ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF NUCLEI FROM CHICK EMBRYO LIVER

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ABSTRACT

A procedure is described for the isolation of enzymatically active nuclei from chick embryo liver. It consists of the homogenization of the pooled tissue in 0.32 M sucrose–3 mM $MgCl_2$ followed by a slow centrifugation. The resulting nuclear pellet is then purified further in a discontinuous density gradient composed of sucrose solutions containing Mg^{2+} ions, the lower portion of the gradient being 2.2 M sucrose–1 mM $MgCl_2$. Based on DNA recovery, the nuclear fraction isolated by the procedure described contained an average of 62% of the nuclei in the original filtered homogenate. Light and electron microscope examinations showed that 90% of the isolated nuclei were derived from hepatocytes. They appeared intact with well preserved nucleoplasmic and nucleolar components, nuclear envelope, and pores. The isolated nuclei were quite pure, having a very low level of cytoplasmic contamination as indicated by cytoplasmic enzyme marker activities and electron microscope studies. The nuclear fraction consisted of 19.9% DNA, 6.2% RNA, 74% protein, the average RNA/DNA ratio being 0.32. Biosynthetic activities of the two nuclear enzymes NAD-pyrophosphorylase and DNA-dependent RNA polymerase were preserved. The specific activities of these enzymes were: NAD-pyrophosphorylase, 0.049 μmoles nicotinamide adenine dinucleotide (NAD) synthesized/min per mg protein; Mg^{2+} activated RNA polymerase, 4.3 $\mu\mu$moles UMP-2-C^{14} incorporated into RNA/μg DNA per 10 min; and Mn^{2+}-$(NH_4)_2SO_4$ activated RNA-polymerase, 136 $\mu\mu$moles UMP-2-C^{14} incorporated into RNA/μg DNA per 45 min.

INTRODUCTION

A variety of natural steroids and certain chemicals stimulate porphyrin-heme synthesis in chick embryo liver by inducing the *de novo* formation of δ-aminolevulinate synthetase (ALAS), the rate-limiting enzyme for this pathway (1, 2, 3, 4). To study the molecular mechanism of this induction phenomenon we have focused our recent studies on the nucleus as a possible primary site of action and determined the effect that these compounds have on RNA synthesis and on DNA-dependent RNA polymerase activities during the induction period of ALAS. We describe, in this report, the procedure developed for the isolation of enzymatically active chick embryo liver nuclei used in our studies and present data relating to the chemistry, enzymatic characterization, and electron microscope study of this fraction.

The isolation procedure is based on the principle of the differential flotation technique originally described by others for the isolation of nuclei from rat and guinea pig livers (5, 6) and subsequently modified for the isolation of enzymatically active rat liver nuclei (7, 8).

The method is rapid and gave an average yield of nuclei of 62% based on the recovery of DNA; it also permitted the study of nuclear enzyme activities. To prevent clumping of the nuclei during the course of their isolation (9), Mg^{2+}

ions were added to the various sucrose solutions (10) in substitution for Ca^{2+} (6), which is known to inhibit RNA polymerase activity (11). The presence of a low concentration of a divalent ion is necessary (12, 13, 14); otherwise, cytoplasmic contamination of the nuclear fraction occurs and cannot be removed by repeated washings. The purity of the nuclei isolated from pooled chick embryo liver homogenates by the method described here was relatively good, the criteria for purity being based on the low content of cytoplasmic enzyme markers present in the nuclear preparation and on the examination of the nuclear pellet by electron microscopy.

MATERIAL AND METHODS

Material

Chick embryos (12–17-day old) were killed by decapitation. Cell fractionations were performed with the MSE "Mistral 6L" centrifuge (Measuring & Scientific Equipment Ltd., London, England), and the Spinco Model L ultracentrifuge, using the SW 25.1 rotor for the final purification of the nuclei. Determinations of enzyme activities were performed on a Cary Model 15 (Cary Instruments, Monrovia, Calif.), as well as on a Beckman Model DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) with Gilford attachments for temperature control, automatic cuvette positioner, and recorder (Gilford Instrument Company, Oberlin, Ohio).

Chemicals were reagent grade: sucrose (RNase free) was purchased from Mann Research Labs, Inc., New York; β-NADPH, NAD, nicotinamide mononucleotide (NMN), nicotinamide, adenosine monophosphate (AMP), adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), uridine triphosphate (UTP), and yeast alcohol dehydrogenase from Sigma Chemical Co., St. Louis, Mo.; calf thymus DNA from Worthington Biochemical Corp., Freehold, N. J.; Omnifluor, UTP-2-C^{14}, ATP-8-C^{14} (specific activity 20–30 mCi/mmole and 15–25 mCi/mmole respectively) from New England Nuclear Corp., Boston, Mass.; and Soluene, from Packard Instrument Co., Inc., Downers Grove, Ill.

Methods

Cell Fractionation

Various procedures earlier employed for the isolation of enzymatically active liver nuclei from other species were tested first for applicability to the chick embryo liver (5, 6, 7, 8, 15). The procedure finally developed and used in our experiments is described in detail in the Results section.

Electron Microscopy

Nuclear fractions were prepared for electron microscopy as described by Maggio et al. (6). The pellets were fixed *in situ* according to their procedure "c" with a slight modification: the fixative solutions were prepared in 0.15 M phosphate buffer (pH 7.6) containing 6 mM $MgCl_2$ instead of their buffer. After fixation, the pellets were washed with the same buffer, dehydrated in ethanol and propylene oxide using the rapid dehydration procedure of Coulter (16), and embedded in Epon-812 overnight at 60°C (17) and for 72 hr at 95°C. Ultrathin sections of oriented pellets were mounted on bar grids, stained in uranyl acetate and lead hydroxide–ethanol, and their entire thickness was examined in a Hitachi or a Siemens Elmiskop I electron microscope.

Light Microscopy

The pellets were fixed and embedded in Epon as described above for electron microscopy. Thick sections (0.1 μ) were cut manually with a Porter-Blum microtome, spread on a drop of water, and dried on glass slides. They were stained with a 1% methylene blue–sodium borate solution and photomicrographed in a Carl Zeiss photomicroscope II.

Chemical Determinations

Protein was determined according to the method of Lowry et al. (18) with bovine serum albumin as a standard. DNA and RNA were extracted from tissues and nuclei, as described by Schneider (19). DNA was determined by the procedure of Burton (20) with calf thymus DNA as standard, and RNA by the method of Schmidt-Thannhauser (21). Phosphorus was measured according to the method of Fiske and SubbaRow (22).

