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The Metabolism of Isolated Rat-Liver Nuclei

BY K. R. REES AND G. F. ROWLAND
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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The investigations described in this paper were undertaken as a preliminary to the study of the biochemical changes in the nucleus of the rat-liver cell during the induction of liver cancer. Allfrey (1954) and Stern & Mirsky (1953) described the isolation in aqueous media of nuclei from the thymus gland of the calf. Such preparations *in vitro* incorporated glycine and other amino acids into nuclear proteins (Allfrey, Mirsky & Osawa, 1955), and orotic acid and adenosine into the ribonucleic acid fraction of the nucleus (Allfrey & Mirsky, 1957). No comparable studies have been carried out on rat-liver nuclei. However, Logan, Ficq & Errera (1959), using radioautographic techniques, have shown that isolated rat-liver nuclei will take up phenylalanine and adenine *in vitro*.

There is considerable doubt about the mechanisms whereby the nucleus obtains its energy supply. Evidence so far obtained points to some form of oxidative phosphorylation (Allfrey & Mirsky, 1957), but the nature of the oxidative pathway involved is still unknown.

In this paper the chemical composition of isolated rat-liver nuclei has been determined, and protein and nucleic acid syntheses have been investigated. The effects of anoxia, 2:4-dinitrophenol, chlorpromazine and azide on these reactions have been studied. Investigations of the adenosinetriphosphatase activities of the preparations coupled with the results of the synthetic experiments have thrown further light on the underlying energy mechanisms of the nucleus.

MATERIALS AND METHODS

Animals. Male hooded Norwegian rats, fed on the M.R.C. 41B diet without restriction, were used, weight 200–250 g.

Reagents. Glass-distilled water was used throughout. Cytochrome *c*, adenosine 5′-triphosphate (ATP) and reduced diphosphopyridine nucleotide (DPNH) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Neotetrazolium chloride, sodium azide and dicoumarin were obtained from L. Light and Co. Ltd., Colnbrook, Bucks., England. Chlorpromazine was a gift from May and Baker Ltd., Dagenham, Essex. ^{32}P, [2-^{14}C]glycine, [6-^{14}C]-orotic acid and [8-^{14}C]adenine hemisulphate were obtained from The Radiochemical Centre, Amersham, Bucks. ^{32}P was purified as described by Kennedy (1953).

Tissue preparations. Mitochondria were isolated from rat liver in 0·25 M-sucrose by the method of Schneider (1948). Soluble cytoplasm (cell sap) was prepared from rat liver in 0·25 M-sucrose as described by Zamecnik & Keller (1954).

For the preparation of nuclei a rat was anaesthetized with ether, and the liver was perfused *in situ* through the portal vein with 20 ml. of ice-cold 0·9% (w/v) sodium chloride solution. The inferior vena cava was severed, and the perfusion was continued with 100 ml. of ice-cold 0·25 M-sucrose containing calcium chloride to a final concentration of 1·8 mM. This procedure freed the liver of nearly all the erythrocytes. The liver was removed, weighed and homogenized in 50 ml. of ice-cold 0·25 M-sucrose containing calcium chloride to a final concentration of 5 mM with about 12 strokes in a vertical hand-operated homogenizer with a nylon sphere pestle [modification of the apparatus described by Dounce (1955)]. The homogenate was filtered in the cold through nylon bolting cloth of two mesh sizes (60 and 110 mesh/in.). The filtrate was made to form a layer with an equal volume of ice-cold 0·34 M-sucrose containing a final concentration of 0·2 mM-calcium chloride and centrifuged at 8200 *g* (9000 rev./min.) for 2 min. at 3° in a MSE Angle 13 refrigerated centrifuge. The supernatant was removed, and the loose sediment was resuspended in half the original volume of ice-cold 0·25 M-sucrose, made to form a layer with an equal volume of ice-cold 0·34 M-sucrose and centrifuged at 4800 *g* (7000 rev./min.) for 2 min. at 3°. This procedure was repeated three times more with centrifuge speeds of 2400 *g* (5000 rev./min.), 1600 *g* (4000 rev./min.) and 1000 *g* (3000 rev./min.). The final residue was suspended in 10 ml. of ice-cold 0·25 M-sucrose. A microscopic examination was made of all preparations to ensure freedom from contamination by cellular debris and erythrocytes. Counts of nuclear numbers were made as described by Grant & Rees (1957).

