The Metabolism of Isolated Rat-Liver Nucleoli and other Subnuclear Fractions

THE ACTIVE SITE OF AMINO ACID INCORPORATION IN THE NUCLEUS

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(*Received* 28 *June* 1962)

It was shown by Rees & Rowland (1961) that rat-liver nuclei isolated in 0·25 M-sucrose incorporated amino acids into protein, and nucleotides into RNA. Although the incorporation was inhibited to varying degrees by anoxia and by several inhibitors of oxidative phosphorylation, it was not affected by detergents, by freezing and thawing the nuclei, or by disruption of the nuclei by ultrasonic vibration (Rees, Ross & Rowland, 1961). We have now studied isolated nucleoli and other subnuclear fractions in order to investigate the active site of synthesis of proteins and nucleic acids within the nucleus. In addition the enzymic and chemical composition of the various fractions has been examined.

Part of this work has been published in a preliminary form (Rowland, Rees & Varcoe, 1962).

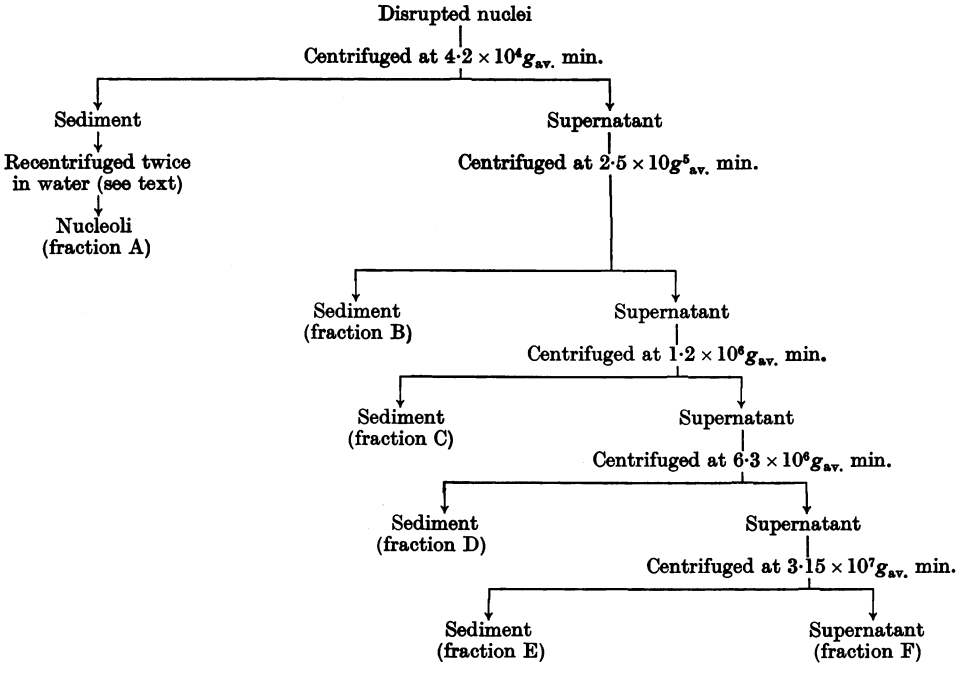
MATERIALS AND METHODS

Animals. Male albino rats, weighing 200–250 g., were used.

Reagents. These were as described by Rees & Rowland (1961). In addition [1-^{14}C]valine and [1-^{14}C]leucine were obtained from The Radiochemical Centre, Amersham, Bucks.

Nuclear and subnuclear preparations. Nuclei were isolated from rat liver in 0·25 M-sucrose as described by Rees & Rowland (1961).

Scheme 1 shows the method used for isolating nucleoli and other subnuclear fractions. Batches of isolated nuclei, suspended in 15–20 ml. of 0·25 M-sucrose, were subjected to ultrasonic vibration (20 kcyc./sec.) at 2° in a MSE ultrasonic disintegrator (60 W) with a titanium probe of 1 cm. diam. until all nuclei were disrupted (usually 7 min.). The resulting suspension was centrifuged for 30 sec. at 2500 *g* to remove probe debris and coagulated protein. The nucleoli



Scheme 1. Procedure for isolating subnuclear fractions of rat-liver nuclei. Full details are given in the Materials and Methods section.