Enzyme Assays

NAD-PYROPHOSPHORYLASE (EC 2.7.7.1): ATP:NMN adenyltransferase was assayed by a modification of the procedure of Kornberg (23, 24, 25). The content of the medium for the first reaction mixture was identical to that described in (25); incubation was carried out for 20 min at 37°C and the reaction was stopped by adding 0.2 ml of 5 N $HClO_4$ at 0°C. NAD was assayed spectrophotometrically (24, 26) and enzyme activity defined as μmoles of NAD formed/min per mg protein.

RNA POLYMERASE (EC 2.7.7.6): Activities of DNA-dependent ribonucleoside triphosphate–ribonucleic acid nucleotidyltransferase were determined essentially according to the procedure of Hamilton et al. (27) as follows: incubation time for the Mg^{2+} assay was 10 min at 37°C, and for the Mn^{2+} assay 45 min. The reaction was stopped at 0°C by adding 0.5 ml of cold 0.1% albumin as carrier and 1 ml of 10% trichloroacetic acid (TCA); the precipi-

tates isolated by centrifugation were washed four times with 4 ml of 5% TCA at 0°C, and the ^{14}C-labeled RNA was solubilized by adding 0.5 ml of Soluene. Radioactivity was determined in a Packard Scintillation Spectrometer with a solution of Omnifluor in toluene. Counting efficiency was 74% in the cold. Units of RNA polymerase activity were expressed as $\mu\mu$moles (or cpm) of ^{14}C-UMP incorporated into RNA/min per μg DNA.

GLUCOSE-6-PHOSPHATASE (EC 3.1.3.9) AND 5′-NUCLEOTIDASE (EC 3.1.3.5): Activities were assayed according to previously described methods (28, 29). Glucose-6-phosphatase was assayed at pH 6.5 with 0.015 M glucose-6-phosphate as substrate, and 5′-nucleotidase at pH 7.5 with 0.5 mM AMP in the presence of 0.01 M Mg^{2+}. Units of activities were expressed as μmoles of inorganic phosphate liberated/min per mg protein.

GLUTAMATE DEHYDROGENASE (EC 1.4.1.2): Activity was determined according to Hogeboom et al. (30) and expressed as μmoles of NADH oxidized/min per mg protein.

CYTOCHROME C REDUCTASES (EC 1.6.2.1) AND (EC 1.6.2.3): $NADH_2$ and $NADPH_2$:cytochrome *c* oxidoreductase activities were determined as described earlier (31), with scaling down of the incubation mixture to 0.6 ml. Activities of the reductases were expressed as μmoles of cytochrome *c* reduced/min per mg protein (32).

RESULTS

Preliminary Experiments

The choice of a procedure to isolate enzymatically active chick embryo liver nuclei was somewhat complicated, a large number of methods being published that relate to other species. Since none was available for our particular system, we examined in early experiments the applicability of certain of these methods to the chick embryo liver.

Three procedures describing the isolation of nuclei in the absence of Ca^{2+} were tried (7, 8, 15); the sucrose solutions in these methods contained Mg^{2+} instead of Ca^{2+}. The nuclear fractions obtained from chick embryo liver by these procedures were contaminated by cytoplasmic fragments and the yield was poor, thus restricting biochemical investigations.

Since these methods could not be used in our studies, we decided to improve them in order to increase the recovery of nuclei from avian embryo livers and to obtain preparations free of cytoplasmic contamination. Various other useful isolation methods employ sucrose solutions containing Ca^{2+}, and we felt that keeping certain favorable features of these methods and replacing Ca^{2+} by Mg^{2+} might increase the yield and the purity of our nuclear preparation. We tested in this manner the procedure of Maggio et al. (6), believed to be one of the best available (33), in which the various nuclear preparations obtained from guinea pig liver were monitored very carefully, by electron microscopy and chemical analyses, but the recovery of nuclei that we initially obtained from avian liver was inadequate.

Subsequently, we tested a first homogenization in 0.32 M sucrose–3 mM $MgCl_2$ at 1:5, 1:10, and 1:20 dilutions (w/v) with respect to the original weight of the liver, using the medium employed by other workers (7, 8). Homogenization of the liver in a 1:5 dilution followed by a dilution to 1:10 before filtration through flannelette gave better yield of nuclei, without affecting their structures. A short centrifugation of this homogenate at low speed, followed by a second purification of the nuclear pellet through a 3-layer discontinuous sucrose density gradient composed of 0.88, 1.5, and 2.2 M sucrose, again gave a better yield. If the gradient centrifugation was carried out in a swinging bucket rotor, the contamination was further reduced. To prevent clumping and to preserve morphology of the nuclei, we added decreasing amounts of Mg^{2+} to the sucrose solutions used for the preparation of the gradient as was done with Ca^{2+} (6, 13). We tested first the addition of 1.5 mM $MgCl_2$ to the 0.88 M sucrose and 1.0 mM $MgCl_2$ to the 1.5 and 2.2 M sucrose, and then later 3 mM $MgCl_2$ to the three sucrose solutions. Both procedures gave good results. A preliminary examination by phase contrast microscopy of the nuclear fraction obtained by our experimental procedures indicated that the preparation was homogeneous and relatively pure. The yield (DNA recovery) was near 45%. In subsequent experiments, we examined in the electron microscope nuclear pellets obtained in the presence of 1.0 mM and 3.0 mM $MgCl_2$ and confirmed that the nuclear preparations were quite pure and that the nuclei were not damaged by the isolation procedure. They showed well preserved nuclear envelopes and nucleoplasm; however, some nuclei had a slightly crenated contour and at high magnification the density of their nucleoplasm was somewhat diminished (pale). These nuclear pellets had been prepared for electron microscopy by procedure "c" of Maggio et al. (6) without

modifications. We tried adding Mg^{2+} to the fixative solutions and found that increasing the concentration to 6 mM improved the appearance of the chromatin and of the nuclear substructures when examined in the electron microscope (34). We also found that the use of 0.15 M phosphate buffer instead of 0.10 M to prepare the fixatives and wash solutions prevented the slight crenation of nuclei which was observed with the lower concentration.

