity somewhat. The osmotic values are referred to 0.15 M NaCl. It must be emphasized that these solutions are to a certain extent arbitrarily devised and may need to be altered as better criteria of unclear preservation are worked out.

Sodium chloride (0.15 M) has been used for perfusion. Inasmuch as heparin releases nucleic acids from isolated cell components (3) and citrate removes calcium and magnesium from perfused tissues (20) these anticoagulants have not been employed.

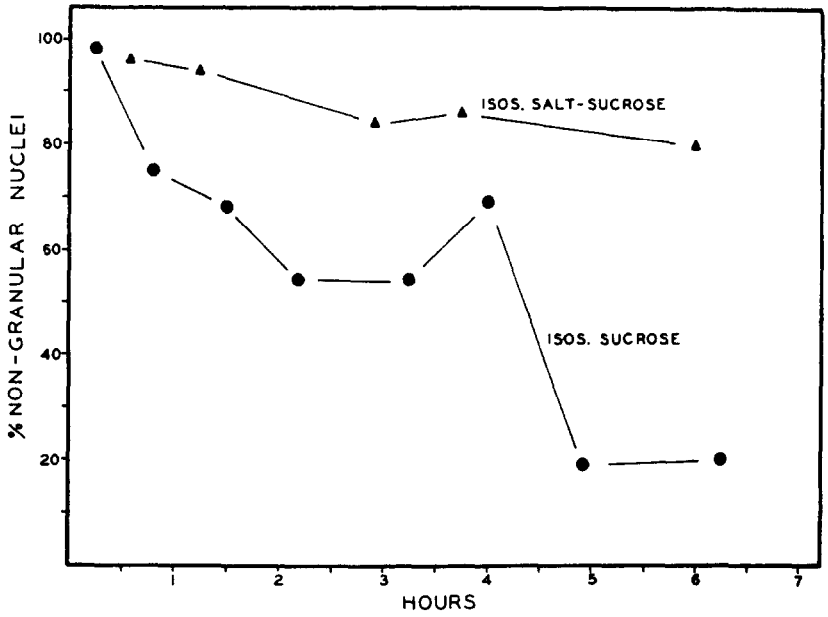

Fig. 3. Increase in granularity of isolated rat liver nuclei with time. Each point represents the results of examination of approximately 100 nuclei. Temp. 0°. Sucrose-salt mixture: 0.06 M sucrose; 0.03 M KH_2PO_4; 0.07 M K_2HPO_4; pH 7.1. Isosmotic sucrose, 0.29 M; pH 6.3. Solution I gave better preservation than either of these solutions.

2. *Nuclear isolation procedure*

The centrifugal procedure is illustrated in Fig. 1. Centrifugal forces are calculated at the bottom of the tube.

1. *Homogenization of rat liver.* 5 ml of solution I added per gram fresh liver. Homogenized with 70 strokes in hand homogenizer. Strained through 10XX bolting silk or four layers of cheesecloth.
2. *Removal of whole cells.* 15–20 ml of homogenate carefully layered over 30 ml of solution II in a 50 ml conical centrifuge tube (see Fig. 1). Centrifuged 8 minutes at 34 × g, observing the precautions for acceleration and deceleration mentioned under centrifugal procedures above.
3. *Sedimentation of nuclei.* Entire upper layer (15–20 ml) removed by sucking up into layering device shown in Fig. 1 and relayered over 30 ml of solution III. Centrifuged 8 minutes at 475 × g. Entire supernate discarded.
4. *Resedimentation of nuclei.* Nuclei resuspended in solution II (5 ml) and layered over solution III in a 15 ml centrifuge tube with a bulb pipette. Centrifuged 6 minutes at 135 × g. Step 4 repeated twice when highest purity is required, centrifuging 6 minutes at 135 × g and 6 minutes at 53 × g.

The entire procedure can be carried out in about 90 min.