Table 1. *Percentage recovery of nitrogen and chemical composition of fractions obtained from rat-liver-cell nuclei*

The fractionation procedure and the analytical methods are given in the Materials and Methods section. The results are the means of six similar experiments, with the ranges given in parentheses.

Fraction	Percentage recovery of nuclear N	Chemical composition (μg./mg. of protein N)		
		RNA P	DNA P	Phospholipid P
Intact nuclei	100	36·7 (25·8–52·3)	113 (77·3–124)	39·5 (28·5–45·0)
A (nucleoli)	5·5 (4·5–7·3)	65·5 (60·0–73·5)	131 (113–142)	37·8 (35·2–41·8)
B	24·2 (16·0–38·6)	45·8 (32·9–58·0)	186 (170–221)	31·8 (17·5–45·7)
C	54·1 (46·9–61·5)	35·9 (23·5–42·0)	106·5 (88·8–129)	39·0 (30·2–46·8)
D	8·1 (7·0–13·8)	34·4 (21·9–43·8)	36·0 (13·3–51·6)	61·3 (53·5–68·8)
E	3·0 (2·1–4·7)	14·9 (9·5–18·8)	17·3 (9·4–25·0)	91·4 (84·2–102·5)
F	4·7 (2·3–7·0)	7·8 (1·6–11·8)	6·3 (3·9–7·8)	13·8 (9·5–19·3)

(fraction A) were then isolated by centrifuging the supernatant at 2100*g* for 20 min. (3000 rev./min. in a MSE Major refrigerated centrifuge at 2°). The nucleolar sediment was washed, twice, by resuspending it in glass-distilled water, and centrifuging at 2100*g* for 15 and 10 min., discarding the supernatant each time. Fraction A was resuspended in water by gentle homogenization in a vertical-action hand-operated homogenizer (Rees & Rowland, 1961). Fractions B–F were isolated from the original supernatant remaining after the nucleoli had been removed. This supernatant was centrifuged at 10 000*g* for 25 min. (10 000 rev./min. in a MSE Angle 13 refrigerated centrifuge at 2°) to yield a sediment (fraction B) and supernatant. The supernatant was then centrifuged at 105 000*g* for 12 min. (40 000 rev./min. in a Spinco model L ultracentrifuge) to sediment fraction C. Fraction D was sedimented by centrifuging the supernatant at 105 000*g* for 1 hr. The supernatant was recentrifuged at 105 000*g* for 5 hr. to yield a sediment (fraction E) and a supernatant (fraction F). Fractions B–E were resuspended in cold 0·25 M-sucrose.

Analytical methods. DNA, RNA, phospholipid and nitrogen were determined by the methods used by Rees & Rowland (1961), modified as follows for application to the small amounts of material obtained in some of the subnuclear fractions. A suspension of material was precipitated with 10% (w/v) trichloroacetic acid and washed twice with 5% (w/v) trichloroacetic acid, and the precipitate was extracted for the determination of phospholipid as described by Wheeldon & Collins (1957) except that acetone was used once and chloroform–ethanol (2:1, v/v) three times. The residue was extracted with 5% trichloroacetic acid at 90° and the extract divided into two for the determination of DNA by the diphenylamine method of Dische (1955) and of RNA by the orcinol method of Mejbaum (1939). The residual protein was analysed for nitrogen by the micro-Kjeldahl method.

Cytochrome oxidase, succinoxidase, dihydronicotinamide–adenine dinucleotide–cytochrome *c* reductase and dihydronicotinamide–adenine dinucleotide–neotetrazolium reductase were determined as described by Rees & Rowland (1961), and lactate dehydrogenase was determined by the method of Kornberg (1955).

The incorporation of radioactivity *in vitro* was measured as described by Rees & Rowland (1961), the incubation mixture being: 50 μmoles of sodium phosphate buffer, pH 7·4; 50 μmoles of NaCl; 1 μc of ^{14}C-labelled substrate; 1·5 ml. of subnuclear fraction suspended in 0·25 M-sucrose; water to 2·5 ml. The temperature of incubation was 38°.

Histochemical methods. Reactions with acid haematin after various types of fixation to show the presence of phospholipid associated with heterochromatin were as described by La Cour, Chayen & Gahan (1958).