Final Procedure

Livers from 12–17-day old chick embryos were dissected, rinsed in 0.9% NaCl, and 6–8 g of tissue were pooled in cold 0.32 M sucrose–3 mM $MgCl_2$, the homogenizing medium. All procedures subsequent to the dissection of embryos were performed at 0°–4°C. The livers were blotted gently on a soft paper towel, weighed in a known volume of fresh homogenizing medium, and passed through a stainless steel tissue press provided with holes of ~1 mm diameter. The resulting tissue pulp was collected, homogenizing medium was added to make a 1:5 dilution (w/v) with respect to the original weight of the liver, and the mixture was homogenized (at 500 rpm) with ten slow up-and-down movements by means of a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa., with an 0.007 inch clearance). The volume of the homogenate was adjusted with the same medium to give a 1:10 dilution (w/v) and filtered through flannelette to remove tissue debris. The filtered homogenate H_1 was centrifuged for 7 min at 900 *g* in a swinging bucket rotor of an M.S.E. centrifuge. The resulting nuclear pellets were weighed and resuspended in 0.88 M sucrose–1.5 mM $MgCl_2$ to a final 1:10 dilution (w/v) with respect to the weight of the pellet, and were very gently homogenized (below 100 rpm) by 5 up-and-down strokes. A 20 ml sample of this nuclear suspension was placed at the bottom of each of the three tubes of the Spinco SW 25.1 rotor, and the discontinuous density gradient was prepared by layering underneath 5 ml samples each of 1.5 M sucrose–1.0 mM $MgCl_2$ and of 2.2 M sucrose–1.0 mM $MgCl_2$ with a syringe and 13 gauge needle (3.5 inch long with a straight tip), the heavier sucrose solution forcing the lighter fraction upward in the tube. The gradient was centrifuged for 1 hr at 54,000 *g* in the Spinco Model L preparative ultracentrifuge. The pellets obtained from this second centrifugation were colorless and slightly opalescent. They sedimented in the shape of a thin halo at the bottom of each tube, and will be referred to as "pellet H_3" in the text. In addition, two distinct layers were noticeable, a zone of turbidity near the top of the original gradient and a reddish layer containing some nuclei, intact cells and heme pigments at the interphase between the 1.5 and 2.2 M sucrose. The entire supernatant was decanted and the nuclear pellet was then transferred with a small spatula and with several washings of 0.25 M sucrose–1.0 mM $MgCl_2$–1.0 mM β-mercaptoethanol to a loose fitting hand-type Dounce glass homogenizer and mixed very gently. The resulting nuclear suspension, referred to as H_3, was used for all the chemical and enzymatic studies described in the text. Preliminary examination of H_3 in the phase microscope indicated that the preparation was quite homogeneous and was composed of intact and unclumped nuclei. A few were of large size, and most contained two or three nucleoli. A sample of H_3 was centrifuged down in the MSE centrifuge at 1200 *g* for 15 min. This fraction referred as "pellet H_4" was used, as well as pellet H_3, for the electron microscope studies presented below. A representative large field of a photomicrograph of nuclear pellet H_3 obtained by this procedure is shown in Fig. 1.

Electron Microscopy

NUCLEAR FRACTION: The purified pellets H_3 or H_4 obtained by the procedure described above were homogeneous and consisted entirely of nuclei (Figs. 1 and 2); most of these were derived from hepatocytes. The nuclei appeared of uniform size with circular profile; some were larger, however, and contained multiple nucleoli; rarely, a nucleus from a Kupffer cell or from a leukocyte was encountered. The purity of these preparations was quite good since very little cytoplasmic debris was visible. Contamination by elements of the endoplasmic reticulum consisted chiefly of ribosomes attached to the outer membrane of the nuclei (Fig. 3). Very few cytoplasmic membranes, either free or still partly attached to a nucleus, were observed when the pellets were examined throughout their entire thickness. Mitochondria or mitochondrial fragments were only occasionally seen among the nuclei and were present in the amount of about one for 40–50 nuclei, which is quite good since Maggio et al. (6) give a count of

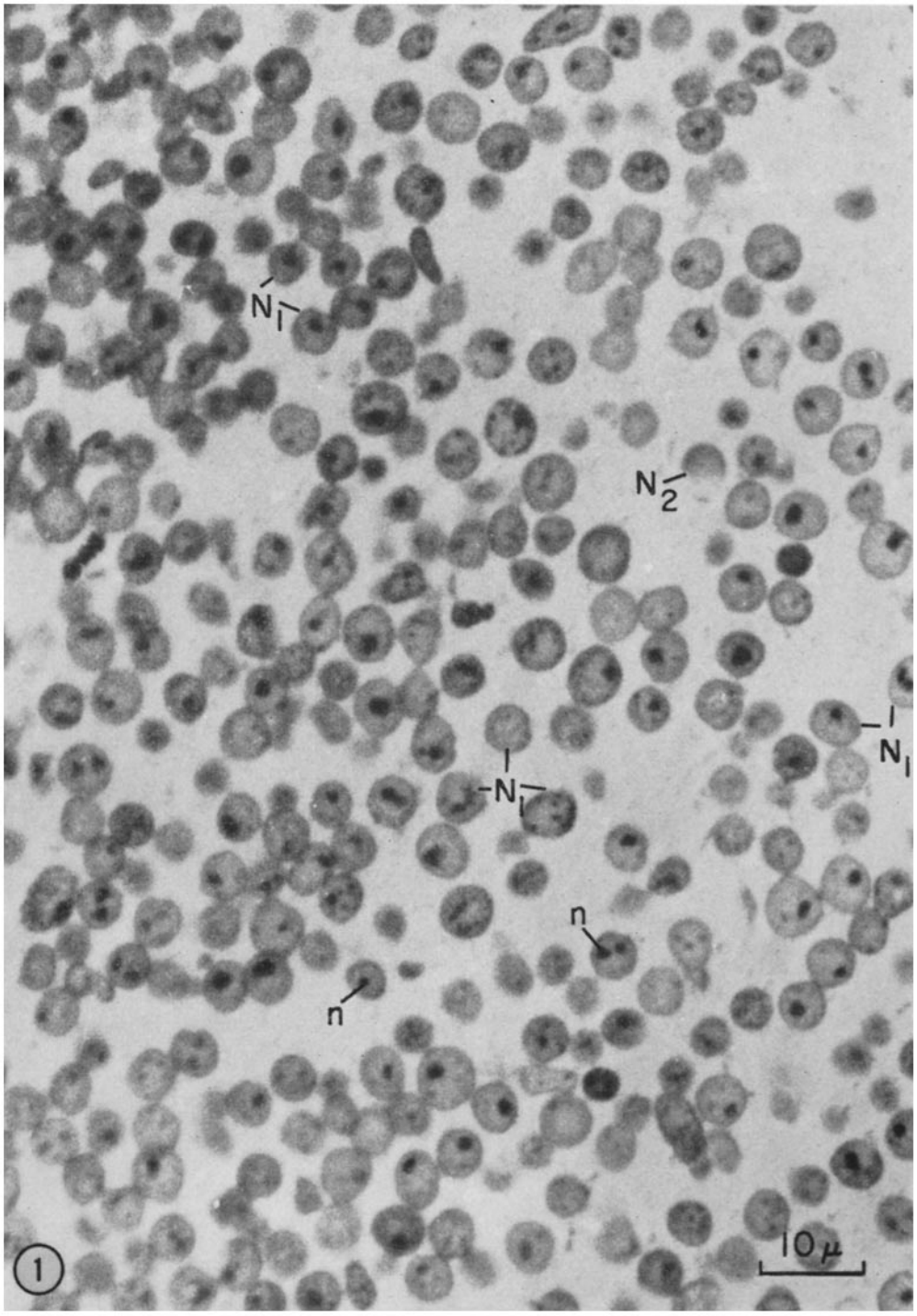

FIGURE 1 Nuclear "pellet H_3" isolated from a pooled homogenate (of 16-day old chick embryo livers) by our final procedure, examined in a Carl Zeiss Photomicroscope II.

