ISOLATION AND PROPERTIES OF LIVER CELL NUCLEOLI*

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INTRODUCTION

The first reports of progress towards the isolation of nucleoli from mammalian tissues by physicochemical techniques were made by Krakauer (1, 2), who used the Waring blender for homogenization with sirupy sucrose as the medium. Liver cells were used as starting material. Specific gravity flotation as well as differential centrifugation was employed in the isolation procedure. The concentrates of nucleoli finally obtained were stated never to be more than 85 per cent pure, and the work apparently has not been continued. One drawback of the procedure was that the nucleoli were obtained directly from the first homogenate, without an intervening isolation of cell nuclei, so that the possibility of confusion between cytoplasmic particulates and nucleoli must be considered. The nuclei were disrupted together with the cells.

Vincent (3) was the second to report on the isolation of nucleoli. Starfish eggs were the source of material, and the nucleoli were isolated directly from an homogenate made by forcing a suspension of the material in a hypodermic syringe through the needle. These nucleoli were analyzed and were found to consist mainly of protein. In addition they contained a small percentage of RNA (but no detectable DNA or histone), small amounts of lipide, and soluble nucleotides. A certain amount of protamine-like material was also said to be present. Phosphorus was found in the protein fraction in a concentration of less than 1 per cent. Of four enzymes tested, acid glyceryl phosphatase was the only one found present, and it was thought that even this might have arisen from contamination by cytoplasmic material. This work has very recently been extended by Baltus (4), who isolated starfish egg nucleoli in sucrose solution and found them to contain nucleoside phosphorylase and an enzyme which synthesizes diphosphopyridine nucleotide.

The third report of the isolation of nucleoli was made by Litt, Monty, and Dounce (5), who used previously isolated liver cell nuclei for starting material. The nuclei were ruptured by means of a sonic oscillator and the liberated

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nucleoli were isolated from the suspension by gravity sedimentations in special sedimenters, followed by differential centrifugation. These nucleoli were found to contain a relatively large concentration of DNA (12 to 18 per cent), and a small percentage of RNA. Histone-like protein was also found to be present.

It is the purpose of the present paper to present in detail a method for obtaining the last mentioned variety of nucleoli, and to outline some new findings concerning certain enzymes present in them. The histochemistry of these nucleoli will also be discussed and possible interpretations of the findings in respect to nucleic acid content will be given. The possible function of nucleoli will be touched upon briefly.

Before entering into the details of the work, it is necessary to describe as accurately as possible just what we mean by the term *nucleolus*, since it has become apparent that there must exist at least two and possibly three or more distinct types of nucleoli. Much of the argument that occurred when our report on nucleoli was first presented arose, in our opinion, from failure to recognize the existence of more than one type of nucleolus.

The two types of nucleoli that are most familiar in the animal kingdom are exemplified by the type that can be seen in large numbers in the nuclei of egg cells of various amphibia, and by the distinctly different type present in far lesser numbers in somatic mammalian cells such as the parenchymal cells of liver. It is of course possible that amphibian eggs may also contain the latter kind of nucleoli in addition to the former type. The former nucleoli are apparently free floating bodies which move to the nuclear membrane and extrude their contents into the cytoplasm (6), thus serving to transport material, probably protein, from the nucleus to the cytoplasm, and therefore might be thought of as analogous to the secretory granules of the acinar cells of the pancreas. The liver cell type of nucleolus on the other hand is not free floating, but is attached to a chromosome (see Discussion). It is this kind of nucleoli that we have studied. The number of nucleoli in liver cells is said to depend upon the ploidy of the cell (7). In order to be certain that no confusion shall arise as to what sort of intranuclear bodies we have designated as liver cell nucleoli, the reader is referred to Fig. 1, which is a photograph of liver cell nuclei isolated by a method described elsewhere, in which 0.44 M sucrose containing 0.005 M calcium chloride is used as the medium of homogenization. The nuclei are subsequently washed in sucrose at pH 6.0 and finally in 1 per cent gum arabic solution. In these nuclei, the nucleoli are especially prominent and can be made to appear black (as in the photograph), without staining, by simply turning the microscope very slightly out of focus. Anyone who has looked at liver cell nuclei isolated in a similar manner cannot fail to recognize the nucleoli which stand out prominently against the nearly optically empty background of the nuclei. In the photograph, one nucleolus appears to be escaping from within a nucleus to the outside.
Fig. 1. Photomicrograph of cell nuclei showing nucleoli. Note the nuclei with ruptured membranes from which nucleoli can be seen emerging. The membranes have been broken in this case by the effect of room temperature. The same sort of effect is produced by the sonic oscillator but to a much greater extent. These nuclei were made in 15 per cent sucrose containing 0.005 M CaCl₂ with two final washings in 1 per cent gum arabic to remove erythrocytes. 4 mm. objective, unstained. Magnification 1,100.

Fig. 2.‡ Photomicrograph of isolated nucleoli. Oil immersion objective, unstained. Magnification 2,560.