The methyl green–pyronin reaction was as described by Darlington & La Cour (1960).

RESULTS

Chemical composition. From the analytical results in Table 1, the nucleoli may be seen to represent about 6% of the nuclear nitrogen, whereas nearly 80% is recovered in fractions B and C. Also shown in Table 1 are the ratios of RNA phosphorus to protein nitrogen, DNA phosphorus to protein nitrogen, and phospholipid phosphorus to protein nitrogen for each fraction. Of the fractions, the nucleoli have the highest RNA:protein ratio, fraction B the highest DNA:protein ratio, and fractions D and E, which are low in nucleic acids, have very high phospholipid:protein ratios. Fraction F, the supernatant, is mainly protein and is very low in nucleic acids and phospholipids.

Enzyme studies. As in previous papers (Rees & Rowland, 1961; Rees, Ross & Rowland, 1962) the activities of various enzymes in the nuclear preparations have been determined and an attempt has been made to localize the site of the enzymes within the nucleus. Succinoxidase determinations were carried out as an indicator of mitochondrial contamination of the intact nuclei. In many preparations no succinoxidase activity was detected, but, where present, mitochondrial contamination was calculated to be less than 2%. Succinoxidase activity was not detected in any of the subnuclear fractions even when these were isolated from nuclei which showed slight activity.

Table 2 shows the activities of various enzymes in intact nuclei, disrupted nuclei, nucleoli and sub-

nuclear fractions. Apart from cytochrome oxidase, where there is a definite increase, ultrasonic disruption does little to alter the enzyme activities. The nucleoli have clearly very little enzyme activity and the low values may be due to slight contamination with material from the other fractions. The results also show that fractions D and E are generally the most active fractions with respect to the enzymes studied, fraction F also being rich in cytochrome oxidase and lactate dehydrogenase.

Synthetic reactions. Intact nuclei, nucleoli and the other fractions were each incubated with various ^{14}C-labelled amino acids or orotic acid for various times in the simple medium as used by Rees & Rowland (1961). The results of three typical experiments are shown in Table 3 rather than a mean of all experiments, since the level of incorporation varied from preparation to preparation. However, the pattern of ability to incorporate by the various fractions was always the same. The nucleoli (fraction A) and also fractions D and E are many times more active in incorporating amino acids and orotic acid than the intact nuclei, whereas the other fractions are generally somewhat less active than the original nuclear preparation. Another feature is that fraction E is always more active than the nucleoli in a given experiment.

On the basis of these experiments it appears that there are two main 'sites' (fraction A and fractions D plus E) for the incorporation of both amino acids and nucleotides. Chemical analysis indicates that fractions D and E are not just disrupted nucleoli, but the question arises whether the two 'sites' are structurally associated in the intact nucleus. To investigate this possibility batches of nuclei were divided in two. One half of the batch was subjected to ultrasonic vibration for the minimum time to give full nuclear breakage (1½–2 min.) and

Table 2. *Activity of enzymes in isolated rat-liver nuclei, ultrasonic extracts of nuclei, nucleoli and subnuclear fractions*

The fractionation procedure and the enzyme-assay systems are given in the Materials and Methods section. The results are the means of at least four experiments, with the ranges given in parentheses. In all cases the necessary blanks have been subtracted and enzyme activity shown to be linearly related to enzyme concentration.

Fraction	Cytochrome oxidase (μl. of O_2/hr./mg. of N)	NADH–cytochrome *c* reductase (μmoles of cytochrome *c* reduced/hr./mg. of N)	NADH–neotetrazolium reductase (mg. of formazan produced/hr./mg. of N)	Lactate dehydrogenase (units/mg. of protein)
Intact nuclei	350 (284–472)	21 (18–27)	6·1 (4·8–7·7)	0·29 (0·21–0·37)
Disrupted nuclei	650 (506–800)	28 (24–35)	5·8 (4·6–7·9)	0·30 (0·24–0·35)
A (nucleoli)	0	3 (1–4)	0·3 (0·1–0·4)	0·11 (0·09–0·15)
B	423 (277–570)	15 (12–19)	2·0 (1·2–2·9)	
C	280 (251–323)	19 (17–20)	2·4 (2·0–2·8)	0·25 (0·17–0·36)
D	1090 (1020–1200)	32 (26–40)	4·6 (3·6–6·2)	
E	2260 (1900–2700)	15 (11–19)	3·9 (2·3–5·8)	0·60 (0·48–0·87)
F	1490 (840–1870)	8 (6–10)	0·5 (0·3–0·8)	0·57 (0·43–0·74)