The preparation consists of intact nuclei (N_1) containing one or more nucleoli (n). Few nuclei are damaged (N_2) by packing during centrifugation. The top and bottom of the pellet are the areas at the right and left of the picture, respectively. The pellet is fixed in 3.3 M HCHO, postfixed in 0.039 M OsO_4 (both in 0.15 M phosphate buffer [pH 7.6] containing 6 mM $MgCl_2$) and embedded in Epon 812. Section is stained with 1% methylene blue–1% Na borate solution. × 1500.

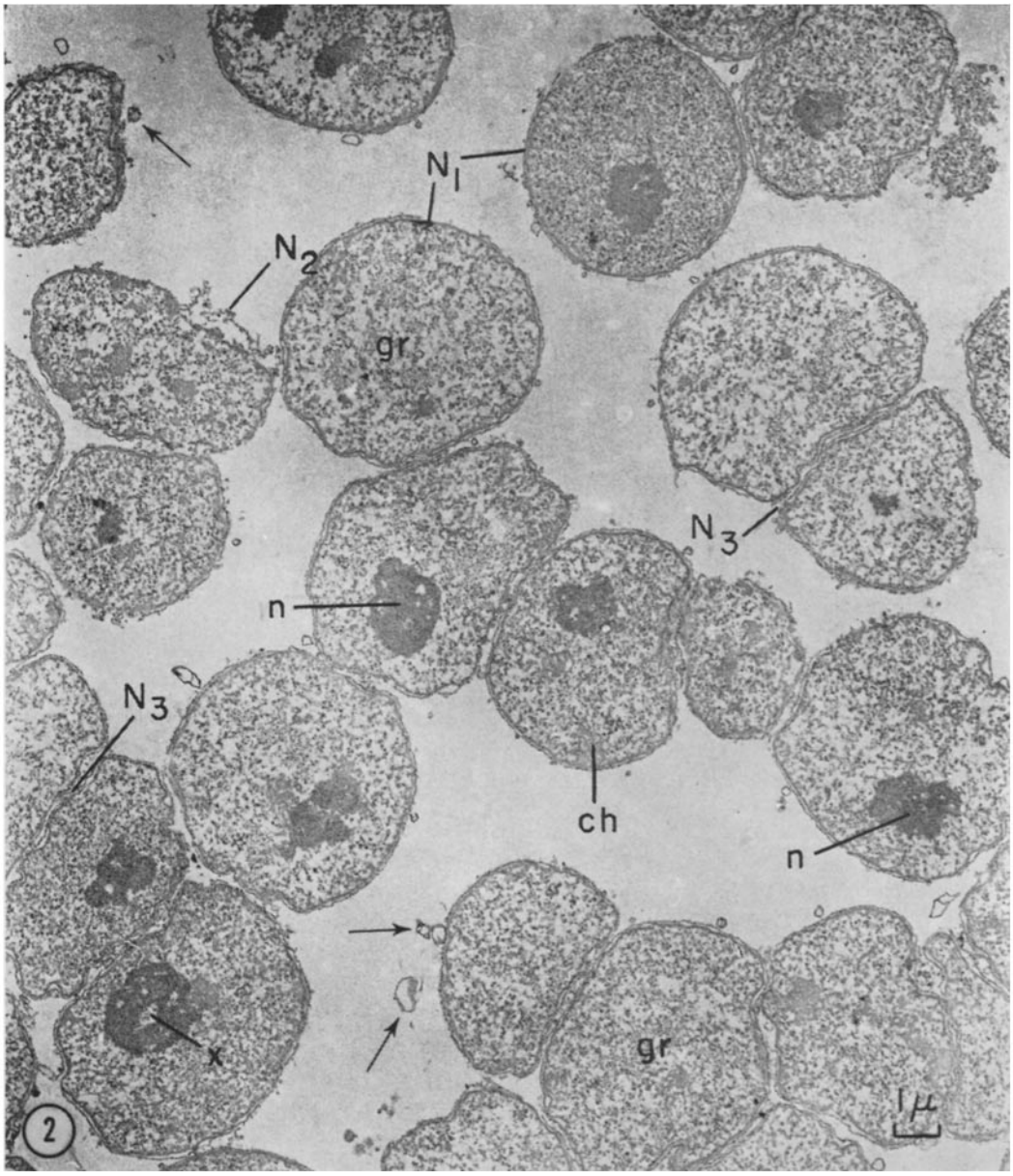

FIGURE 2 Nuclear "pellet H_4" isolated from a pooled homogenate (of 16-day old chick embryo livers) by our final procedure, examined in a Siemens Elmiskop I electron microscope.

The preparation consists of intact (N_1), partially damaged (N_2) nuclei. Some are slightly deformed (N_3) by packing during centrifugation.

Cytoplasmic contamination is minimal and consists of membranes from the endoplasmic reticulum (microsomes), some of them still attached to the nuclear envelope (*arrows*). A certain amount of differentiation is visible in the nucleoplasm, fine condensed chromatin regions (*ch*), and small granules (*gr*) heterogeneous in size. Nucleoli (*n*) are present in several nuclei and show their dense granular structures; others have lacunae (*x*), which appear as areas of lighter content. Nuclear envelopes can be seen at the periphery of the nuclear profiles.

Pellet fixed in 3.3 M HCHO, postfixed in 0.039 M OsO_4 (both in 0.15 M phosphate buffer [pH 7.6] containing 6 mM $MgCl_2$) and embedded in Epon 812. Sections are stained with uranyl acetate and lead citrate. × 6000.