‡ Reprinted through the courtesy of Academic Press Inc., from Dounce (9), page 146.
The nuclei used as a source of the nucleoli described in this paper were isolated by a different procedure from that just mentioned, but the nucleoli derived from them have the same general appearance as the nucleoli in the nuclei shown in Fig. 1. It is difficult or impossible to obtain good photographs showing distinct nucleoli in nuclei isolated in gum arabic at pH 6.0 (which were the type used in isolating nucleoli) because of the obscuring mass of condensed chromatin in which they are embedded. After this chromatin has been dispersed by means of the sonic oscillator, however, the liberated nucleoli immediately become very prominent. The liver cell type of nucleolus appears definitely to be a chromosomal appendage, as is the rat fibroblast nucleolus according to Lewis (8). If this view is correct, it should not seem surprising to find that in chemical composition this type of nucleolus resembles the rest of the chromosome. The work outlined in this paper shows that apparently this is indeed the case with liver cell nucleoli.

Experimental Procedures

Procedure for Isolation of Nucleoli.—

The following method has been applied to nuclei isolated in 1 per cent gum arabic at pH 6.0, from rat liver and from cat liver (9), and also to nuclei isolated at pH 4.0 in very dilute citric acid from rat liver (9). All operations are carried out as close to 0°C. as possible. The nuclei isolated from 200 gm. of liver are suspended in approximately 35 ml. of 1 per cent gum arabic of pH 6.3, and the pH of the suspension is then readjusted if necessary to pH 6.25-6.30 with very dilute NaOH, care being taken to avoid local excesses.

The resulting suspension is then divided roughly into two equal portions, and each portion (approximately 18 to 20 ml.) is subjected to sonic oscillation for 7 to 7.5 minutes. The required duration of the treatment in the oscillator is determined by microscopic examination of samples withdrawn at intervals from the oscillator chamber. When enzyme studies were being carried out on the nucleolar preparations, a sample of the suspension which was withdrawn after oscillation and labelled “nuclear homogenate” was set aside for subsequent analysis.

The material taken out of the sonic oscillator is next subjected to centrifugation in two 50 ml. cylindrical centrifuge tubes (each tube containing about 18 ml.) for 20 minutes at 1200 R.P.M. The supernatant fluid from the centrifugation ought to be nearly free of nucleoli, since at this stage of the preparation it is desirable to lose as few nucleoli as possible. This supernatant fluid contains the bulk of the chromosomal material, much of which is in a very finely divided form. It is set aside to be later separated into two fractions by centrifugation.

The sediment from the first centrifugation is next suspended in 25 ml. of ice cold distilled water, and then is allowed to sediment by gravity in a specially designed 50 ml. sedimentor (10) for 15 hours. The 1 ml. of material in the bottom section of the sedimentor is discarded. (This discarded fraction contains nuclear membrane material, unbroken nuclei, rubber from the oscillator gasket, fibrous material, and other unidentifiable fragments.) The supernatant from the sedimentor after being carefully and evenly resuspended (without further change in volume) is again subjected to sedimentation by gravity in the 50 ml.
sedimentation, this time for 7 hours. One ml. in the bottom section again is discarded. The resulting supernatant fluid should be practically free of large particulate contamination, and microscopically should contain almost exclusively nucleoli and chromosomal material, with only very occasionally observable small nuclei.

The supernatant suspension from the second sedimentation is transferred to a 50 ml. centrifuge tube, and the concentration with respect to gum arabic is brought up to 2 per cent by the addition of an appropriate volume of 10 per cent gum arabic solution which has previously been adjusted to pH 6.0. The suspension is then centrifuged at 1,000 R.P.M. for 20 minutes (the total volume is approximately 30 ml.). The pellet is resuspended in 8 to 10 ml. of 2 per cent gum arabic of pH 6.0, and the resulting suspension is centrifuged in a

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
</table>

Fractionation of the Nuclear Homogenate

All steps carried out at 0-3°C.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Medium</th>
<th>Container</th>
<th>Centrifuge speed</th>
<th>Duration of operation</th>
<th>Location of nucleoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 35-40 ml.</td>
<td>1 per cent gum arabic pH 6.3</td>
<td>2-50 ml. cylindrical centrifuge tubes</td>
<td>1200 R.P.M.</td>
<td>20 min.</td>
<td>Sediment</td>
</tr>
<tr>
<td>2. 25 ml.</td>
<td>Distilled water pH 6.0-6.2</td>
<td>50 ml. sedimenters</td>
<td>0 (gravity alone)</td>
<td>15 hrs.</td>
<td>Supernatant fluid</td>
</tr>
<tr>
<td>3. 24 ml.</td>
<td>Distilled water</td>
<td>50 ml. sedimenters</td>
<td>0 (gravity alone)</td>
<td>15 hrs.</td>
<td>Supernatant fluid</td>
</tr>
<tr>
<td>4. 30 ml.</td>
<td>2 per cent gum arabic pH 6.0</td>
<td>50 ml. cylindrical centrifuge tube</td>
<td>1000 R.P.M.</td>
<td>15 min.</td>
<td>Sediment</td>
</tr>
<tr>
<td>5. 8-10 ml.</td>
<td>2 per cent gum arabic pH 6.0</td>
<td>15 ml. conical centrifuge tube</td>
<td>900 R.P.M.</td>
<td>15 min.</td>
<td>Sediment</td>
</tr>
<tr>
<td>6. (Optional) 8 ml.</td>
<td>2 per cent gum arabic pH 6.0</td>
<td>15 ml. conical centrifuge tube</td>
<td>900 R.P.M.</td>
<td>15 min.</td>
<td>Sediment</td>
</tr>
</tbody>
</table>

15 ml. conical centrifuge tube at 900 R.P.M. for 15 minutes. These centrifugations in gum arabic cause the sedimentation of the nucleoli, leaving the finely divided chromosomal contamination in suspension. Generally these two centrifugations provide nucleoli nearly free of chromosomal contamination. However, careful microscopic examination must be relied upon to determine whether a third centrifugation in 2 per cent gum arabic for 10 minutes at 900 R.P.M. is advisable. Table I gives a flow-sheet summarizing the various steps of the isolation procedure.

