A Procedure for the Isolation of Enzymically Active Rat-Liver Nuclei

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The current concept of the genetic control of protein synthesis has focused attention on the metabolic activities of the cell nucleus, and current work in this Laboratory on the hormonal regulation of nucleic acid and protein synthesis (Widnell & Tata, 1963; Tata, 1963; Freeman, Roodyn & Tata, 1963) necessitated the isolation of nuclei under optimum conditions.

When reviewing the methods available for the isolation of liver nuclei, Roodyn (1963) concluded that the criteria for assessing preparations of nuclei should be their morphological appearance, together with the demonstration of both nuclear enzyme activity and low activities of cytoplasmic enzyme markers. NAD pyrophosphorylase (EC 2.7.7.1) (Hogeboom & Schneider, 1952) and DNA-dependent ribonucleotide triphosphate-ribonucleic acid nucleotidyltransferase (RNA polymerase; EC 2.7.7.6) (Weiss, 1960) were selected as the two exclusively nuclear activities so far reported, and cytochrome oxidase (EC 1.9.3.1), NADH-cytochrome oxidoreductase (EC 1.6.2.1), glucose 6-phosphatase (EC 3.1.3.9) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as the cytoplasmic enzyme markers.

The use of organic solvents and citric acid for isolation of nuclei was avoided since their presence may damage membrane structures (Keir, Smellie & Siebert, 1962; Gurr, Finean & Hawthorne, 1963), and Ca^{2+} ions were omitted from the medium since they inhibit RNA polymerase (Weiss, 1960).

Although nuclei isolated in 2.2M-sucrose are relatively free from cytoplasmic contamination (Chauveau, Moulé & Rouiller, 1956), the high viscosity of the suspension involves the danger of heating during homogenization; in the method described below, therefore a crude nuclear fraction isolated in 0.25–0.32M-sucrose–3 mM-magnesium chloride (Stirpe & Aldridge, 1961) is purified by resuspension in 2.2M-sucrose and centrifugation. A preliminary communication of our results has been presented (Widnell, 1963).

EXPERIMENTAL

Animals. Male Wistar rats, weighing about 150 g., bred at this Institute and given food and water *ad libitum*, were used throughout.

Reagents. NAD, NMN, ATP, GTP, CTP, UTP and deoxyribonuclease were obtained from the Sigma Chemical

Co., St Louis, Mo., U.S.A.; nicotinamide was from Roche Products, Welwyn Garden City, Herts.; yeast RNA and yeast alcohol dehydrogenase were from Boehringer und Soehne G.m.b.H., Mannheim, Germany; yeast DNA was from L. Light and Co. Ltd., Colnbrook, Bucks.; [$8\text{-}^{14}\text{C}$]ATP (specific activity 2.3 mc/m-mole) was from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A.

Other materials. Nylon bolting cloth (110 mesh) was obtained from John Stanier Ltd., Manchester.

Isolation of nuclei. All procedures were performed at 0–4°. Livers from at least three animals were pooled and weighed in 0.32M-sucrose containing MgCl_2 (3 mM) (homogenizing medium); the tissue was minced finely with curved scissors and rinsed once with homogenizing medium before preparing a 25% homogenate (1 part liver, 3 parts medium) in a Potter-Elvehjem-type homogenizer of the same dimensions as that described by Aldridge, Emery & Street (1960) for rat liver. Homogenization was complete after 25 slow up-and-down movements, with a pestle speed of 1200 rev./min. The homogenate was filtered through two layers of nylon bolting cloth to remove clumps of connective tissue attached to unbroken cells; 12.5 ml. samples of homogenate were diluted to 20 ml. with homogenizing medium, and then to a final sucrose concentration of 0.25 M with water. Homogenizing medium (20 ml.) was layered underneath, and a crude nuclear fraction isolated as the pellet obtained by centrifugation at 700g for 10 min.

For nuclear preparation A, this pellet was washed twice by resuspending in 20 ml. of the homogenizing medium and resedimenting as above. The preparation was finally suspended to a volume of 2 ml. in 0.25 M-sucrose containing MgCl_2 (1 mM).

For nuclear preparation B, the pellet from the first low-speed centrifugation was resuspended to a volume of 13 ml. in 2.4 M-sucrose containing MgCl_2 (1 mM) by one or two strokes in a hand-operated ground-glass homogenizer with a loose-fitting pestle. The suspension was centrifuged for 1 hr. at 50000g in the no. 40 rotor of the Spinco model L preparative ultracentrifuge, during which time the nuclei sedimented to the bottom, and contaminating erythrocytes, whole cells and mitochondria formed a plug at the top of the tube, which was removed with a spatula. The nuclear pellet was resuspended to a final volume of 1 ml. in 0.25 M-sucrose containing MgCl_2 (1 mM).