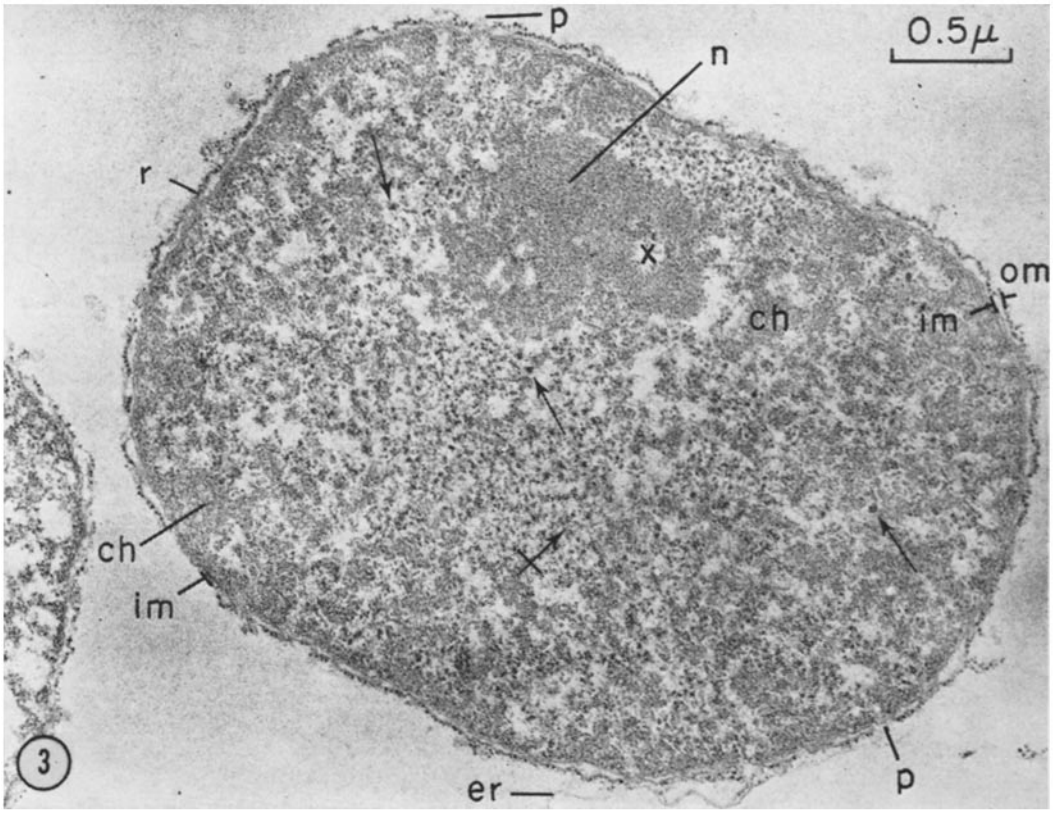

FIGURE 3 Two profiles of liver cell nuclei in a nuclear fraction H_3 isolated by our final procedure and examined at a higher magnification in a Hitachi electron microscope.

In the nucleoplasm of the central profile one large nucleolus (*n*) is visible and shows the characteristic dense granular texture of its content as well as several lacunae (*x*), which appear as lighter areas. Regions of fine textured chromatin (*ch*) are located at the periphery of the nucleus near the inner membrane of the nuclear envelope (*im*) and around the nucleolus. Interchromatin (+⟶) and perichromatin (⟶) granules are visible, as well as a few nuclear pores (*p*). A small piece of membrane from the endoplasmic reticulum (*er*) is still attached to the outer nuclear membrane (*om*). On the latter, ribosomes (*r*), which appear in rows, are clearly distinguishable. Preparation of the pellet for electron microscopy as for Fig. 2. × 32,000.

1 per 10 nuclei and Hogeboom et al. (13) 1.5 per nucleus, with their isolation procedures. Nuclei showed occasionally blebs attached to their nuclear envelope; structural damage otherwise appeared minimal. Very few nuclear profiles showed complete rupture of the nuclear envelope and loss of their nucleoplasm or nucleoli.

MORPHOLOGY OF ISOLATED NUCLEI: The chick embryo liver nuclei from our purified preparations appeared well preserved when doubly fixed in HCHO and OsO_4 and they resembled nuclei from liver cells *in situ*. The ultrastructural elements of their nucleoplasm and nucleoli, as well as nuclear pores, were quite distinguishable (Fig. 3).

In the nucleoplasm, the chromatin known to contain deoxyribonucleoproteins (35, 36) showed a fine, even texture in most nuclei. In addition, granular and fibrillar elements of varying size and staining intensities were present; these were widely distributed between the nucleoli and the nuclear envelope. The smaller interchromatin granules (37) were randomly distributed as clusters of particles among the chromatin, whereas the larger perichromatin granules (38) were seen at the periphery of the chromatin clumps. These two types of nuclear granules have been shown to consist of ribonucleoproteins and to carry RNA (38, 39). The filamentous elements, possibly the perichromatin fibrils (40), were faintly visible.

These structures, as well as others not visible in Fig. 3, are difficult to identify unless special staining procedures are used.

Most of the isolated nuclei contained nucleoli (Fig. 2). These appeared as densely stained large masses in the nucleoplasm and were composed of fibrillar and granular materials with lacunae of lighter content, the texture of which resembled that of chromatin (41, 42, 43). The principal nucleolar components (43, 44) could be identified in various nuclear preparations. The closely packed nucleolar granules (Fig. 3) located along the strands of the fibrillar network are ribonucleoprotein in nature; they contain in addition the ribosomal genes, and precursor rRNA molecules are known to be synthesized in such structures (45, 46, 47). The fibrillar centers (44) were visible in some nucleoli, located either at the periphery of the nucleoli and protruding out in contact with the chromatin or enclosed in the dense fibrillar nucleolonema.

The nuclear envelope composed of a double membrane containing nuclear pores (6, 48) was usually identifiable around each nucleus (Figs. 3 and 4). These pores presumably allow passage of nuclear components to the cytoplasm as well as entrance of various materials to the nuclei; however, concrete evidence of this transport mechanism is absent. Blebs and small pieces of membranes still attached to the nuclear envelope and in continuity with the endoplasmic reticulum were occasionally seen. The outer membrane, to which ribosomes are attached, was sometimes broken.

Chemical Determinations

NUCLEAR RECOVERY: The nuclear fraction H_3 obtained by our procedure contained, on the average, 62% of the DNA from the initial filtered homogenate H_1 as shown in Table I. An increase in nuclear recovery was obtained by increasing, previous to centrifugation in the SW 25.1 rotor, the 1:10 dilution of the crude nuclear pellet to 1:20 with the 0.88 M sucrose solution. The yield of nuclei in pellet H_3 was improved only slightly by prolonging the centrifugation time of the sucrose gradient to 90 min.