The gum arabic is removed from the isolated nucleoli by at least two washings with small volumes (8 to 10 ml.) of distilled H2O. However, even three or four such washings do not leave the nucleoli completely free of gum arabic.

The original supernatant suspension remaining after the first centrifugation of nucleoli was fractionated in an arbitrary manner, by centrifugation at full speed for 30 minutes using the high-speed attachment of a refrigerated International centrifuge (speed approximately 17,000 R.P.M.). The sediment, labelled "chromosomal fraction," consisted mainly of whole and fragmented chromosomes, chiefly the latter. The supernatant fluid, which was of a
slightly milky appearance, contained very finely divided chromosomal material in colloidal suspension and presumably a small amount of material brought into solution by treatment in the oscillator. No particulate matter could be seen on microscopic examination. This fraction was labelled "chromosomal supernatant." The two fractions were later analyzed separately.

Notes Concerning the Isolation Procedure.—

It should be pointed out that earlier work in this laboratory on the isolation of nucleoli had met with failure only because of the difficulty of removing chromosomal contamination from the nucleolar preparations by centrifugation in water. The use of gum arabic has greatly minimized this problem, with the result that chromosomal fragments are now less difficult to remove from the nucleoli than are the very small nuclei which inevitably are present in suspensions of cell nuclei isolated from liver. These "micronuclei," which evidently come from non-parenchymal cells of the liver, are very resistant to sonic oscillation, and difficulty in removing them from the nucleolar preparation necessitates the occasional discarding of a preparation of isolated nucleoli as being unfit for analysis. Little or no difference can be seen between the effects of oscillation in water and oscillation in gum arabic solution, but oscillation in the gum solution is more convenient by virtue of its buffering action.

Histochemical Studies.—

(a) Pyronine-Methyl Green Staining.—Isolated nuclei and isolated nucleoli at various stages of the isolation procedure were subjected to staining with a pyronine-methyl green stain made up according to the method of Kay (11).

(b) Staining with the Feulgen Reagent.—Inasmuch as the color shown by cytological structures with the methyl green-pyronine stain seems to depend more on the degree of polymerization of the nucleic acid responsible for the staining (12-15), or possibly on the manner in which this nucleic acid is attached to protein (16), than on the specific type of nucleic acid in question, the behavior of nucleoli towards the Feulgen stain was investigated.2

Very thin sections of rat liver (approximately 1 micron in thickness) were prepared from liver fixed in buffered osmic acid dehydrated in ascending concentrations of alcohol, and embedded in methyl-methacrylate. The plastic was removed in part by extraction with chloroform before staining with the Feulgen reagent.

Nuclei isolated from rat liver by the gum arabic procedure, and nucleoli which had been isolated from such nuclei were centrifuged into pellets and fixed in one of a number of fixatives (Bouin's, Zenker's, acid alcohol, formaldehyde, or buffered osmic acid), dehydrated by passing through ascending concentrations of alcohol, and embedded in paraffin. Sections of these preparations were subsequently freed of the embedding material before staining. Specimens of isolated nuclei and nucleoli were also prepared by air drying fresh smears over a flame. The samples prepared by these techniques were passed through ascending concentrations of ethanol, and steps for the removal of lipide were taken by subsequently extracting with alcohol-ether and chloroform-methanol solutions. Rehydration was effected by passing the slides through descending concentrations of ethanol into water.


The assistance of Dr. V. Emmel and Dr. H. Parks of the Department of Anatomy, Miss Mary Darrow of the Biological Stain Commission, and Dr. Michael Watson of the Atomic Energy Project, The University of Rochester, in various parts of these experiments is gratefully acknowledged.
The specimens were stained by the Feulgen procedure as modified by Conn and Darrow (17). The Feulgen reagent was made up according to the method of Lilie (18). As controls, smears or sections were processed exactly as were the test sections, with the exception that the hydrolysis in HCl was omitted. No color was ever observed in the nucleoli of the control preparations.

**Analysis of Nucleoli for DNA (Desoxyribonucleic Acid) by the Schneider Technique.**—

DNA was determined by the technique of Schneider (19), by subjecting the hot trichloracetic acid extract to the diphenylamine reaction of Dische (20). In this work we have used a standard curve made from a hot trichloracetic solution of thymus DNA isolated by the method of Kay, Simmons, and Dounce (21). Aliquots of this solution were allowed to react with the diphenylamine reagent of Dische and color was read with a Klett-Summerson colorimeter using a No. 54 filter. Using the standard curve thus obtained, amounts of DNA varying from 0.6 to 5.0 mg. could be determined easily and with reasonable precision.

The quantities of DNA in given aliquots of aqueous suspensions of nucleoli were then determined, and aliquots of equal volumes were dried to constant weight in an oven at 105°C. for dry weight determinations. Pipettes with coarse tips were used in this work.