Microscopic observation. Preparations were examined and photographed with Cook, Troughton and Sims phase-contrast equipment and counting was carried out in a B.S. specification haemocytometer with a Thoma ruling. In the preliminary experiments developing the method, each stage was checked by microscopic examination of the suspensions.

Enzyme assays. RNA polymerase was determined essentially according to the method of Weiss (1960). The medium contained (in 0.5 ml.): 50 μmoles of tris-HCl

buffer, pH 8.1; 2.5 μ moles of $MgCl_2$; 35 μ moles of KCl; 3 μ moles of NaF; 10 μ moles of cysteine; 0.3 μ mole each of GTP, CTP and UTP; 0.02 μ mole of [^{14}C]ATP; 0.1 ml. of nuclear suspension. After incubation in air for 15 min. at 37°, the reaction was stopped by the addition of 5 ml. of 0.5 N-HClO₄ at 0°; 2 mg. of carrier RNA in an aqueous solution, pH 7, was then added. The precipitate, isolated by centrifugation, was washed twice with 4 ml. of 0.2 N-HClO₄ and twice with a mixture of redistilled ethanol and ether (3:1, v/v), all operations being carried out at 0°. The resulting ^{14}C -labelled RNA was extracted from the precipitate by two successive treatments with 10% NaCl containing 0.5 mg. of carrier RNA/ml. at 100° for 30 min. RNA was then precipitated from the combined supernatants by the addition of 2 vol. of redistilled ethanol at 0°. The pellet was dissolved in a small volume of 2 N-NH₃, deposited on Whatman no. 1 filter paper and the radioactivity determined in a Packard Tri-Carb liquid-scintillation spectrometer (see Wang & Jones, 1959). The counting efficiency under identical conditions for a standard Na₂ $^{14}CO_3$ source was 44%. The specific activity of RNA polymerase was defined as μ moles of [^{14}C]ATP incorporated into RNA/15 min./mg. of DNA in the nuclear suspension.

NAD pyrophosphorylase was determined by a modification (Branster & Morton, 1956) of the original method of Kornberg (1950). The medium contained (in 0.8 ml.): 0.3 ml. of 0.25 M-glycylglycine buffer adjusted to pH 7.4 with KOH; 5 μ moles of ATP; 3 μ moles of NMN; 300 μ moles of nicotinamide; 0.2 ml. of nuclear suspension. After incubation in air for 20 min. at 37°, the reaction was stopped by the addition of 0.2 ml. of 5 N-HClO₄ at 0°. The precipitate after centrifugation at 0° was washed with 0.5 ml. of N-HClO₄ and the combined supernatants, adjusted to pH 8.0 with NaOH (with phenol red as an internal indicator), were diluted to 2.0 ml. The NAD content was determined in 0.5 ml. samples according to the method of Kornberg (1950). The specific activity of NAD pyrophosphorylase was defined as μ m-moles of NAD synthesized/20 min./mg. of DNA in the suspension.

Cytochrome oxidase was determined by the macro-method of Cooperstein & Lazarow (1951), and the specific activity expressed as μ m-moles of cytochrome *c* oxidized/min./mg. of protein at the initial rate of reaction.

NADH-cytochrome *c* oxidoreductase was determined by following the reduction of cytochrome *c* at 550 m μ in a Unicam SP. 600 spectrophotometer according to the method of Tata *et al.* (1963). The specific activity was expressed as μ m-moles of cytochrome *c* reduced/min./mg. of protein at the initial rate of reaction.

Glucose 6-phosphatase was determined according to the method of Tata *et al.* (1963) and the specific activity expressed as μ m-moles of inorganic phosphate liberated/min./mg. of protein.

Glucose 6-phosphate dehydrogenase was determined according to the method of Glock & McLean (1953).

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as the standard. For estimation of DNA and RNA 0.2 ml. samples of nuclear preparation and 0.5 ml. samples of filtered homogenate were precipitated and washed three times with 10 ml. of 0.2 N-HClO₄ at 0°. Nucleic acids were extracted from the precipitates twice with 2 ml. of 0.5 N-HClO₄ at 75° for 10 min., conditions described by Løvtrup (1962) to result in minimal

destruction of DNA. In the combined supernatants DNA was estimated by the method of Burton (1956) and RNA by that of Ceriotti (1955).