Variations in the recovery of nuclei were obtained in several experiments. These can be related in part to the original weight of pooled liver tissue processed each time, 3–5 g of tissue giving the greatest recovery as shown in Table I.

CHEMISTRY: The amounts of DNA, RNA, and protein found in five different H_3 fractions, prepared according to our final isolation procedure, are given in Table I. On the average, DNA made up 19.9%, RNA 6.2%, and protein 74.0% of the nuclear fraction, when the sum of these three constituents was taken as 100. These values are very close to those reported by Maggio et al. (6) for guinea pig liver. The average ratio of DNA to protein was 0.264, RNA to protein 0.084, and RNA to DNA 0.317. A small fraction of the RNA in our nuclear preparation was due to the ribosomes attached to the outer membrane. Other constituents such as lipids and carbohydrates were not determined.

Nuclear Enzyme Activities

Two enzyme activities previously reported to be exclusively located in the nucleus are NAD-pyrophosphorylase (7, 24, 33, 49, 53) and DNA-dependent RNA polymerase (8, 11, 27); the latter is probably also present in mitochondria (49, 50, 51, 52). Activities of these two enzymes in H_3 preparations are shown in Table II. The average specific activity for NAD-pyrophosphorylase in H_3 was 0.049 μmoles NAD/min per mg protein. The chick embryo liver enzyme was activated by Mg^{2+} and inhibited by Ni^{2+}; the latter metal was reported to be an absolute requirement for this enzyme in the rat liver (54). The average recovery of NAD-pyrophosphorylase, as a nuclear enzyme marker, was calculated to be 78% (Table II).

RNA polymerase activities of various nuclear fractions H_3 were determined in the presence of either Mn^{2+} ions and $(NH_4)_2SO_4$ at pH 7.5 or Mg^{2+} ions alone at pH 8.5. These polymerase activities, located respectively in the nucleoplasm and the nucleoli, synthesize different RNA species (6, 27). The data reported in Table II show in vitro incorporation of UMP-2-C^{14} into RNA, synthesized on a chick embryo liver nuclear DNA template at 37°C. The average specific activity for the Mg^{2+} activated enzyme was 4.3 $\mu\mu$moles/μg DNA per 10 min, and for the Mn^{2+}–$(NH_4)_2SO_4$ activated enzyme 136 $\mu\mu$moles/μg DNA per 45 min. The enzyme reactions were dependent on the presence of all four nucleoside triphosphates, a DNA template, a divalent ion (either Mg^{2+} or Mn^{2+}), and were inhibited by RNase, DNase, and actinomycin D (Table III). The reaction was also directly proportional to the DNA present in

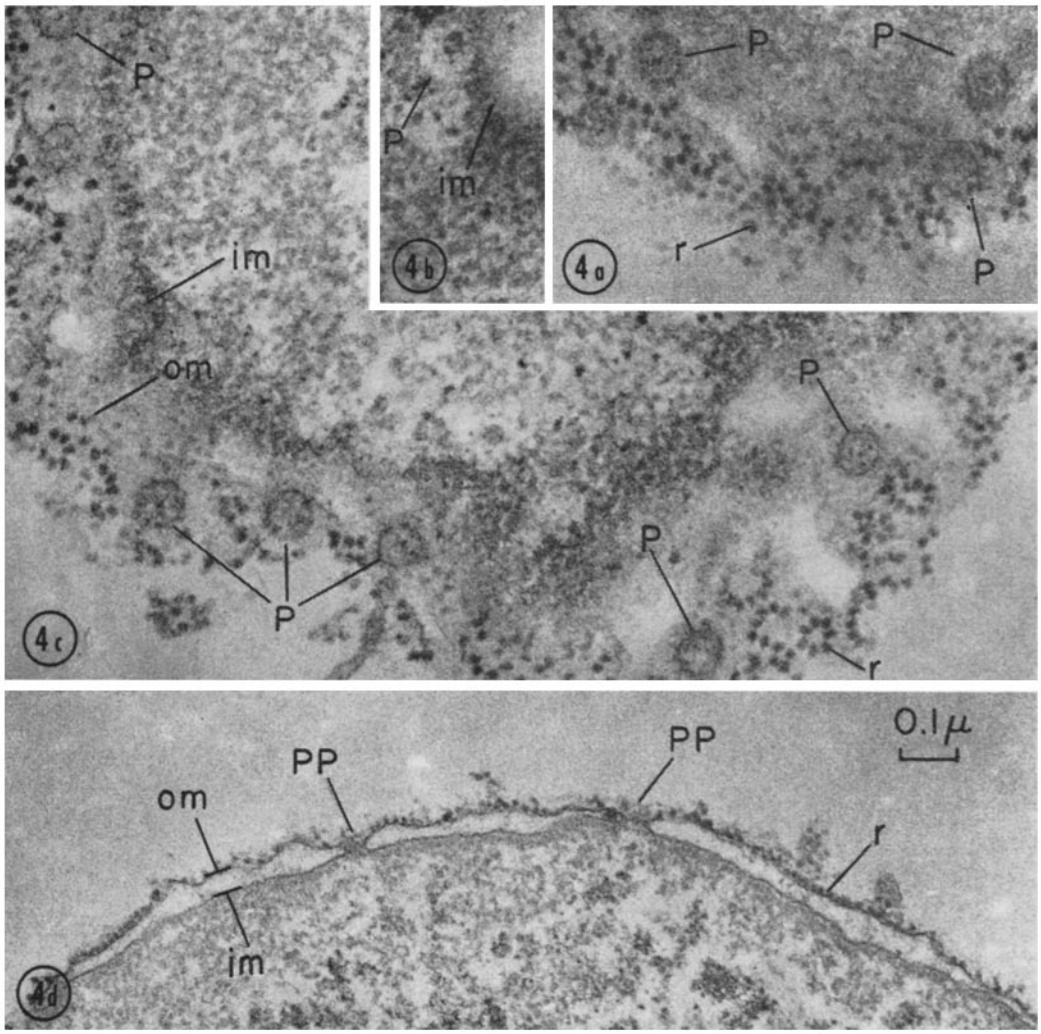

FIGURE 4 Three grazing sections at the top (4a, 4b, 4c) and a normal section at the bottom (4d) showing structural details at the periphery of four liver cell nuclei in nuclear "pellet H_4" isolated by our procedure.