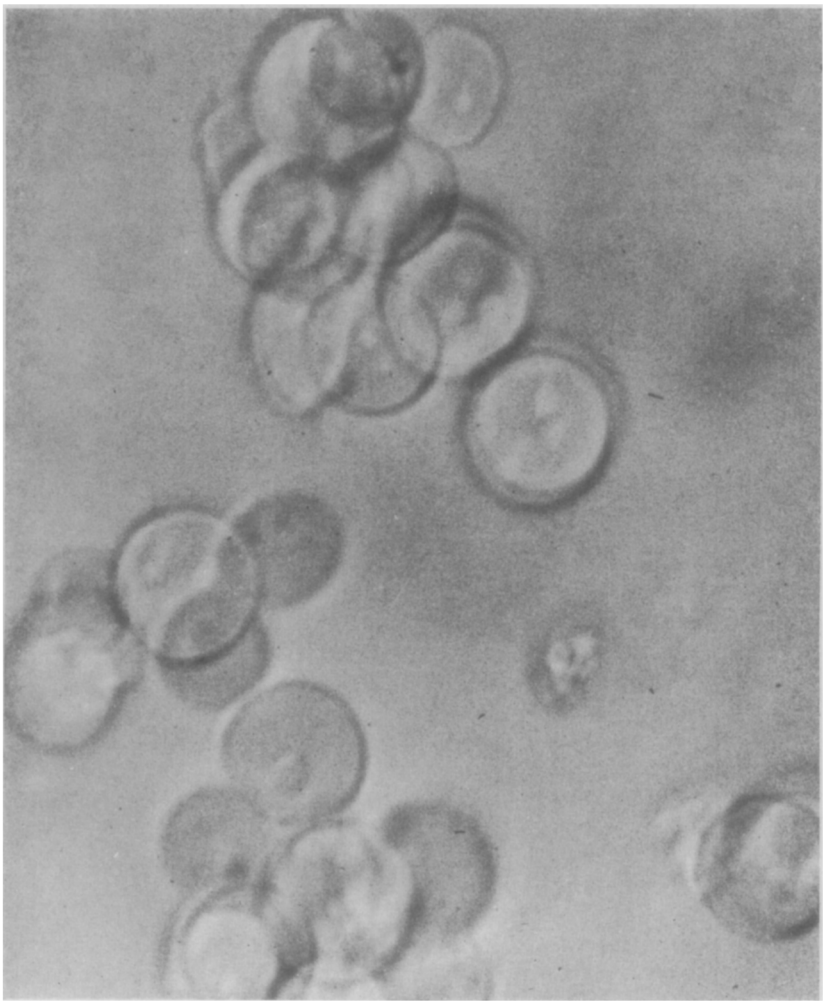

Fig. 2. Fresh rat liver nuclei showing nucleoli and absence of granulation.

RESULTS

Microscopic observations.

The isolated nuclei are similar in optical appearance to those observed in the fresh intact cell, though very slightly larger on the average. They are smooth hyaline spheres free of granulation when observed under the light microscope (Fig. 2). One to three nucleoli are readily visible. Under dark

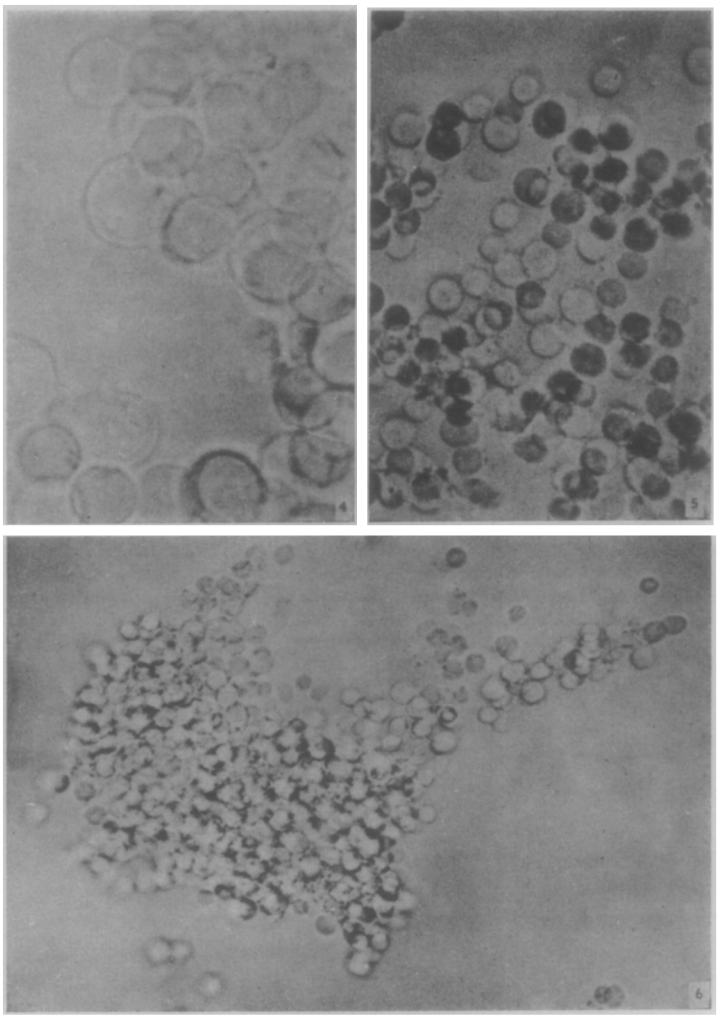

Fig. 4. Fresh rat liver nuclei isolated by procedure described in text. Photographed by dir illumination in Solution II described on page 54. Note occasional mitochondria appear as small dark bodies between nuclei.
Fig. 5. Preparation similar to that shown in Fig. 6, but photographed by oblique illuminati
Fig. 6. Isolated rat liver nuclei showing clumping which occurs in neutral solutions.

field the nuclei are either optically empty or show a few small granul With methylene blue a uniform light stain is observed, while the nucle stain somewhat more darkly.