RESULTS

Critical aspects of the isolation procedure. It is necessary during homogenization to compromise between incomplete cell breakage and structural damage to the nuclei; although a homogenizer with a more tightly fitting pestle than the one used ensured complete cell breakage, microscopic examination of the crude nuclear fractions showed that many of the nuclei were of irregular shape. Similarly, when the concentration of tissue in the homogenate was decreased from 25%, the damage to nuclei increased; homogenate concentrations greater than 25% required more than 30 strokes for the same degree of cell breakage, during which time there was a tendency for the temperature to rise above 4°. Homogenization in 0.32 M-sucrose was found to result in less damage to nuclei than did 0.25 M-sucrose.

Suspension of the crude nuclear pellet in 2.4 M-sucrose under the conditions described above result in a final sucrose concentration of 2.2 M; at lower concentrations of sucrose, erythrocyte contamination became significant, whereas at higher concentrations the nuclei became shrivelled. Cooling the ultracentrifuge rotor below 0° caused a considerable loss in the yield of nuclei, presumably by altering the viscosity of sucrose, and it was found that an ultracentrifuge rotor with an angle of greater than 25° was necessary for the formation of a clean pellet at the bottom of the tube.

Although the morphological appearance of the crude fraction was best with 3 mM-magnesium chloride in the homogenizing medium, this concentration in the 2.4 M-sucrose resulted in contamination of the nuclear pellet with whole cells, whereas 0.4 mM-magnesium chloride caused some nuclei to agglutinate. Neither contamination nor agglutination was observed with 1 mM-magnesium chloride, the concentration adopted for the 2.4 M-sucrose and final resuspension medium.

Morphological appearance of nuclei. Phase-contrast micrographs of the preparations are shown in Plate 1: (a) and (b) show a large field of nuclear preparations A and B respectively, and clearly demonstrate the decrease in cytoplasmic contamination in preparation B; Plates 1(c) and 1(d) show the same at higher magnification, indicating that suspension in 2.2 M-sucrose has had little, if any, effect on the morphological appearance. Whole-cell contamination in routine preparations of nuclear preparation B was never greater than 1 per 500 nuclei and erythrocyte contamination never greater than 1 per 200.

Phase-contrast micrographs of nuclear preparations A and B: (a) Nuclear preparation A $\times 315$; (b) nuclear preparation B $\times 315$; (c) nuclear preparation A $\times 715$; (d) nuclear preparation B $\times 715$.

Chemical analyses. Table 1 shows the protein, DNA and RNA contents of the nuclear preparations A and B; centrifugation in high-density sucrose of the nuclei obtained as a crude pellet in iso-osmotic sucrose resulted in a twofold decrease in the protein and RNA contents relative to DNA. The recovery of DNA in nuclear preparation B depended on the efficiency of dispersion of the crude pellet in 2.2M-sucrose.

Nuclear enzyme activities. The specific activities of NAD pyrophosphorylase and RNA polymerase in nuclear preparations A and B are shown in Table 2. A further purification of nuclear preparation A did not increase the specific activity of NAD pyrophosphorylase/mg. of DNA, but increased the specific activity of RNA polymerase nearly fourfold. To determine whether qualitative differences existed in the NAD-pyrophosphorylase activities of the two preparations, in that NADP might have been formed in addition to NAD, NADP was estimated, with glucose 6-phosphate dehydrogenase, in the perchloric acid extract of the NAD-pyrophosphorylase assay; it was not detected in either preparation. Fig. 1 shows the time-course of RNA polymerase in nuclear preparation B and its dependence on DNA as seen from the effect

of adding deoxyribonuclease at different time-intervals. Buffering the isolation media at pH 5.8, the pH at which nuclear protease activity is minimal (Dounce & Umana, 1962), or at pH 6.8 (Sporn, Wanko & Dingman, 1962) resulted in much more variable enzyme activities, which were never greater than those observed in nuclear preparation B. Perfusion of the liver immediately after killing the animal had no effect on the specific

Fig. 1. Time-course of DNA-dependent RNA polymerase in nuclear preparation B and the effect of deoxyribonuclease. The continuous line denotes the time course under incubation conditions described in the text; the broken lines denote the effect of adding deoxyribonuclease (25 μ g.) at the times arrowed during incubation for 15 min.

Table 1. *Protein and nucleic acid contents of rat-liver nuclei*

Experimental details are given in the text.