**Analysis of RNA by Spectrophotometry.**—

The procedure involves spectrophotometric determination of the absorption at 260 μm due to total nucleic acid with subsequent subtraction of the absorption due to DNA, leaving as a remainder the absorption due to RNA. In order to acquire the data necessary for the calculations, the extinction coefficients at 260 μm (the absorption maximum) were determined for samples of purified DNA and RNA which had been subjected to treatment with hot trichloracetic acid. (Acid hydrolysis increases the absorption coefficients of both DNA and RNA.) The DNA used for this purpose was that isolated according to the method of Kay, Simmons, and Dounce (21) and the RNA according to that of Kay and Dounce (22).

Carefully dried and weighed samples of DNA and RNA were treated with hot trichloracetic acid in the exact manner as used in the Schneider procedure (19) (see under Analysis for DNA). Aliquots of the solutions were then extracted five times with 5 ml. portions of ethyl ether to remove the trichloracetic acid (which has a high absorption in the ultraviolet region of the spectrum), care being taken to recover the aqueous phase quantitatively each time. The extracted solutions were heated to 60°C for 1 hour to remove ether, and then were made up by adding the required amounts of distilled water to volumes appropriate for reading in the spectrophotometer (generally 10 ml.). The values for absorption at 260 μm were then read in terms of optical density in the Beckman model DU spectrophotometer, and from these values and the corresponding dry weights of material, together with the dilutions, values of \( E_{260}^{\text{per mg}} \) for the trichloracetic acid-treated DNA and RNA samples were calculated. It was found that the value of \( E_{260}^{\text{per mg}} \) for DNA after treatment with hot trichloracetic acid was 246, and for RNA the corresponding value of \( E_{260}^{\text{per mg}} \) was 203.

To determine the RNA content of the nucleoli, the total absorption at 260 μm of an aliquot of the hot trichloracetic extract was measured (after removing the trichloracetic acid with ether), and the fraction of this absorption due to DNA was calculated from the DNA content as measured by the Schneider-Dische technique (see under Analysis for DNA). (The nucleoli had been subjected to preliminary extractions with cold 10 per cent trichloracetic acid before the hot trichloracetic was used, according to the directions of Schneider (19).)

The fraction of the absorption of the aliquot due to DNA was then calculated by subtracting the absorption due to DNA from the total absorption of the aliquot, and from this the amount of RNA in the same aliquot was obtained using the previously determined value of \( E_{260}^{\text{per mg}} \) for the trichloracetic acid–treated RNA. The per cent RNA was finally obtained...
by relating the amount of RNA in the aliquot to the dry weight of nucleoli corresponding to the aliquot (see Analysis for DNA).

**Text for Histone with Isolated Nucleoli.**

An aliquot of nucleoli containing a known dry weight was extracted with cold 0.1 N HCl. To the 1 ml of extract was added two drops of 1 to 5 ammonia. A precipitate which formed was collected after a time, by centrifugation, and after one washing with distilled H2O was dried at 105°C in the oven and weighed (dry weight 0.6 mg.). The ammoniacal supernatant fluid showed no further precipitation with (NH4)2SO4 or sulfosalicylic acid.

**Enzyme Studies.**

(a) **Aldolase.** Aldolase was determined by the method of Dounce et al. (23), wherein the triose phosphate is converted to acetaldehyde and determined colorimetrically by a modification of the Barker-Summerson method for lactic acid. Blanks were run for all samples, and both blanks and experimental samples were run in triplicate at least. The average value of the colorimeter readings obtained from a series of identical determinations was used in the calculations of enzyme activity, except that occasionally an individual colorimeter reading which was completely out of line with other members of the same series was discarded. The same procedure was used for determining the blank values. Aldolase activities are expressed in arbitrary units which are related to the amount of triose phosphate produced per milligram of suspension of sample (dry weight) per 15 minutes at 25°C. These units were calculated by dividing the value of the difference between the average colorimeter reading for the experimental tubes and that for the appropriate blank tubes by the dry weight of the samples used.

(b) **Arginase.** Arginase was determined by the method of Dounce and Beyer (10). This procedure involves the gravimetric determination as dixanthyleurea of the urea formed by the action of arginase on arginine, the substrate. Arginase activities are expressed as k per gram of tissue (dry weight).

\[ k = \frac{1}{t} \log \frac{A}{A - X} \]

\[ t = \text{reaction time in minutes}, \ A = \text{milligrams of dixanthyleurea resulting from complete hydrolysis of substrate}, \ X = \text{milligrams of dixanthyleurea actually recovered after time } t. \]

(c) **Catalase.** Catalase was determined manometrically in an apparatus devised by Dounce (24). Catalase activity is expressed in arbitrary units as cubic millimeters of oxygen evolved per milligram of sample (dry weight) per 15 minutes.

**RESULTS**

**Microscopic Appearance.**

The microscopic appearance of the nucleoli is shown by Fig. 2, which is a photomicrograph of an unstained preparation taken with the oil immersion objective. The vermiform internal structure is just suggested in this photograph. It should be noted that the sonic oscillator has broken off the chromosomes from the nucleoli, but a suggestion of a chromosomal stub attached to one or two of the nucleoli can be seen.