The inner and outer membranes are marked (*im*) and (*om*) respectively. In the grazing sections, these two membranes overlap to give an area of moderate density around the nuclear profile. Also, in these sections some of the ribosomes (*r*) still attached to the outer membrane appear arranged in spirals, whereas in the medial section below they appear in rows. A number of pores are visible in the three sections and are marked (P); some seem plugged at (PP). In the grazing sections (4c) certain pores appear as small circles; others have definite hexagonal profiles in (4a) in contrast to an octagonal shape previously reported (63). They are plugged with dense material, and some contain small, denser granules in their center, whereas in the normal section they show areas of lighter content directly under them which look like channels through the peripheral chromatin.

Preparation of the pellet for electron microscopy as for Fig. 2. All four sections × 77,000.

the reaction mixture, in the range 50 and 200 μg DNA, added as 0.1 ml of nuclear fraction H_3. If the specific activities are expressed as μμmoles of UMP-2-C^{14} incorporated into RNA/minute per μg DNA, the Mn^{2+}–$(NH_4)_2SO_4$ activated RNA polymerase activity was three times greater than the Mg^{2+} activated one. Recovery of RNA polymerase activities in nuclear fraction H_3

TABLE I

Nucleic Acid and Protein Contents of Hepatic Nuclear Fractions (Chick Embryo)

The nuclear fractions H_3 were isolated by the procedure described in the text. DNA, RNA, and protein were determined as indicated in the Methods section.

Experiment	Wet weight processed	Yield (recovery of DNA)	DNA		RNA		Protein		DNA/protein	RNA/protein	RNA/DNA
	g*	%	mg/g‡	%§	mg/g‡	%§	mg/g‡	%§			
1	6.50	47	0.665	20.3	0.208	6.4	2.40	73.5	0.277	0.087	0.312
2	10.5	45	0.625	24.2	0.160	6.2	1.79	70.0	0.349	0.089	0.255
3	3.57	62	0.430	16.5	0.157	6.0	2.05	77.5	0.209	0.077	0.365
4	2.50	68	0.880	—	—	—	3.77	—	0.233	—	—
5	5.50	86	0.650	18.7	0.218	6.3	2.60	75.0	0.250	0.084	0.335
Averages		62	0.650	19.9	0.186	6.2	2.52	74.0	0.264	0.084	0.317

*Pooled 16-day old chick embryo livers.
‡Per gram of original tissue.
§DNA + RNA + proteins = 100.

TABLE II

Specific Activities of NAD-Pyrophosphorylase and RNA Polymerase in Hepatic Nuclear Fractions (Chick Embryo)

The homogenates H_1 were the initial filtered homogenates of the pooled livers. The nuclear fractions H_3 were isolated by the procedure described in the text. NAD-pyrophosphorylase and RNA polymerase activities were determined according to the assay procedures described in the Methods section. The RNA polymerase reaction mixture contained 95×10^3 cpm of UTP-2-C^{14} and 0.1 ml of nuclear fraction H_3, representing 45–66 μg DNA. The specific activities for the Mg^{2+} and Mn^{2+}-$(NH_4)_2SO_4$ assays are expressed as μμmoles of UMP-2-C^{14} incorporated into RNA at 37°C per 20 min and per 45 min, respectively.

Experiment	NAD-pyrophosphorylase						DNA-dependent RNA polymerase	
	Specific activity		Total activity				Specific activity	
	μmoles NAD/min per mg protein × 10^{-3}		μmoles NAD/min per mg protein per fraction		Recovery		Mg^{2+} assay	Mn^{2+}-$(NH_4)_2SO_4$ assay
							μμmoles of ^{14}C-UMP incorporated into RNA per μg DNA	
	H_1	H_3	H_1	H_3	H_1	H_3	H_1	H_3
					%	%		
1	—	—	—	—	—	—	5.2	88.5
2	6.0	62.3	0.360	0.425	100	115	—	—
3	—	—	—	—	—	—	3.6	150
4	7.8	40.2	0.430	0.302	100	70	—	—
5	—	—	—	—	—	—	5.2	132
6	8.9	43.7	0.525	0.250	100	48	—	—
7	—	—	—	—	—	—	3.9	121
8	—	—	—	—	—	—	3.8	186
Averages	7.6	48.7	0.437	0.329	100	78	4.3	136

TABLE III

Nucleotide and Ionic Requirements and Inhibitory Effects of Various Compounds in the RNA Polymerase Assay

The standard RNA polymerase assay conditions were used and are described in the Methods section. For experiment A, the reaction mixture for each assay contained 92×10^3 cpm of UTP-2-C^{14} and 0.10 ml of nuclear fraction H_3, representing 72.5 μg of DNA. For experiment B, the reaction mixture for each assay contained 74×10^3 cpm of UTP-2-C^{14} and 0.10 ml of nuclear fraction H_3, representing 57.2 μg of DNA. The enzyme activities are expressed as net cpm of ^{14}C-UMP incorporated into RNA per 45 min for the Mn^{2+}-$(NH_4)_2SO_4$ assay and per 5 min for the Mg^{2+} assay.

		Mn^{2+}-$(NH_4)_2SO_4$ assay		Mg^{2+} assay	
Experiment	Reaction mixture	cpm	% of ^{14}C-UMP Incorporated	cpm	% of ^{14}C-UMP Incorporated
A	Complete incubation medium	4251	100	148.7	100
	− ATP, CTP, GTP	69.3	1.6	78.7	53
	− ATP	206.9	4.9	62.4	42
	− CTP	1066	25	38.5	26
	− GTP	73.5	1.7	41.6	28
	+ 5 μg Actinomycin D	595.4	14	32.1	22
	+ 200 μg RNase	59.6	1.4	20.3	14
	+ 50 μg DNase	23.6	0.55	45.8	31
B	Complete incubation mixture containing 1.0 μmole of $MnCl_2$	2160	100	—	—
	− $MnCl_2$	131.1	6.1	—	—
	Complete incubation mixture containing 0.5 μmole of $MgCl_2$	—	—	85.0	100
	− $MgCl_2$	—	—	40.2	47

could not be determined, since these enzyme activities could not be accurately determined in homogenate H_1. These values for NAD-pyrophosphorylase and RNA polymerase activities are difficult to compare with other values reported in the literature for various tissues since assay conditions are different and enzyme activities were not expressed similarly (27, 54, 55).

Cytoplasmic Enzyme Marker Activities

The specific activities of five enzymes, markers for different cytoplasmic organelles, were determined in various isolated nuclear fractions H_3, and the percentages of homogenate H_1 activities ultimately recovered in the isolated nuclear fractions H_3 are shown in Table IV. Contamination of the nuclear fraction by these enzymes was very low, indicating substantially less cytoplasmic contamination than has previously been reported (56).