Analytical methods

Breakdown of ATP was studied in the system: ATP, 5 mM; $MgSO_4$, 10 mM; KCl, 37·5 mM; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·2, 50 mM; 0·5 ml. of nuclear suspension and water to 2·0 ml. The system was incubated at 28° for 20 min. The inorganic phosphate liberated was determined by the method of Fiske & Subbarow (1925).

Deoxyribonucleic acid (DNA) was determined by the method of Dische (1955). Determination of DPNH–cytochrome *c* reductase was by the method described by Dawkins, Judah & Rees (1959). The DPNH–neotetrazolium-reductase system was determined by the method described by Slater (1959). Ribonucleic acid (RNA) and phospholipid were determined as described by Davidson, Frazer & Hutchinson (1951). Nitrogen was determined by the micro-Kjeldahl method.

Table 1. *Recovery of nuclei from rat-liver homogenates*

Results are expressed per 100 mg. wet wt. of liver. Results are the means of ten concordant experiments.

	Rat-liver homogenate	Nuclear preparation	Recovery (%)
Nuclei (no.)	17×10^5	8×10^5 ($7{\cdot}2 \times 10^5$–$9{\cdot}6 \times 10^5$)	47 (42–56)
Nitrogen (mg.)	3·19	0·164 (0·151–0·179)	5·2 (4·7–5·6)
DNA phosphorus (μg.)	21	9·8 (9·0–12·2)	46 (43–58)

Cytochrome oxidase was measured manometrically in the following system: sodium phosphate buffer, 16 mM, pH 7·4; cytochrome *c*, 167 μM; ascorbate, 10 mM; ethylenediaminetetra-acetic acid (EDTA), 0·3 mM; 0·25 ml. of nuclear suspension in 0·25 M-sucrose (frozen and thawed twice); final volume, 3 ml. The temperature of the bath was 38°. The EDTA was added to decrease the metal-catalysed auto-oxidation of the ascorbate.

Succinoxidase was determined manometrically in the following system: sodium phosphate buffer, 33 mM, pH 7·4; sodium succinate, 33 mM; cytochrome *c*, 33 μM; 0·5 ml. of nuclear suspension in 0·25 M-sucrose solution (frozen and thawed twice); final volume, 3 ml. The temperature of the bath was 38°.

Incorporation of radioactivity in vitro

Incorporation of ^{32}P into RNA was based on the method of Siebert, Lang, Lucius & Rossmüller (1953). The system used was: tris–HCl buffer, pH 7·2, 20 mM; KCl, 75 mM; $MgSO_4$, 5 mM; 1·0 ml. of nuclear suspension in 0·25 M-sucrose; ^{32}P, 0·4 mM with an activity of 5 μC; water to 3·0 ml. The temperature was 30°. The reaction was stopped by the addition of 2·0 ml. of 20% (w/v) trichloroacetic acid. The RNA was extracted from the residue and purified by paper electrophoresis to remove inorganic phosphate by the method of Graymore (1956). Radioactivity was determined by elution of the paper with water and counting the solution in a liquid counter (20th Century Electronics Ltd., thin-walled B6). Measurements are corrected for isotope decay, background and dead time of the instrument.