Fig. 3 shows an electron micrograph of a nucleolus within a whole nucleus (isolated at pH 6.0 in very dilute citric acid) and three free nucleoli. The vacuolation is abnormal for these nucleoli, and is probably produced by the
fixative, but nevertheless the photographs indicate strongly that the isolated nucleoli are of the same nature as the nucleolar body within the nucleus.¹

**Information Obtained from Staining Procedures.**—

(a) **Pyronine-Methyl Green Stain.**—When the stain was applied to isolated nuclei, the over-all staining was a deep greenish blue, while some of the nucleoli contained within the nuclei took a pink stain, whereas others appeared to be unstained or stained a blue color. When fresh smears of isolated nucleoli were stained, individual nucleoli were found to vary in color from pink to blue, with many showing an intermediate color. It was obvious that there was a wide divergence in staining properties of the individual nucleoli.

(b) **The Feulgen Reagent.**—The Feulgen-stained liver sections were examined by means of both ordinary and phase microscopy. In these thin sections (1 to 2 microns) the nucleus stains only faintly. There could be no doubt that most of the nucleoli were Feulgen-positive, but a wide divergence in staining intensity was seen for individuals. A few nucleoli were found to stain either not at all or so faintly that a positive reaction could not be established against the faintly pink nuclear background. Essentially the same impressions were obtained from an examination of nucleoli within isolated nuclei.

The examination of the staining properties of the isolated nucleoli was unhindered by a pink nuclear background. The isolated structures displayed the same divergence in staining intensity observed in the fixed liver sections.

¹ These electron micrographs were taken by Dr. Michael Watson.
and in the isolated nuclei, with individuals ranging in color from deep red to faint pink. In this case it was not possible to conclude that any nucleoli were truly Feulgen-negative although a few individuals stained very weakly.

In none of these experiments was there found any evidence of a perinucleolar ring of Feulgen-positive material, but many of the nucleoli observed within liver sections or isolated nuclei displayed one or two strongly Feulgen-positive protuberances, which were thought to be the zones of attachment to chromosomes. Although the vermiform intranucleolar structure seemed to be most intensely stained by the Feulgen reagent, this material is at the limit of resolution of the light microscope, so that this observation might be inaccurate.

### Table II

**Per Cent of DNA on a Dry Weight Basis in Rat Liver Nucleoli and in the “Chromosomal Supernatant” Fractions**

Each figure represents the average of at least two determinations.

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Nucleoli</th>
<th>Chromosomal fraction</th>
<th>Chromosomal supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>17.2</td>
<td>11.4</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>18.1</td>
<td>12.5</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>17.5</td>
<td>11.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Average ..........</td>
<td>17.6</td>
<td>11.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

### Results of Nucleic Acid Determinations

(a) *Analysis for DNA.*—Repeated analyses of five different preparations of isolated rat liver nucleoli for DNA gave values between 16 and 19 per cent for the DNA concentrations on a dry weight basis, with an over-all average of 17.5 per cent. Analyses of the “chromosomal fraction” and the “chromosomal supernatant” showed that both contained DNA in very appreciable concentration, but in neither case in as high concentration as in the nucleolar fraction. The results of the analyses were rather consistent in spite of the arbitrary way in which the last two fractions were separated.

Table II gives the results of analyses of three samples of nucleoli, with average values included. The average per cent DNA differs by 0.1 per cent from the over-all average of all analyses of nucleoli isolated from rat liver.

In the course of analyses of preparation 2 of Table II, the total dry weights recovered in each of the three fractions were determined, and it therefore became possible to calculate the percentage of the total recoverable nuclear DNA contained in each fraction, as well as the percentage of total recoverable nuclear mass recovered in each fraction. The results of these calculations are shown in Table III.

It will be noted that in addition of the columns in Table III necessarily
gives 100 per cent, since the individual values contained therein are based on the total recoverable DNA and total recoverable mass respectively. It has been found in other experiments that the total recoverable mass is 90 per cent or better of the actual nuclear mass. Although losses must necessarily occur in purification of the original nucleolar sediment, this sediment itself constitutes only a rather small fraction of the total nuclear material, the bulk of the chromosomal material remaining in suspension during the first centrifugation. The recovery of the nuclear mass can therefore be high in spite of the loss of a considerable amount of the nucleolar fraction during purification of the nucleoli. It must be kept in mind that the first nucleolar sediment is actually very impure, since there is such a small mass of nucleoli present in nuclei (a few per cent of the total nuclear mass) that a very small proportion of the

<p>| TABLE III |
| Percentages of Total Recoverable Nuclear DNA and Total Recoverable Nuclear Mass Recovered in the Three Fractions of Preparation 2 of Table I |
|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Per cent of total recovered nuclear DNA</th>
<th>Per cent of total recovered nuclear mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoli</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>Chromosomal fraction</td>
<td>68</td>
<td>50</td>
</tr>
<tr>
<td>Chromosomal supernatant</td>
<td>23</td>
<td>45.5</td>
</tr>
</tbody>
</table>

total chromosomal material when mixed with them can cause a high degree of contamination.

The data in Table III permit the calculation that the isolated nuclei from which the nucleoli were obtained contain about 10 per cent DNA. This is in reasonable agreement with the value of about 11 per cent obtained by direct analysis of the nuclei.

It should be noted that the relatively high content of DNA in the "chromosomal supernatant" is an indication that some of the chromosomal material has been divided into particles of colloidal dimensions by the treatment in the sonic oscillator. This colloidal material can be centrifuged down in the Spinco preparative ultracentrifuge at 75,000 g in 30 minutes or less, taking the DNA quantitatively out of the suspension.