DISCUSSION

Method

The method described here permits the isolation of nuclear fractions from chick embryo liver in high yield with very low levels of cytoplasmic contamination. In addition, enzymatically active nuclei are obtained which retain their capacity to carry out RNA synthesis. The nuclear preparation thus obtained from a homogenate of pooled 12–17-day old chick embryo livers therefore permits biochemical investigations on this hormone-responsive tissue which would otherwise not be possible.

Our procedure borrows certain essential features from methods developed previously for the isolation of nuclei from other tissues. It retains the suspension medium, composed of sucrose solutions containing Mg^{2+}, that was used by other investigators in their studies on RNA synthesis (7, 8,

TABLE IV

Specific Activities of Cytoplasmic Enzyme Markers Present in Nuclear Preparations H_3 (Chick Embryo)

Standard assays were used to determine enzyme activities according to procedures described in the Methods section. Per cent was determined by calculating per cent of the total activity of homogenate H_1 found in nuclear fraction H_3.*

Experiment	Glucose-6-phosphatase			5′-Nucleotidase			Glutamate dehydrogenase			$NADPH_2$-Cytochrome *c* oxidoreductase			$NADH_2$-cytochrome *c* oxidoreductase		
	μmoles of P_i liberated per min/mg protein × 10^{-2}		Total activity found in H_3	μmoles of P_i liberated per min/mg protein × 10^{-3}		Total activity found in H_3	μmoles of NADH oxidized per min/mg protein × 10^{-3}		Total activity found in H_3	μmoles cytochrome *c* reduced per min/mg protein × 10^{-3}		Total activity found in H_3	μmoles cytochrome *c* reduced per min/mg protein × 10^{-3}		Total activity found in H_3
	H_1	H_3	*per cent*	H_1	H_3	*per cent*	H_1	H_3	*per cent*	H_1	H_3	*per cent*	H_1	H_3	*per cent*
A	7.2	0.13	0.23	10.3	0.60	0.50	12.4	2.1	2.0	—	—	—	—	—	—
B	8.9	—	—	9.4	—	—	9.7	2.0	0.6	—	—	—	—	—	—
C	9.4	—	—	13.7	—	—	13.0	1	1	—	—	—	—	—	—
D	—	—	—	9.6	3.6	2.9	—	—	—	—	—	—	—	—	—
E	7.8	0.09	0.10	10.2	0.1	1	—	—	—	8.10	3.57	5.0	89.5	33.0	4.2
F	—	—	—	—	—	—	—	—	—	9.15	2.52	3.8	72.8	23.1	4.3
G	—	—	—	—	—	—	—	—	—	5.15	2.02	3.8	49.6	20.6	4.0
H	—	—	—	—	—	—	—	—	—	10.7	1	1	98.0	29.4	1.3
I	6.7	—	—	10.4	2.0	5.0	—	—	—	—	—	—	—	—	—
Averages	8.0	0.11	0.17	10.6	1.6	2.1	11.7	1.4	0.87	8.27	2.03	3.1	77.5	24.0	3.5

*This figure is based on *total* activities in the H_1 and H_3 fractions (specific activities × total volume in each fraction).

27, 57, 58). The preliminary filtration of the homogenate, followed by a low speed centrifugation, allows a gross separation of the nuclei from the various other components of the cell and contributes to the high yield obtained in our procedure. Finally, the purification of the crude nuclear pellet so obtained through a discontinuous sucrose density gradient, the lower part of which contains 2.2 M sucrose, removes cytoplasmic contamination most efficiently.

Nuclear Fraction

PURITY: The degree of purity of the nuclear preparations isolated by the procedure described in this report was evaluated by phase and electron microscopy and by determinations of their content of several cytoplasmic enzyme markers.

Examinations of the entire thickness of several of our pellets indicated that our nuclear preparations were quite pure. The nuclei retained their outer nuclear membrane to which ribosomes are attached, this being an unavoidable but negligible contamination when nuclei are isolated in aqueous media. This outer nuclear envelope is cytoplasmic in nature and is part of the endoplasmic reticulum (59). It can be removed by washing the nuclei with mild detergent solutions, but with variable loss of soluble components from the nucleoplasm. For our studies, the small RNA contamination introduced by the ribosomes attached to this membrane was not significant.

To confirm the low level of cytoplasmic impurities observed by electron microscopy, we determined the concentration of five cytoplasmic enzyme marker activities on the same nuclear pellets. These activities associated with our nuclear preparations would represent either microsomal, mitochondrial, or plasma membrane contaminations. In the several preparations studied, the levels of these marker enzyme activities were extremely low or not detectable (Table IV).

MORPHOLOGY: The nuclear preparations obtained by our procedure were homogeneous in their composition and consisted mostly of nuclei, of which more than 90% were derived from parenchymal liver cells. Electron microscope examination showed that the nuclei were well preserved, most of them having circular profiles with a well defined nuclear envelope. Components of the nucleoplasm stained more intensely when nuclei were fixed in the presence of 6 mM $MgCl_2$ and appeared, by electron microscope examination, to have more contrast. No evidence for nucleoplasmic loss through the nuclear pores during the isolation procedure was detected. The content of the nucleoplasm was well preserved and the chromatin in most nuclei appeared homogeneous.

CHEMISTRY: The chemical composition of our H_3 fractions can be compared to that of the hepatic nuclear fractions obtained by Maggio et al. (6) for guinea pig, since we expressed our data in the same manner. The yield, based on the recovery of DNA, was improved from about 30 to about 60%. The DNA, RNA, and protein contents of the isolated nuclei in our preparations were, in varying amounts, slightly higher than those reported by Maggio et al. (6), and others (5, 13, 60).

NUCLEAR ENZYMES: The nuclei of our isolated fractions (H_3) were enzymatically active as indicated by the presence of significant activities of NAD-pyrophosphorylase and DNA-dependent RNA polymerase (Table II). Loss of proteins from the nuclei was minimal, since 78% of the total activity of NAD-pyrophosphorylase present in the original homogenate was still found in the isolated nuclear fraction. The RNA species synthesized by the isolated nuclei, as judged by labeling experiments with UTP-2-C^{14} (Tables II, III), were mostly due to the presence of the Mn^{2+}-$(NH_4)_2SO_4$ stimulated RNA polymerase. Some of the RNA species synthesized in the nucleoplasm by this enzyme are of the messenger type, whereas the Mg^{2+} stimulated RNA polymerase, located in the nucleolus, synthesizes the ribosomal RNA precursor molecules (27, 61, 62).

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