For incorporation of [6-^{14}C]orotic acid and [8-^{14}C]adenine into RNA the system used was: sodium phosphate buffer, pH 7·4, 20 mM; glucose, 12 mM; NaCl, 10 mM; 1 μC of [^{14}C]adenine or [^{14}C]orotic acid; 1·5 ml. of nuclear suspension in 0·25 M-sucrose; water to 2·5 ml. The temperature was 30°. The reaction was stopped by the addition of 2·0 ml. of ice-cold 10% (w/v) perchloric acid. The precipitate was washed four times with 2·0 ml. of ice-cold 2% (w/v) perchloric acid, twice with ethanol and twice with ether. The radioactivity of the dried protein was determined. In experiments where indicated in the text, the RNA was extracted, purified and hydrolysed (Graymore, 1956). The hydrolysate was run on Whatman no. 3 MM paper between water-cooled plates in 0·05 M-citrate buffer (pH 3·5) at 6 kv and 50 mA for 40 min. The nucleotide areas were identified by viewing in ultraviolet light. Each compound was eluted from the paper with water, and the eluate was dried on a plastic planchet of surface area 1 cm.^{2} for determination of radioactivity.

For incorporation of [2-^{14}C]glycine into nuclear protein the system used was: sodium phosphate buffer, pH 7·4, 20 mM; NaCl, 20 mM; [^{14}C]glycine, 1 μC; 1·5 ml. of nuclear suspension in 0·25 M-sucrose; water to 2·5 ml. The temperature was 38°. The reaction was stopped by the addition of 2·0 ml. of 20% trichloroacetic acid. The precipitate was extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 20 min. at 90°. The protein residue was then washed successively with 5 ml. of 5% trichloroacetic acid containing 1% (w/v) of glycine and 5 ml. of 5% trichloroacetic acid, then twice with ethanol and twice with ether. The protein residue was dried at room temperature.

Table 2. *Chemical composition of rat-liver nuclei isolated in* 0·25 M-*sucrose*

Results are the means of ten concordant experiments.

	μμg./nucleus	μg./mg. of nuclear nitrogen
Nitrogen	164 (153–171)	—
Phosphorus		
DNA	690 (600–760)	61 (53–70)
RNA	670 (580–720)	44 (42–48)
Phospholipid	590 (550–740)	37 (34–44)

Preparation of dried protein residues for measurement of radioactivity

The protein residue was ground in a pestle and mortar, and then compressed with a stainless-steel pestle on a planchet of 1 cm.^{2} surface area. All samples were counted for 5 min. in a 20th Century Electronics gas-flow counter, Type WF2. The results were corrected for background and adjusted for infinite thickness.

RESULTS

Chemical composition

The yield of nuclei from rat liver was followed by counting the nuclei and by determination of the DNA and nitrogen content, both in the homogenate and in the nuclear preparations. Table 1 gives the mean result of ten experiments. The DNA, RNA, nitrogen and phospholipid contents of such nuclear preparations are given in Table 2.

Synthetic reactions

The nuclear preparations incorporated [2-^{14}C]-glycine into nuclear proteins, and [6-^{14}C]orotic acid, [8-^{14}C]adenine and ^{32}P into nuclear RNA *in vitro* (Table 3). The rate of incorporation of all these compounds was approximately linear over 90 min.

In the above experiments with [^{14}C]orotic acid and [^{14}C]adenine, the radioactivity of the dried nuclear protein had been determined. To verify that these substances had been used in true syn-

Table 3. *Incorporation of* [2-^{14}C]*glycine* in vitro *into nuclear protein and of* [6-^{14}C]*orotic acid,* [8-^{14}C]*adenine and* ^{32}P *into nuclear ribonucleic acid*

System used was as described in the Materials and Methods section. Results are those of a representative experiment of a group of four similar experiments.

Time of incubation (min.)	^{32}P (counts/min./μg. of RNA P)	Counts/min. at infinite thickness		
		Glycine	Adenine	Orotic acid
30	34	63	57	48
60	77	92	115	97
90	108	151	181	145

Table 4. *Distribution of* ^{14}C *in nucleotide fractions of ribonucleic acid after incorporation* in vitro

Purification of RNA, hydrolysis and electrophoretic separation of nucleotides were as described in the Materials and Methods section. Results are those of a representative experiment of a group of four similar experiments.