Table 3. *Incorporation* in vitro *of* ^{14}C*-labelled amino acids into protein and of* [6-^{14}C]*orotic acid into ribonucleic acid by nuclei, nucleoli and subnuclear fractions of rat liver*

The system used was as described in the Materials and Methods section. The results are those of three representative experiments of a group of nine similar experiments.

Expt. no.	Substrate	Incubation time (hr.)	Radioactivity in nuclei, nucleoli and sub-nuclear fractions (counts/min./cm.^{2} at infinite thickness)						
			Nuclei	A (nucleoli)	B	C	D	E	F
1	[2-^{14}C]Glycine	1	104	1240	99	92	780	2820	54
		2	134	3350	165	185	2050	8000	99
	[1-^{14}C]Valine	1	12	464	27	37	323	1600	7
		2	20	1290	34	63	710	3650	10
	[6-^{14}C]Orotic acid	1	166	510	139	115	286	915	58
		2	208	1310	316	207	467	2500	115
2	[2-^{14}C]Glycine	1	152	316	81	227	273	565	122
		2	213	501	150	438	593	1590	136
	[6-^{14}C]Orotic acid	1	135	127	71	111	95	398	56
		2	266	334	142	186	170	1082	37
3	[2-^{14}C]Glycine	3	310	1237	443	570	950	2077	177
	[1-^{14}C]Leucine	3	389	717	205	405	644	1971	127

the remainder for 10 min. Nucleoli and subnuclear fractions were then isolated from each batch of disrupted nuclei and their ability to incorporate [1-^{14}C]leucine was studied. Table 4 shows that increasing the time that nuclei are subjected to ultrasonics results in a decrease in the incorporation by the nucleoli, and in an increase in that by fractions D and E. Preliminary results of chemical analysis suggest that a longer period of disruption results in a decrease in the lipid content of the nucleolar fraction and in a corresponding rise in fractions D and E. These results support the contention that the two synthetic sites are closely related structures within the nucleus.

Further information on the localization of these fractions was obtained by histochemical techniques. Confirmation that fraction A corresponds to the nucleolus was obtained by staining with methyl green–pyronin (Table 5). When intact nuclei are treated with this stain the nucleoli stain pink and the remainder of the nucleus stains green. The structures visible under the microscope in fraction A also stain pink. La Cour *et al.* (1958) have shown that phospholipid of the heterochromatin stains black with acid haematin when tissues are fixed with Lewitzsky's fluid [1 % (w/v) chromium trioxide–10 % (v/v) formalin (1 : 1, v/v)], but not when fixation was carried out in Baker's solution [1 % (w/v) calcium chloride in 4 % (v/v) formalin which has been kept over marble chips]. These reactions, when applied to fractions A and E (Table 5), suggest that fraction E contains large quantities of heterochromatic phospholipid and that the nucleoli also contain a small amount of this lipid. These results indicate that fractions D and E represent heterochromatin which in the intact nucleus is associated with the nucleolus and which has become separated during disruption of the nuclei.

Table 4. *Effect of different ultrasonic-disruption times on the ability of subnuclear fractions of rat-liver nuclei to incorporate* [1-^{14}C]*leucine into protein*

The system used was as described in the Materials and Methods section. Incubation was for 3 hr. The results are those of a representative experiment of a group of three similar experiments.

Fraction	Radioactivity in subnuclear fraction (counts/min./cm.^{2} at infinite thickness): Isolated from nuclei disrupted for 1¾ min.	Isolated from nuclei disrupted for 10 min.
A (nucleoli)	974	384
B	228	192
C	225	204
D	1432	2000
E	1556	1946
F	162	258

The experiments described above were all based on separation of subnuclear material before the incorporation of labelled substrates. Table 6 shows the results of fractionation of the nuclei after the incorporation of [1-^{14}C]leucine *in vitro*. A very high specific activity was obtained in the protein of the nucleoli in comparison with that of the other fractions, D and E being particularly low.