The preparations are of a high degree of purity. A few mitochondria are trapped between the nuclei in the final preparation (Figs. 4–6) and an occasional whole cell is found. The ratio of whole cells to nuclei in an average preparation is considerably less than 1 : 100. Exact counts of nuclei are difficult to make since the nuclei form large aggregates (fig. 6).

As a measure of the maintenance of normal appearance at low temperature, determinations were made of the percentage of nuclei showing distinct granulation in a salt-sucrose solution and in pure sucrose at intervals after the completion of the isolation procedure. The results are shown in Fig. 3 (see legend). In isosmotic sucrose-salt mixture approximately 80 per cent still maintain their normal appearance after six hours at 0° C. The results are similar for solutions of widely different tonicity. At 37° granules quickly develop. The percentages of nuclei of normal appearance are smaller in pure sucrose solutions at 0°.

Ion effects on the isolated nucleus will be considered in a separate communication. Reference will be made here only to the surface deformations brought about in isosmotic NaCl and KCl solutions (2) and to a marked and rapid shrinkage resulting from the addition of 0.001 M $CaCl_2$ to nuclei in an isosmotic salt-sucrose solution (see legend for Fig. 3).

Nucleic acid analyses.

The nuclear yield in the isolation procedure has been determined by analysis of the desoxyribonucleic acid (27, 7) in the original homogenate and in the purified nuclei. The yield is about 5 per cent. This can be increased by a briefer centrifugation in Step 2 but at the expense of an increase in the proportion of whole cells.

Analysis of 5 preparations of nuclei gave a protein-desoxyribonucleic acid ratio of 5.1 with a range of 2.7 to 8.9. DNA was determined by the diphenylamine reaction and protein was calculated from total nitrogen after removal of lipids and nucleic acids. Protein-DNA ratios of 6.5 to 20 have been obtained in absorption studies of nuclei stained by the Feulgen technique (24). The lower values found in the present study might be thought to indicate protein loss during isolation—and such may indeed occur. However, in view of the uncertainty of the determinations by optical absorption (26, 23), protein loss from nuclei isolated as described above cannot be inferred. The problem may perhaps be attacked by the differential centrifugal isolation and chemical analysis of fixed nuclei.

DISCUSSION

The primary purpose of the present investigation has been to obtain in quantity nuclei which retain certain of their normal properties. Using the method here described, hyaline, nongranular nuclei resembling those within the living cell have been isolated. However, optical similarity offers no guarantee that changes may not have occurred. In fact, there is some reason to believe from the slight size difference of intra- and extracellular nuclei that changes have taken place during isolation. Indeed, it would be surprising if the simple salt-sucrose solution used in isolation could satisfactorily substitute for the cytoplasm and leave the nucleus unchanged. Nonetheless, the method of isolation here described makes available for biochemical and physiological study nuclei which have been subjected to relatively gentle treatment and are essentially free from cytoplasmic constituents. The preparation has proved useful in studies of nucleoprotein gels using the solutions mentioned (3). For other studies new media will be needed, their composition depending upon the purposes of the experiment. This should require no basic change in the method of isolation.

SUMMARY

A method is presented for isolating nuclei of rat and hamster liver in a high state of purity and in a condition optically similar to nuclei within living cells.

The isolation procedure consists in the homogenization and differential centrifugation of fresh liver at 0–5° in a salt-sucrose solution buffered at pH 7.1. By layering the material to be centrifuged over a relatively large volume of a slightly denser solution the purification can be carried out in 4 or 5 centrifugations. The entire procedure can be completed in about 90 minutes. The yield as determined by measurements of desoxyribonucleic acid is about 5 per cent.

The solutions contain KH_2PO_4, K_2HPO_4, $NaHCO_3$, and sucrose. The sucrose concentration is varied to give density differences required for layering. Salt-sucrose solutions maintain a large proportion of the isolated nuclei in a nongranular condition for 6 hours at 0°. Pure sucrose is less satisfactory for maintenance.

The protein-desoxyribonucleic acid ratio for the isolated nuclei averages 5.1 with a range of 2.7 to 8.9.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Mrs. Sara M. Bond and Mr. Max V. Skeen. The present investigations were supported by a grant from the Atomic Energy Commission.

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