Source of ^{14}C	Counts/min. at infinite thickness				
	Hydrolysed RNA used	Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
[^{14}C]Adenine	117	0	52	2	6
[^{14}C]Orotic acid	98	21	7	9	44

Table 5. *Effects of adenosine triphosphate and cell sap on the incorporation of* [^{14}C]*glycine* in vitro *into nuclear protein*

System was as described in the Materials and Methods section. Results are those of a representative experiment of a group of three similar experiments.

Additions	Counts/min. at infinite thickness	Inhibition (%)
Nil	467	—
ATP (10 μmoles)	287	38·5
Cell sap (0·5 ml.)	318	32·0
Cell sap (0·5 ml.) + ATP (10 μmoles)	279	30·0

Table 6. *Effect of anoxia, azide and* 2:4-*dinitrophenol on the incorporation of glycine into nuclear protein and of adenine and orotic acid into nuclear ribonucleic acid*

Systems were as described in the Materials and Methods section. Additions are recorded as final concentrations. Incubation time was 90 min. Results are those of a representative experiment of a group of four similar experiments.

Additions or treatment	Counts/min. at infinite thickness		
	Glycine	Adenine	Orotic acid
None	185	181	143
Dinitrophenol			
0·1 mM	132	—	118
0·3 mM	94	102	104
0·6 mM	75	82	70
1·0 mM	55	53	51
Azide			
0·6 mM	70	—	48
0·9 mM	56	8	41
Anoxia	83	61	22

thesis of RNA, the nuclear RNA was isolated, purified and hydrolysed. The hydrolysate was subjected to paper electrophoresis, and the constituent nucleotides were separated and counted. The results of such an experiment are given in Table 4. With orotic acid, the highest activity was in the uridylic acid fraction, and some was also found in cytidylic acid. With adenine by far the greatest activity was in the adenylic acid fraction. In the ^{32}P-incorporation experiments, the RNA was isolated and purified by electrophoresis.

Allfrey *et al.* (1955) demonstrated that replacement of sodium ions by potassium ions in the incubation medium decreased the incorporation of [^{14}C]glycine into thymus nuclear protein. Similarly, Breitman & Webster (1959) found decreased incorporation of glycine and alanine into RNA, DNA and protein of thymus nuclei, but the incorporations of formate, adenine, guanine and adenosine were unimpaired. In view of these findings, the replacement of sodium ions by potassium ions in the incubation medium was examined in the incorporation of [2-^{14}C]glycine into protein and [6-^{14}C]orotic acid into RNA by isolated liver-cell nuclei, but was found to be without effect.

The incorporation of amino acids *in vitro* by microsomal preparations is considerably increased by the addition of cell sap, and by cell sap plus ATP or an ATP-generating system (Zamecnik & Keller, 1954). In the nuclear preparations such additions proved inhibitory but the effects were not additive (Table 5).

Allfrey *et al.* (1955) have suggested that the energy system of thymus nuclei depends on oxidative mechanisms. The effect of anoxia was tested

on the synthesis of RNA and protein by liver-cell nuclei, and the results are given in Table 6. Anoxia decreased the incorporation of glycine into protein by about 40%, the incorporation of adenine by about 60% and that of orotic acid by about 90%.

The action of 2:4-dinitrophenol and of sodium azide was investigated on protein and RNA synthesis (Table 6). The degree of inhibition rose with increasing concentrations of azide and 2:4-dinitrophenol. The latter at a final concentration of 1·0 mM gave a 60% inhibition of protein and RNA synthesis.

Since both dinitrophenol and azide are inhibitors of oxidative phosphorylation, such results may indicate that the energy system in the nucleus is similar to the oxidative phosphorylation mechanisms of the mitochondrion. Chlorpromazine has been shown to inhibit some of the phosphorylation steps associated with oxidative phosphorylation (Dawkins *et al.* 1959). This compound inhibited both protein and RNA synthesis by liver-nuclear preparations (Table 7).