DISCUSSION

The site of protein synthesis within the nucleus was once a subject of interest (Caspersson, 1947), but has subsequently been overshadowed by the interest in cytoplasmic protein synthesis. Recent techniques permitting the isolation of metabolic-

Table 5. *Staining reactions of subnuclear fractions A and E from rat-liver nuclei*

The fractionation procedure and the staining techniques are given in the Materials and Methods section.

Staining reaction: Stain	Staining reaction: Reaction	Fixation of smear before staining: Fixative	Fixation of smear before staining: Reaction	Colour reaction of subnuclear fraction: A (nucleoli)	Colour reaction of subnuclear fraction: E
Acid haematin	Phospholipids stain dark brown to blue–black	None	—	Light brown	Brown–black
		Baker's soln. (1 % $CaCl_2$ in 4 % formaldehyde)	Extracts phospholipid associated with heterochromatin	None	Pale yellow
		Lewitsky's fluid [1 % chromium trioxide–10 % formalin (1 : 1, v/v)]	Retains phospholipid associated with heterochromatin	Brown–black	Intense black
Methyl green–pyronin	RNA stains pink (due to pyronin) DNA stains green (due to methyl green)	None	—	Pink (as in intact nuclei)	Slightly pink

Table 6. *Fractionation of rat-liver nuclei after incorporation of* [1-^{14}C]*leucine* in vitro

The system used was as described in the Materials and Methods section. The reaction was stopped after 3 hr. by cooling to 0°, and the nuclei were washed several times with cold 0·25M-sucrose containing unlabelled leucine, subjected to ultrasonics for 7 min.; the fractions were then isolated as described in the Materials and Methods section. Results are those of a representative experiment of a group of three similar experiments.

Fraction	Radioactivity of protein (counts/min./cm.^{2} at infinite thickness)
Nuclei	70
A (nucleoli)	1225
B	400
C	121
D	44
E	15
F	188

ally active preparations of nuclei (Allfrey, Mirsky & Osawa, 1957; Rees & Rowland, 1961) open up the possibility of reinvestigating this problem.

The only structure visible in the resting nucleus is the nucleolus, and so the method for fractionation was begun with the isolation of this subnuclear component from disrupted nuclei. The isolation of nucleoli from disrupted rat-liver nuclei has been described by Monty, Litt, Kay & Dounce (1956). The procedure, however, involved a sedimentation step of over 12 hr. and was carried out with 1 % (w/v) gum arabic. Such a lengthy procedure is unnecessary since a satisfactory isolation may be achieved by differential centrifuging. The subsequent isolation of nuclear material from the nucleoli-free supernatant was based on a series of arbitrary centrifugings in which the centrifugal force is increased fivefold each time. Although this fractionation was arbitrary the results of chemical analysis indicate that a separation of four distinct nuclear constituents, including the nucleoli, was achieved. Evidence that fraction A is truly nucleolar comes from microscopic examination of the fresh material, from the staining reaction and from the chemical composition. Chemical analysis of nucleoli isolated by Monty *et al.* (1956) showed a similar picture to that reported in this paper. Of particular interest are their RNA:DNA ratios, which indicate a definite amount of DNA associated with the nucleolus. This is confirmed by the analyses reported in this paper. Fractions B and C, which comprise almost 80 % of nucleus, are considered on the basis of chemical composition to represent the chromosomal material.

The high lipid : protein ratios found in fractions D and E suggest that they are a single nuclear component and may represent the heterochromatin (La Cour *et al.* 1958). This leaves the supernatant (fraction F) which is low in nucleic acids and lipids and which is considered to be the nuclear sap.