	Nuclear prep. A	Nuclear prep. B	Homogenate
Protein:DNA ratio	9.7	4.8	65.6
RNA:DNA ratio	0.48	0.22	3.7
Recovery of DNA	89 %	62 %	—

Table 2. *Specific activities of nicotinamide-adenine dinucleotide pyrophosphorylase and deoxyribonucleic acid-dependent ribonucleic acid polymerase in nuclear preparations A and B*

Experimental details are given in the text. NAD-pyrophosphorylase activity is expressed as μ m-moles of NAD synthesized/20 min., and RNA-polymerase activity as μ moles of [14 C]ATP incorporated into RNA/15 min.

		Enzyme activity			
		Nuclear prep. A		Nuclear prep. B	
		(per mg. of protein)	(per mg. of DNA)	(per mg. of protein)	(per mg. of DNA)
NAD pyrophosphorylase	Medium				
	Expt. 1				
	Complete incubation medium	43	416	89	428
Expt. 2	NMN omitted	1	7	2	9
	Complete incubation medium	45	434	86	413
	NMN omitted	0.5	4	1	5
RNA polymerase	Expt. 1				
	Complete incubation medium	16	157	118	569
	GTP, CTP, UTP omitted	1.5	15	2	11
	Expt. 2				
	Complete incubation medium	18	173	123	591
	GTP, CTP, UTP omitted	2	17	2	9
	UTP omitted	—	—	19	93
	GTP omitted	—	—	24	114
	CTP omitted	—	—	22	106

Table 3. *Specific activities of cytoplasmic enzyme markers in nuclear preparations A and B*

Experimental details are given in the text. The values of percentage activity in homogenate found in nuclei are corrected for the recovery of DNA.

	Cytochrome-oxidase activity		NADH-cytochrome c-oxidoreductase activity		Glucose 6-phosphatase activity		Glucose 6-phosphate-dehydrogenase activity
	(μ m-moles of cytochrome c oxidized/min./mg. of protein)	(% of activity in homogenate found in nuclei)	(μ m-moles of cytochrome c reduced/min./mg. of protein)	(% of activity in homogenate found in nuclei)	(μ m-moles of inorganic phosphate liberated/min./mg. of protein)	(% of activity in homogenate found in nuclei)	
Nuclear prep. A	88	6.5	1085	4.6	996	13.2	Not detected
Nuclear prep. B	11.7	0.27	456	0.62	366	1.6	Not detected
Homogenate	157	—	2710	—	855	—	—

activity of either NAD pyrophosphorylase or RNA polymerase.

Cytoplasmic enzyme activities. Table 3 shows the specific activities of cytochrome oxidase, NADH-cytochrome c oxidoreductase and glucose 6-phosphatase in nuclear preparations A and B, together with the percentage of the activity in the homogenate found in the nuclear fraction; nuclear preparation B is much less contaminated than nuclear preparation A. These cytoplasmic marker enzymes were not assayed in the plug that floated to the surface during centrifugation in 2.2M-sucrose, so that recovery of these activities from the crude fraction cannot be expressed. Further, the high concentration of nuclei from preparation B required for detectable activity render low precision in the assays inevitable. Glucose 6-phosphate dehydrogenase was not detected in either preparation.

DISCUSSION

The method described above for the isolation of nuclear preparation B results in a preparation which is made up of at least 80 % intact nuclei, uncontaminated by whole cells and erythrocytes, as judged by phase-contrast microscopy, with high activity retained of two exclusively nuclear enzymes and with low cytoplasmic enzyme contamination. It fulfilled the requirements for studying the role of the nucleus in the regulation of protein synthesis for which the method was devised. In the absence of functional criteria to adopt when assessing preparations of nuclei, the combination of morphological appearance, nuclear enzyme activity and low cytoplasmic enzyme activity suggested by Roodyn (1963) would seem the most useful.

Relatively little is known, however, about nuclear enzymes, as some of the activities described are difficult to assess in the light of cytoplasmic contamination in the preparations (see Roodyn, 1963). Moreover, nuclei isolated in organic solvents (see Siebert, 1961), although free from cytoplasmic contamination, have high activities of enzymes associated with the soluble fraction of the cell, and

it is possible (Keir *et al.* 1962) that in the intact cell there are soluble enzymes common to the nucleus and cytoplasm which are leached out during homogenization in iso-osmotic sucrose. This would be consistent with our results for glucose 6-phosphate dehydrogenase; the enzyme was not detected in our nuclear preparations but has been described in nuclei isolated in organic solvents. Further, nuclear RNA polymerase is firmly bound to DNA (Weiss, 1960; Goldberg, 1961), and NAD pyrophosphorylase is associated with nuclear ribosomes, as seen after disruption of the nuclear membrane (Traub, Kaufmann & Ginsburg-Tietz, 1964). It is also likely that many low-molecular-weight compounds are leached out during our isolation procedure (see Mirsky, 1961), and it may be necessary, therefore, to use different methods of preparation of nuclei for the investigation of other problems.