Investigations of energy mechanisms

The inhibitory effects of chlorpromazine, azide, 2:4-dinitrophenol and anoxia on the synthetic reactions suggest the presence of an oxidative phosphorylation mechanism in the nucleus. Extraction of normal liver-nuclei preparations with ice-cold 2% perchloric acid did not reveal the presence of acid-soluble nucleotides.

Liver-nuclei preparations did not incorporate ^{32}P into added ATP, and so an alternative approach was made to study the energy mechanisms of the nuclei by examining the properties of the nuclear adenosine triphosphatase.

There is much evidence to suggest that the magnesium-activated adenosine triphosphatase of mitochondria is a reversal of some of the terminal steps of oxidative phosphorylation in preparations no longer able to couple electron transport with the phosphorylation of adenosine diphosphate (ADP) (Siekevitz, Löw, Ernster & Lindberg, 1958). Schneider (1946) found that 27% of the adenosine triphosphatase of rat-liver homogenate was in the nuclear fraction. Such nuclear fractions were by virtue of their method of preparation contaminated with cell-wall debris, whole cells and possibly mitochondria. An examination of nuclei isolated by the technique given in this paper revealed the presence of an adenosine triphosphatase which is stimulated by magnesium (Table 8). Also included for comparison are results for mitochondrial adenosine triphosphatase.

The effect of azide, dinitrophenol and chlorpromazine on the magnesium-activated adenosine triphosphatase of the nuclei was determined (Table 9). Dinitrophenol stimulated breakdown of ATP by about 30%, and this effect was abolished by azide and chlorpromazine. Since similar results have been obtained with mitochondrial preparations (Dawkins, Judah & Rees, 1960), the possibility of mitochondrial contamination in the nuclear preparation was investigated. Although no mitochondria could be found on microscopic

Table 7. *Effect of chlorpromazine on the incorporation of glycine into nuclear protein and orotic acid and adenine into nuclear ribonucleic acid*

System was as described in the Materials and Methods section; incubation time, 90 min. Additions are recorded as final concentrations. Results are those of a representative experiment of a group of three similar experiments.

	Counts/min. at infinite thickness		
Additions	Glycine	Adenine	Orotic acid
None	400	173	250
Chlorpromazine			
0·15 mM	157	137	208
0·3 mM	78	53	70

Table 8. *Comparison of adenosine-triphosphatase activity of nuclei and mitochondria in 0·25 M-sucrose*

Medium: ATP, 5 mM; $MgSO_4$, 10 mM; KCl, 37·5 mM; tris buffer, pH 7·2, 50 mM. Nuclei and mitochondria were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 2·0 ml.; incubation was for 20 min. at 25°. Dinitrophenol was added to 0·03 mM final concentration. Results are the means of six experiments and the ranges are given.

	P liberated (μmoles/mg. of N/20 min.)	
	Mitochondria	Nuclei
Medium	1·3 (1·1–1·5)	5·3 (4·5–5·6)
Medium – Mg^{2+} ions	1·0 (0·9–1·1)	3·2 (3·0–3·5)
Medium + dinitrophenol	5·1 (4·5–5·3)	7·2 (6·2–7·6)

Table 9. *Action of azide, 2:4-dinitrophenol and chlorpromazine on the adenosine triphosphatase of nuclei in 0·25 M-sucrose*

The medium and conditions were as in Table 8. Nuclei were added in 0·5 ml. of 0·25 M-sucrose. Results are the means of six experiments and the ranges are given. Additions are recorded as final concentrations.

Additions	P liberated (μmoles/mg. of N/20 min.)
Nil	5·3 (4·5–5·6)
Dinitrophenol, 0·03 mM	7·2 (6·2–7·6)
Chlorpromazine, 0·2 mM	5·1 (4·2–5·4)
Azide, 1·0 mM	4·3 (4·0–4·4)
Dinitrophenol, 0·03 mM + chlorpromazine, 0·2 mM	4·4 (4·1–4·7)
Dinitrophenol, 0·03 mM + azide, 1·0 mM	5·3 (4·7–5·6)

Table 10. *Cytochrome-oxidase and succinoxidase activity of nuclear and mitochondrial preparations*

Mitochondria and nuclei suspensions in 0·25 M-sucrose were frozen and thawed twice before use. Results are the means of six experiments and the ranges are given.