(b) Results of Spectrophotometric Analyses for RNA.—The technique employed appears to give quite precise results in the determination of RNA, but since 8 to 10 mg. of nucleoli (dry weight) was required for a single complete determination, only a few analyses were made. The percentages of RNA varied from 1.0 to 1.5 per cent, with an average of 1.3 per cent for the three nucleolar preparations studied. It seems safe to conclude that the isolated nucleoli contain no more than 2 per cent RNA.

No attempt has been made to estimate the intranuclear distribution of
RNA quantitatively, but it seems obvious from these analyses that the isolated nucleoli contain only a very small fraction of the total nuclear RNA. For the nuclei used as starting material, the RNA concentration is around 2 to 3 per cent (9). The idea held by some that all of the nuclear RNA is in the nucleolus seems completely unfounded in the case of liver cell nuclei, since this could hardly be the case even if the nucleoli were nearly pure RNA.

Histone Content of Isolated Nucleoli.—

If we accept the very probable conclusion that the material extracted from the nucleoli with cold 0.1 N HCl and precipitated with ammonia is histone,

<table>
<thead>
<tr>
<th>Table IV</th>
</tr>
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<tbody>
<tr>
<td>Specific Activities of Enzymes of the Subnuclear Fractions of Rat and Cat Liver Nucleoli</td>
</tr>
<tr>
<td>Source</td>
</tr>
<tr>
<td>Rat liver nuclei pH 6.0</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Cat liver nuclei pH 6.0</td>
</tr>
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</tbody>
</table>

Each of the results appearing in the table represents the average of at least triplicate determinations on a given preparation of nucleoli. One preparation of rat liver nucleoli was used for determining the enzymes aldolase and catalase, while the enzyme arginase was determined on a different preparation. All analyses of cat liver subnuclear fractions were made on a single preparation.

then from the results of the single experiment described on the estimation of this material, the histone content of the nucleoli is in the neighborhood of 22 per cent.

Enzyme Determinations.—

The results of the enzyme studies are summarized in Tables IV and V. The specific activities are shown in Table IV, where data obtained from cat liver nucleoli as well as from rat liver nucleoli are included. It can be seen that only in the case of aldolase is the specific activity of the nucleoli higher than that of the other two subnuclear fractions. The relatively high catalase activities of the subnuclear fractions of cat liver nuclei is rather surprising.

In Table V are given data showing the approximate distributions of the
amounts of the various enzymes in the subnuclear fractions of rat liver nucleoli. These distributions of amounts of enzyme are calculated as percentages of total recoverable enzyme, the latter being obtained by summing the amounts recovered in each fraction, so that the percentages in the first three rows of any column of Table V must necessarily add to 100 per cent. The actual total recoveries for the various enzymes generally were not as good as for deoxyribonucleic acid and dry weight, possibly because of decay phenomena. We have quantitative recovery data only for aldolase and catalase. For the former 66 per cent of the total activity of the suspension after sonic oscillation was recovered by summing the total activities of the three subnuclear fractions, and for the latter 86 per cent was recovered. For technical reasons

![Table V](https://www.bspartners.com/)

**Enzyme Distribution* in Subnuclear Fractions**

<table>
<thead>
<tr>
<th>Source Fraction</th>
<th>Aldolase</th>
<th>Arginase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoli</td>
<td>11.5</td>
<td>3.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Chromosomal fraction</td>
<td>30.0</td>
<td>59.3</td>
<td>22.6</td>
</tr>
<tr>
<td>Chromosomal supernatant</td>
<td>58.5</td>
<td>37.5</td>
<td>74.5</td>
</tr>
<tr>
<td>Actual total recoveries</td>
<td>66.0</td>
<td>—</td>
<td>86.0</td>
</tr>
</tbody>
</table>

* Enzyme distributions are calculated in the following manner. The specific activity of the enzyme in each fraction is multiplied by the total dry weight of that fraction, thus giving a measure of the total enzyme contained in each fraction. The sum of the products obtained from all three fractions is a measure of the total enzyme recovered from nuclear homogenates after the fractionation procedure. The percentage contributions of each fraction to these totals are the figures appearing in the table.

In considering the results of enzyme determinations, it becomes necessary to take into account the time intervals involved in the rather lengthy procedure for isolating the nucleoli, since it is possible that autolytic changes and decay of enzyme activities might occur during the course of these prolonged experiments. Isolation of the nucleoli was completed approximately 32 hours after the first homogenization of the liver was effected. The determinations of aldolase, arginase, and nucleic acids were essentially complete 40 hours after the start of the experiment. Catalase determinations were complete within 52 hours from the starting time.

It will be noted that all three enzymes tested are enzymes of the soluble aqueous phase of the cell. The recently discovered interruptions in the nuclear membrane (25) and the apparent permeability of the nuclear membrane to protein (26) make it seem likely that enzymes of the soluble aqueous phase should occur both in cytoplasm and nucleus. The likelihood that the DNA of the nucleus binds protein during the isolation of liver cell nuclei in aqueous medium has been discussed previously
DISCUSSION

In this work we have chosen to use already isolated cell nuclei as starting material for the isolation of nucleoli. This has been done in order to minimize the effects of cytoplasmic enzymes on the nucleoli and also in order to lessen doubt as to what organelles were really being isolated. The question therefore naturally arises as to what sort of nuclei are the most suitable for isolation of nucleoli.