All the subnuclear fractions incorporated amino acids into protein to some extent, but it is clear that two main components of high activity have been isolated from the nucleus. Since an arbitrary fractionation scheme was adopted it is considered that the activity that persists in the intermediate fractions is due to contamination by material from the active components. Caspersson (1947) suggested that a region of chromatin associated with the nucleolus (heterochromatin) secreted substances of a protein nature, and Sirlin (1958), using radioautography, has produced evidence that ^{14}C-labelled amino acids are incorporated by nucleoli and their associated chromatin. La Cour *et al.* (1958) have shown the presence of a lipid material in chromosomes and in particular in the heterochromatin. Since fractions D and E are rich in phospholipid and show a high synthetic ability it seemed likely that these fractions contained heterochromatin. Conceivably these fractions could have become separated from the nucleoli during the ultrasonic disruption of the nuclei. For this reason an attempt was made to reduce the disruption time to the point where nucleoli and heterochromatin were still structurally associated. This was not completely achieved, although with a reduced disruption time the activity of the nucleoli was higher and that of fractions D and E lower. In addition there appeared to be a transfer of lipid material from the nucleolus to fractions D and E with a prolonged disruption time.

Fractions D and E possess similar staining properties to the lipid material surrounding the nucleoli in intact nuclei. It is concluded from this evidence that fractions D and E are probably the heterochromatin long recognized by the cytologist, and that in the liver-cell nucleus it is structurally associated with the nucleolus.

The fractionation of nuclei after incubation at 37° with [1-^{14}C]leucine does not follow the same pattern as that obtained with freshly isolated nuclei. This is borne out by the finding that the quantities of nuclear material recovered in the fractions differ markedly depending on whether preincubated or fresh nuclei are used. This may be the explanation for the high labelling in the nucleolus and the low activity in fractions D and E, and, if correct, it must be assumed that the heterochromatin has not been separated from the nucleolus during the disruption of the nuclei in this type of experiment.

The question remains whether there are two different sites of incorporation of amino acids in the nucleus, the nucleolus and heterochromatin, or whether the nucleolar activity can be explained in terms of residual heterochromatin not separated

during nuclear disruption. This latter view is supported by the results of a prolonged period of disintegration in which nucleolar activity was considerably decreased. In addition, the staining reactions of nucleoli with acid haematin indicate the presence of some heterochromatic phospholipids even after such a prolonged disruption time. It is concluded therefore that, although the site of incorporation of amino acids in the nucleus is in the region of the nucleolus, it is probably in the associated heterochromatin and not the nucleolus itself. Whether this state of affairs is true for the incorporation of orotic acid into RNA is not yet known, but, since the pattern of incorporation of orotic acid in the subnuclear fractions follows that of incorporation of amino acids, it may well prove to be localized in the heterochromatin region also.

The possibility of a close structural association between the sites for nuclear protein synthesis and RNA synthesis is of interest when considering whether nuclear protein synthesis depends on the presence of ribonucleoprotein particles as are present in microsomes. Such microsomal particles or ribosomes are characterized by their RNA and protein composition, their sedimentability and their ability to incorporate amino acids into protein when fortified with 'pH 5 enzymes' from cell sap, ATP, GTP, Mg^{2+} ions and an ATP-generating system. Various groups of workers have described nuclear ribonucleoprotein particles but it is difficult to compare their results since different methods of fractionation have been used.

Frenster, Allfrey & Mirsky (1960) described the isolation of a range of ribonucleoprotein particles from thymus-cell nuclei with certain similarities to cytoplasmic ribosomes. The greatest incorporation into both protein and RNA occurred in ribonucleoprotein particles that had a very low RNA:protein ratio, a situation resembling that described in this paper more closely than that in microsomes.

Szafranski, Wehr & Golaszewski (1961) obtained three fractions from guinea-pig-liver nuclei of which one was described as consisting of ribonucleoprotein particles. Although this fraction incorporated amino acids in a system without an added energy source, the supernatant fraction (similar to fractions D and E described in the present paper) was three times as active as their so-called ribonucleoprotein particles.

Rendi (1960) isolated subnuclear fractions from rat-liver by using deoxycholate and Lubrol to disrupt the nuclei. However, when the nuclei were fractionated after the incorporation of amino acids, the most active fraction was not that consisting of the so-called ribonucleoprotein particles but was one without RNA or DNA. This fraction, moreover, incorporated amino acids without the addition of an external energy source.

It would appear, therefore, that the active fractions described in the present work do not correspond to the nuclear ribonucleoprotein particles described by other workers since they are very low in RNA. It appears, however, that similar subnuclear material, low in nucleic acids, has been isolated by these other workers and that this material is often more active after incorporation than the ribonucleoprotein particles. Unfortunately, none of the other workers have analysed their subnuclear fractions for phospholipid, which we have found to be a major constituent of the active fractions D and E.