Preparation	Succinoxidase activity (μl. of O/mg. of N/hr.)	Cytochrome-oxidase activity (μl. of O/mg. of N/hr.)	Cytochrome oxidase/ succinoxidase
Mitochondria	2000 (1800–2100)	5000 (4800–5300)	2·5
Nuclei	110 (100–135)	535 (520–570)	4·8

examination, the succinoxidase activity of the nuclear preparations was determined, and a small but definite activity was found (Table 10). Succinoxidase is a solely mitochondrial enzyme system (Schneider, 1946). Assuming mitochondrial contamination, the succinoxidase activity found in nuclei indicates the presence of 0·075 mg. of mitochondrial nitrogen in each 1·0 mg. of nuclear nitrogen. The magnesium-activated adenosine triphosphatase equivalent to 0·06 mg. of mitochondrial nitrogen is 0·1 μmole of phosphate liberated/20 min., and in the dinitrophenol-stimulated system it is 0·3 μmole of phosphate. Thus after correction for mitochondrial contamination, the nuclear magnesium-activated adenosine-triphosphatase activity in Table 8 is 5·2 μmoles of phosphate and 6·9 μmoles of phosphate in the dinitrophenol-activated system.

If the energy system of the nucleus is similar to that of the mitochondrion, the phosphorylation reactions would be coupled to an electron-transport system. The inhibitory effects of chlorpromazine on synthetic reactions suggest that a flavoprotein is involved, e.g. DPNH–cytochrome *c* reductase (Dawkins *et al.* 1959, 1960; Siekevitz *et al.* 1958). The nuclear preparations were assayed for this enzyme which was found to be present [7·8 (7·1–8·8) μmoles of cytochrome *c* reduced/hr./mg. of nuclear N]. The presence of this enzyme was confirmed by the finding that nuclear preparations could couple the oxidation of DPNH with the electron acceptor neotetrazolium [15·9 (14·9–17·0) mg. of formazan formed/hr./mg. of nuclear N]. The nuclear activity of this system was about 50 % of that of the mitochondrial activity. This reaction was inhibited by dicoumarin, as was the reaction in the mitochondrial system (Slater, 1959). This suggests that a quinone such as ubiquinone is involved in the oxidation of DPNH. The preparations, however, would not oxidize DPNH in the absence of added electron carriers.

DISCUSSION

The methods of preparation based on the use of 0·25 M-sucrose (Dounce, 1955) have been successfully applied to the isolation of nuclei from calf thymus by Allfrey *et al.* (1955). This tissue has a high nuclear to cytoplasmic ratio and is relatively free of blood. It is, however, difficult to obtain thymus gland immediately after the death of the animal and Logan *et al.* (1959) have pointed out that these thymus nuclear preparations are liable to contamination with thymocytes.

For our investigation, rat liver was used because further work is proposed on nuclear changes in hepatocarcinogenesis. The nuclear to cytoplasmic ratio is lower than in thymus gland and there is a large quantity of blood in the normal liver. The liver was perfused *in situ* to remove the blood. Five washes and centrifugings were used to remove cell debris. The preparation was completed in 45 min. by using high-speed centrifuging for short times, and was as free from contamination as those prepared with low-speed centrifugings.