We have recently compared various types of nuclei isolated in aqueous media, and have concluded that the nuclei used in our isolation of nucleoli have been subjected to a certain type of degradation by an intramitochondrial enzyme. This enzyme gains access to the nuclei if the mitochondria are ruptured during the isolation procedure, and rapidly diffuses into them. It has been found that if isolated nuclei can form gels in alkaline solution, this is an indication that the mitochondrial enzyme in question has not acted, whereas if no such gels are found, the enzyme definitely has acted (28).

The nucleoli isolated as described in this paper do not form gels in alkaline solution (at pH 8-10) in spite of their high DNA content. If the DNA is of the type similar to that of the DNA of the chromosome and if it is bound to nucleolar protein, it follows that the mitochondrial enzyme must have penetrated into the nucleoli and detached the nucleolar DNA from nucleolar protein. If on the other hand the DNA is of a low polymer type (see below), the conclusion is not necessarily valid.

In order to throw further light on the problem, preliminary experiments were done in an attempt to obtain nucleoli from nuclei isolated at pH 4, which do form gels in alkaline solution. It was found that if the pH of the suspension of nuclei isolated at pH 4 is subsequently raised to 6.2 with very dilute alkali, nucleoli can be liberated from these nuclei by use of the sonic oscillator, although larger fragments of chromosomes remain in the solution than when nuclei isolated at pH 6.0 are used.

The suspension was capable of forming a gel immediately after being removed from the sonic oscillator, but the nucleoli finally isolated did not form a gel. Thus at first sight it looks as though there might be a difference between the state or type of DNA in nucleoli and in chromosomes proper, but much more work will be necessary before this point can be decided with certainty, since so much time elapses during isolation of the nucleoli that the small amount of residual mitochondrial enzyme which can be present in nuclei isolated at pH 4.0 might be able to produce a considerable effect after adjustment of the pH of the nuclear material to 6.2-6.3.

We do not yet know what type of isolated nuclei would in theory be best
for use in the isolation of nucleoli. When it becomes possible to isolate nuclei in 0.44 M sucrose at pH 6.0 on a large scale without rupture of the mitochondria, these nuclei may be the ones of choice to use in isolating nucleoli, since such nuclei form gels in alkaline solution, and contain firmly bound DNA. For the present, however, one must be content to use nuclei isolated from liver in very dilute citric acid at pH 6.0 or 4.0. The latter offer more difficulties in work with nucleoli than the former.

The next point we shall consider is the meaning of the high percentage of DNA in our isolated nucleoli. The fact that all the isolated nucleoli are Feulgen-positive would seem to indicate the presence of DNA or DNA-like material in all the particles; but the results with pyronine-methyl green staining might be interpreted to mean the absence of DNA from an appreciable fraction of the nucleoli. The high content of DNA obtained by chemical analysis would argue against the presence of an appreciable proportion of DNA-lacking nucleoli. The concentration of DNA in the nucleoli was in fact higher than in any of the other nuclear fractions isolated from the sonically oscillated nuclei.

The knob or horn-like protuberances which can be seen on nucleoli in thin sections of liver or in isolated nuclei stain more intensely with the Feulgen stain than do most of the nucleoli proper, and hence must contain a very high concentration of DNA. We cannot exclude the possibility that these protuberances may contribute to the DNA content of our isolated nucleoli, although we see very few of them attached to the isolated nucleoli. It looks as though the action of the sonic oscillator strips the majority of the protuberances from the nucleoli.

We have been unable to confirm the presence of complete rings of Feulgen-positive material (9) around nucleoli by observing thin sections of Feulgen-stained whole liver cells or isolated liver cells. We are inclined therefore to regard such rings as being made up of the protuberances, in part, and of chromatin in the vicinity of the nucleolus. It seems possible that if fixatives are used which cause condensation of chromatin, the chromosome to which a given nucleolus is attached may coil around the nucleolus, giving the appearance of a Feulgen-positive ring. We earlier proposed the possibility that the high DNA content of the isolated nucleoli might result from the collapse of Feulgen-positive perinucleolar material upon the nucleolus during the isolation procedure (9). The more recent investigations reported here tend, however, to make this possibility seem somewhat less likely.

A different possible explanation for the partially discordant results is that the nucleoli may contain a low polymer type of DNA, either present originally or developed from high polymer DNA as the result of the isolation procedure.

A particularly easy way to see these protuberances is to examine rat liver nuclei isolated in 0.44 M sucrose solution at pH 6.0 (28) by means of the phase microscope.
If a low polymer type of DNA is present, none of the findings would present any discrepancy.

The concentration of DNA in the isolated nucleoli is so high that it seems difficult to account for its presence on the basis of adsorption from the supernatant fluid.

The cinematographic studies of Warren Lewis (8) appear definitely to identify the nucleolus of the rat fibroblast as a chromosomal appendage. We have seen evidence of attachment of nucleoli to chromosomes in rat liver cells, and Mirsky (29) has published a photograph showing the same thing. Our nucleoli also contain an appreciable quantity of histone or histone-like material. It seems logical therefore to consider the composition of the liver cell nucleolus as similar to that of the chromosomes, in spite of the objections against this view which have been raised by some.

There is certainly no lack of histochemical evidence in the literature indicating the presence of DNA in nucleoli (30-34). The vermiform internal structure of the nucleolus, first described by Estable and Sotelo (35), and recently studied by electron microscopy (36-38), has been stated to be Feulgen-positive by Lettré and Siebs (39).