While our method has been in routine use, two similar methods have been published (Sporn *et al.* 1962; Maggio, Siekevitz & Palade, 1963) in which homogenization in low-density sucrose is followed by purification in hyperosmotic sucrose. Our results for chemical analysis and cytoplasmic enzyme markers are in good agreement with these as well as with methods involving isolation in hyperosmotic sucrose alone (Chauveau *et al.* 1956; Moulé & Chauveau, 1959). Although our morphological examination of nuclei has been restricted to phase-contrast microscopy, there is other evidence (Chauveau *et al.* 1956; Sporn *et al.* 1962; Maggio *et al.* 1963; Gurr *et al.* 1963) that hyperosmotic sucrose has no damaging effect on the morphological fine structure of nuclei.

The activity of DNA-dependent RNA polymerase in nuclear preparation B was higher than that reported by Weiss (1960) in an 'aggregate enzyme' from osmotically broken nuclei, a result consistent with our preliminary observations that osmotic as well as mechanical disruption of the nucleus decreases the activity of the enzyme. This comparison is only based on the incorporation of [14 C]ATP into RNA, and a further comparison with the incorporation of the other three nucleoside triphosphates

would be desirable. However, it is reasonable to conclude, from the dependence of RNA polymerase in nuclear preparation B on the presence of all four nucleoside triphosphates (Table 2), the inhibition by deoxyribonuclease (Fig. 1) and the observation that actinomycin D (1 μ g./mg. of DNA) caused an 85 % inhibition of enzyme activity, that the enzyme activity we have measured is the same as that described by Weiss (1960). A detailed account of the characteristics of this enzyme as observed in our nuclear preparations will be presented elsewhere. The low activity of RNA polymerase in nuclear preparation A is most likely due both to dilution of [14 C]ATP by non-radioactive ATP in the suspension and to adenosine-triphosphatase activity in contaminating mitochondria. The finding that the specific activity of NAD pyrophosphorylase is the same in nuclear preparations A and B is essentially in agreement with the results of Traub *et al.* (1964).

The nature of the studies on RNA polymerase (Widnell & Tata, 1963) for which nuclei were required in our Laboratory made a high recovery of DNA desirable. A DNA loss of about 70 % is unavoidable when preparing highly pure nuclei with a uniform morphological appearance (Maggio *et al.* 1963), and Sporn *et al.* (1962), using essentially the same method as ourselves, reported a recovery of about 50 %. In our routine preparations of nuclei B a consistent recovery of 60–65 % of the DNA has been obtained.

This consideration of the chemical and enzyme properties of our preparations is independent of their physiological significance. We have found that these nuclei retain their functional capacity to the extent that they reflect physiological changes induced in the whole animal. Nuclei isolated from thyroidectomized rats whose rate of growth was stimulated by thyroid hormones (Widnell & Tata, 1963) exhibited a twofold stimulation in the activity of RNA polymerase. Further, after a single replacement dose of these hormones, the stimulation of RNA polymerase preceded the increase in cytoplasmic protein synthesis and enzyme activities in a manner compatible with the current concept of the nuclear regulation of protein synthesis.

SUMMARY

1. A method is described for the isolation of rat-liver nuclei involving homogenization in 0.32 M-sucrose–3 mM-magnesium chloride and purification in 2.2 M-sucrose–1 mM-magnesium chloride.

2. The chemical composition of these purified nuclei has been compared with those isolated in 0.32 M-sucrose–3 mM-magnesium chloride alone.

3. These purified nuclei are virtually free of whole cells and erythrocytes, and have lower

activities of cytochrome oxidase, NADH-cytochrome *c* oxidoreductase and glucose 6-phosphatase (cytoplasmic enzymes used as markers) than the unpurified preparation; glucose 6-phosphate dehydrogenase was not detected in either preparation.

4. The specific activity of NAD pyrophosphorylase, expressed per unit of DNA, was the same in each preparation, but the specific activity/mg. of DNA of DNA-dependent RNA polymerase was more than threefold greater in the purified preparation.

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