Chemical analysis of these preparations gave constant results with a RNA/DNA ratio of 0·97, which is in good agreement with 0·975 obtained by Kay, Smellie, Humphrey & Davidson (1956) for rabbit-embryo liver nuclei in a sucrose medium. However, Naora & Takeda (1954) obtained a ratio of 0·333 for rat-liver nuclei isolated in non-aqueous media (1938) by the Behrens procedure. Kay & Davidson (1955) demonstrated that when liver nuclei isolated by non-aqueous media are treated with sucrose solution, there are losses of protein and RNA. However, the ratio of 0·333 obtained by Naora & Takeda must indicate losses of RNA from the nuclei during non-aqueous isolation, since if the DNA figures obtained in our preparations are adjusted to 100 % recovery and no loss of RNA is assumed, the ratio was 0·45. The findings of Kay & Davidson (1955) may be due to losses of non-nuclear RNA and protein from the heavily contaminated non-aqueous nuclei.

The preparations of rat-liver nuclei described in this paper were found *in vitro* to incorporate [2-^{14}C]glycine into nuclear proteins and [8-^{14}C]-adenine and [6-^{14}C]orotic acid into nuclear RNA. Similar results with rat-liver nuclei and [8-^{14}C]-adenine and [2-^{14}C]phenylalanine have been obtained by Logan *et al.* (1959). Rat-liver nuclei were thus similar in this respect to the preparations from calf-thymus gland. Linear rates of incorporation were obtained with the rat-liver nuclei for periods of up to 190 min. The incorporation rate is

lower in rat-liver nuclei than in calf-thymus nuclei, and there is no change in rates of incorporation when sodium ions in the incubation medium are replaced by potassium ions.

The synthetic reactions described in this paper were inhibited by 2:4-dinitrophenol, azide and anoxia. This would suggest that the energy for these reactions depends on a mechanism such as oxidative phosphorylation. Allfrey, Mirsky & Osawa (1957) came to similar conclusions about thymus nuclei, and Osawa, Allfrey & Mirsky (1957) demonstrated the presence of nuclear adenine nucleotides which became phosphorylated in the presence of oxygen. Naora & Takeda (1954) claim that 45% of the total cell ATP of the rat liver is in the nucleus. However, the nuclei described in this paper did not contain free adenine nucleotides. This may well be a result of the isolation procedure (Keir & Davidson, 1958).

In liver microsomes, incorporation of amino acids is enhanced by the addition of ATP and cell sap (Zamecnik & Keller, 1954), whereas with nuclei these factors are inhibitory. This difference may be caused by structural differences which in the nuclei may result in barriers against the entry of molecules, such as ATP or polynucleotides, which play a role in amino acid incorporation by microsomes (Lipmann, 1958).

The stimulation of nuclear adenosine triphosphatase by 2:4-dinitrophenol and the effects of chlorpromazine support the idea of oxidative phosphorylation. Chlorpromazine is known to act by inhibition of flavoproteins (Dawkins *et al.* 1959, 1960) and the inhibition of the synthetic reactions by this agent suggests that a flavoprotein enzyme is involved in the production of energy for the synthetic reactions. Such a flavoprotein enzyme could be DPNH–cytochrome *c* reductase, which has been shown to be present. The presence of a DPNH–neotetrazolium reductase in the nucleus suggests that the flavoprotein DPNH–cytochrome *c* reductase may be coupled with a quinone like ubiquinone. Martius (1956) has shown that the quinone vitamin K is present in rat-liver nuclei.

It is thus possible to visualize a series of reactions to generate ATP. An electron chain oxidizing DPNH via a flavoprotein enzyme to a quinone could be coupled with a phosphorylation mechanism similar to that in the mitochondrion. This leaves the problem of explaining how the reduced quinone is reoxidized. In theory this could be achieved by an anaerobic mechanism whereby electrons are passed to an acceptor such as unsaturated fatty acids. In view of the inhibitory effects of anoxia in synthetic reactions and the effects of aeration on maintaining the ATP/ADP ratio in thymus nuclei described by Osawa *et al.* (1957), it would seem likely that an oxidative mechanism is involved. However, the fact that DPNH is not oxidized directly by nuclear preparations without the addition of electron acceptors demonstrates that the quinone is not oxidized by atmospheric oxygen. In mitochondria the oxidation of reduced ubiquinone can take place via the cytochrome system (Green, 1959). The results obtained in this paper suggest that cytochrome oxidase is present in the nucleus and hence a similar mechanism of oxidation may exist.