In regard to the enzyme analyses, the results cannot yet be interpreted in a very clear cut manner. Thus far, with one possible exception (40), it seems to be true that enzymes of the soluble phase of the cell are present both in the cytoplasm and in the nucleus. The three enzymes tested by us are all known to be present in the cytoplasm and in isolated nuclei. Of the three, only aldolase was present in higher concentration in the nucleoli than in the other fractions obtained by differential centrifugation of the sonically oscillated nuclei. It seems entirely possible that these enzymes may penetrate the nucleoli by diffusion in vivo and may be held there during the isolation procedure through binding to DNA (see section on Results).

The last point to be discussed is the possible function of the nucleolus in the cell. The nucleoli of amphibian egg cell nuclei seem in a sense to be analogous to secretory granules, since they pass to the surface of the nuclear membrane and appear to empty their contents into the cytoplasm, leaving a residue inside the nuclear membrane (6) (cf. recent studies of Pollister (41). The nucleoli of liver however are attached to chromosomes and apparently maintain their integrity during interphase. A possible clue to the function of the latter type of nucleoli is their disappearance during mitosis.

When the chromatin has condensed into compact chromosomes during mitosis, the latter can hardly be expected to take part in chemical reactions, owing to their presence as an insoluble phase and owing to the great steric hindrance which must be present. Chromosomes are highly dispersed during interphase, however, and are therefore apparently in a favorable state for participating in chemical reactions.

If we apply this same type of reasoning to the nucleolus, we might surmise that the nucleoli should be in a state most favorable for participating in chemical reactions.
when they are dispersed, during mitosis. The assumption is introduced at this point that during mitosis the nucleoli do not dissolve away but merely disperse and become invisible, as do the chromosomes during interphase. It has in fact already been suggested that the nucleolus may function during mitosis (42, 43). But what sort of chemical reactions might occur during mitosis that would not be necessary during interphase?

If we accept the findings presented in this paper that the liver cell nucleolus (Warren Lewis type) is composed of the same material, by and large, that one finds in the chromosome proper (DNA, a little RNA, histone, and other protein), it seems logical that it should function in a manner similar to that in which the chromosome proper must function; i.e., to produce enzymes and proteins either directly or indirectly. According to this concept, the nucleolus would become a sort of auxiliary chromosome, functioning during mitosis at a time when the chromosome proper is not functioning.

Why should there be need within the nucleus of such an auxiliary chromosome? Since amebae can survive for many days without the nucleus (references cited in (27) and (44)), it does not seem likely that loss of chromosomal function during the far shorter period of mitosis could be particularly deleterious to the cell, and hence the function of the nucleolar chromatin need not be expected to duplicate, on a small scale, that of the chromatin of the chromosome proper. But during mitosis there must be a need for special enzymes involved in functions such as dissolving and regenerating the nuclear membrane, and synthesizing and dissolving the special protein of the mitotic apparatus (isolated by Mazia (45)). We should therefore like to propose that the nucleolus of the Warren Lewis type might possibly be an organelle of the nucleus governing the synthesis of such enzymes. In terms of template theory, the nucleolus of the Warren Lewis type would therefore be presumed to contain the special DNA or DNA protein templates necessary for the synthesis of the special enzymes and proteins involved solely in the mitotic process.

The hypothesis just presented does not explain all the observations regarding nucleolar changes within cells, since it does not, for instance, deal with the nucleolar enlargement said to occur in secreting cells such as those of pancreas and salivary glands following secretion (9, 46). It is also reported, however, that nucleoli are commonly enlarged in tumor cells and in liver cells stimulated to divide (7). It is possible that whatever factors cause the latter enlargement also cause the former. The nucleolar enlargement is not understood, and might indicate only a physicochemical type of change such as change in hydration, rather than an actual increase in nucleolar substance. It seems premature to conclude that because certain types of nucleoli enlarge during specific stages in the cycle of protein synthesis, these nucleoli must participate in that protein synthesis.

We do not, however, wish to imply that the nucleoli such as those of the acinar cells of pancreas can have no part in enzyme synthesis by these cells as has been suggested by others (46), but only to suggest that they do not necessarily play a part. We do not know how many different functions might be found for various types of nucleoli, and have presented our hypothesis as one plausible possibility not previously considered to our knowledge by biochemists, and one which might be worth investigating further.
Addendum.—The reader is referred to an article by Gall (47) for a comparison of the nucleoli of amphibian oocytes with those of somatic cells (Warren Lewis type).

SUMMARY

1. The significance of the term nucleolus has been discussed.
2. A detailed method for the isolation of nucleoli from already isolated rat or cat liver nuclei has been presented.
3. The presence of DNA in isolated liver cell nucleoli has been indicated by histochemical methods.
4. The percentages of DNA and RNA in the isolated nucleoli have been determined by chemical analysis.
5. The specific activities of aldolase, arginase, and catalase have been determined for two subnuclear fractions and for the isolated nucleoli of rat and cat liver, and the relative amounts of these enzymes in the same subnuclear fractions and nucleoli of rat liver have been measured.
6. The significance of the above findings has been discussed and consideration has been given to what types of isolated nuclei might best serve as starting material for the isolation of nucleoli.
7. A new hypothesis has been presented that nucleoli of the liver cell type may function primarily in furnishing (directly or indirectly) templates for the synthesis of the particular enzymes that must govern the chemistry of mitosis.

REFERENCES