The presence in nuclei of certain enzymes (Rees & Rowland, 1961) suggested that an electron-transport chain similar to the respiratory chain in mitochondria may exist in rat-liver nuclei and may play a role in the production of energy for synthetic reactions. Since the nucleoli and heterochromatin incorporate actively when isolated from the rest of the nuclear material, they should be rich in such enzymes if these enzymes are involved in energy-yielding reactions necessary for the incorporation. The limited number of enzymes studied in this respect all appear to be concentrated in fractions D and E and all are very low in the nucleoli. Although it appears that the site for incorporation is localized in the heterochromatic material it is difficult to understand why the nucleoli that retain enough of this material to incorporate actively do not also retain high concentrations of the enzymes. It must be concluded that there is insufficient evidence at present to confirm that an oxidative mechanism is responsible for the energy production needed for the incorporation of amino acids into protein in the nucleus.

SUMMARY

1. Rat-liver nuclei isolated in 0·25 M-sucrose were disrupted by ultrasonic vibration and subjected to differential centrifuging to isolate nucleoli. The remainder of the disrupted nuclear material was arbitrarily fractionated by centrifuging into four sedimentable fractions and a supernatant.

2. Determinations of RNA, DNA, phospholipid and protein, together with staining reactions of the subnuclear fractions, suggest that a separation of nucleoli, chromosomal material, a lipid-rich material and nuclear sap was achieved.

3. Although all the subnuclear fractions will incorporate ^{14}C-labelled amino acids into protein and [6-^{14}C]orotic acid into RNA without an additional external energy source, the nucleoli and the lipid-rich material have by far the greatest activity.

4. There is high specific activity of certain

oxidative enzyme systems in the lipid-rich fractions but they appear to be absent from nucleoli.

5. It is concluded that the lipid-rich material may be the heterochromatin associated with the nucleoli and that this is the active site for the incorporation of amino acids by rat-liver nuclei.

We thank Professor C. Rimington, F.R.S., and Dr J. Chayen for helpful discussions, and Dr P. S. Gahan for his help with the histochemistry. The work was supported by a grant from the British Empire Cancer Campaign.

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Physicochemical Studies on Cytochrome b_2

SEDIMENTATION, DIFFUSION AND ELECTROPHORESIS OF THE CRYSTALLINE DEOXYRIBONUCLEOPROTEIN

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(*Received* 4 *May* 1962)

Cytochrome b_2 is the L(+)-lactate–cytochrome *c* oxidoreductase [L(+)-lactate dehydrogenase] of baker's yeast (Bach, Dixon & Zerfas, 1946; Appleby & Morton, 1954; Boeri, Cutolo, Luzzati & Tosi, 1955). The enzyme was obtained as a crystalline, apparently homogeneous, deoxyribonucleoprotein containing equimolecular amounts of riboflavin phosphate and of protohaem (Appleby & Morton, 1954, 1959*a*, *b*, 1960). Both of these prosthetic groups are reduced in the presence of lactate (Appleby & Morton, 1954; Hasegawa & Ogura, 1961).

Further physicochemical studies of the crystalline deoxyribonucleoprotein (now known as Type I cytochrome b_2; Morton, 1961*a*) are described in this paper. The molecular weight of the enzyme in solution has been determined by a number of methods.

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MATERIALS AND METHODS

General

Cytochrome b_2 (*Type I*). This was prepared from dried baker's yeast essentially as described by Appleby & Morton (1959*a*). Solutions were stored under nitrogen at −15°. The enzyme was recrystallized before use, and experiments with any one sample were carried out within 3 days of recrystallization. The enzyme was dissolved in buffer composed of (final concentrations) 0·3M-sodium lactate, 0·05M-tetrasodium pyrophosphate and 0·1 mM-EDTA (disodium salt), adjusted to pH 6·8 with hydrochloric acid. This buffer (*I* 0·63) was used to obtain the high ionic strength necessary to give adequate concentrations of cytochrome b_2 (see Appleby & Morton, 1959*a*) and because it was found that the enzymic activity was retained for long periods in pyrophosphate buffer.