SUMMARY

1. A method for the isolation of rat-liver nuclei in 0·25 M-sucrose has been described, and the nucleic acid and phospholipid content have been determined.

2. The nuclei incorporate ^{32}P, [8-^{14}C]adenine, [6-^{14}C]orotic acid and [2-^{14}C]glycine into their nucleic acids or protein *in vitro*.

3. Anoxia, 2:4-dinitrophenol, azide and chlorpromazine inhibit the incorporation into the protein and nucleic acid.

4. The adenosine-triphosphatase activity of the nuclear preparations has been measured, and the effects of inhibitors have been studied.

5. The presence of cytochrome oxidase, reduced diphosphopyridine nucleotide–cytochrome *c* reductase and reduced diphosphopyridine nucleotide–neotetrazolium reductase have been demonstrated in the nuclear preparations.

6. A possible scheme for the production of adenosine triphosphate in the nucleus is discussed.

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The Reduction of Acetoacetate to β-Hydroxybutyrate in Animal Tissues

BY R. G. KULKA,* H. A. KREBS AND L. V. EGGLESTON
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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Krebs & Eggleston (1948) reported that minced sheep-heart muscle, when suspended in a saline medium, reduced acetoacetate to β-hydroxybutyrate more rapidly under aerobic than under anaerobic conditions. It is somewhat unexpected that oxygen should promote a reduction and the present investigation was undertaken to throw light on the mechanism by which this effect of oxygen is brought about.

EXPERIMENTAL

Materials

Sodium acetoacetate was prepared as described by Krebs & Eggleston (1945). Stock solutions were stored at −18°. Because of its instability the concentration of the acetoacetate solution used was determined for each experiment.

Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd. The product was eluted as a single peak when chromatographed on a Celite column (Utter & Swim, 1957) and recoveries of the eluted free acid, based on titration against standard NaOH, were 103% in two successive experiments. Samples of sodium D(−)- and L(+)-β-hydroxybutyrate were kindly given by Dr G. D. Greville (see Lehninger & Greville, 1953).

Adenosine triphosphate (ATP) was obtained from Schwartz Chemical Co., U.S.A. Dicoumarol was the product of Organon Laboratories Ltd.

* 1851 Overseas Scholar.

Tissue preparations

Homogenates were prepared in a saline medium which consisted of 100 vol. of 0·155M-KCl, 10 vol. of 0·1M-potassium phosphate buffer, pH 7·4, and 1 vol. of 0·1M-$MgCl_2$.

Pig and sheep hearts were obtained from an abattoir. They were placed in ice immediately after removal from the animal and reached the laboratory in 15–20 min. Other tissues were immersed in ice-cooled saline medium immediately after removal from the animal. The tissue was freed from fat and connective tissue, minced in a chilled Fischer mincer (Jouan, Paris) and homogenized in a stainless steel Potter–Elvehjem homogenizer with 6·5 vol. of cold (0°) saline medium.

Incubations were carried out in conical Warburg flasks at 30°. The centre wells of vessels contained 2N-NaOH and filter paper in aerobic experiments, and a stick of yellow phosphorus in anaerobic experiments. The main compartments contained 3·0 ml. of homogenate (approx. 70 mg. dry wt. of tissue). Substrates were placed in the side arm in the form of a 0·1–0·2M-solution of the sodium salt and the total volume was made up to 4·0 ml. with saline medium. The vessels were kept in ice during the preparation of the experiment. Unless stated otherwise the substrates were added after 5 min. incubation at 30° and the vessels were incubated for a further 45 min. At the end of the incubation the vessels were cooled in ice and 2·0 ml. of the contents of each vessel was transferred to a tube containing 0·5 ml. of N-HCl (solution A). These tubes were stored at −18°. To the remaining 2·0 ml. in each vessel 0·5 ml. of 25% (w/v) trichloroacetic acid was added and the