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Protein Synthesis in Mitochondria

4. PREPARATION AND PROPERTIES OF MITOCHONDRIA FROM KREBS II MOUSE ASCITES-TUMOUR CELLS*

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1. Methods of disrupting Krebs II mouse ascites-tumour cells have been studied. After washing the cells free of ions with sucrose solutions, rapid disruption was obtained in sucrose by use of an Ultra-Turrax disintegrator or a Dounce homogenizer. 2. Disruption of cells after osmotic shock led to the loss of proteins, especially cytochrome *c*, from the mitochondria. Such losses did not occur when cells were disrupted by shear in 0·3 M-sucrose. 3. The distribution of protein, RNA, DNA, malate dehydrogenase, cytochrome *c*, cytochrome oxidase and succinoxidase was measured in the various cell fractions after separation by differential centrifuging. 4. The mitochondrial fraction sedimented at 9500*g* was further fractionated by equilibrium sedimentation in a sucrose gradient. The distribution of protein and enzyme activity in the gradient indicated that the 9500 *g* pellet contains other material besides mitochondria. 5. Krebs-cell mitochondria contain up to five times as much RNA as do liver mitochondria. 6. After purification by equilibrium centrifugation Krebs-cell mitochondria still contain traces of DNA.

Studies of protein synthesis in animal cells have shown that radioactive amino acids are most rapidly incorporated into the protein of the microsomal fraction and the ribosomes separated from it (Littlefield, Keller, Gros & Zamecnik, 1955). The mechanisms involved in this incorporation have been examined in detail and it has been shown that the proteins released from cells after synthesis are formed on ribosomes or polysomes. Examples are albumin (Peters, 1962; Hayano, Tsubone, Azuma & Yamamura, 1962) and chymotrypsinogen (Siekevitz & Palade, 1960). In addition, conclusive evidence is available that one of the soluble intracellular proteins, haemoglobin, is synthesized on ribosomes (Schweet, Bishop & Morris, 1961).

Little is known as yet, however, about the sites of synthesis of the proteins characteristic of other cell organelles. In previous work (Roodyn, Reis & Work, 1961*a*; Roodyn, Suttie & Work, 1962; Roodyn, 1962) and in more recent unpublished work from this Laboratory it was shown that the mitochondrial proteins malate dehydrogenase, cytochrome *c* and catalase (of liver) all became rapidly labelled when radioactive amino acids were injected into animals, but that they did not become labelled when mitochondria were incubated *in vitro* under conditions that permit rapid incorporation of radioactive amino acids into the insoluble structural proteins of the mitochondria.

* Part 3: Roodyn (1962).

There is an obvious contradiction between experiments *in vivo* and those conducted with isolated mitochondria, and it seemed advisable therefore to make a more thorough study of the kinetics of labelling of the proteins of mitochondria within the intact cell. To do this it is necessary to have a method of cell disruption and fractionation that so far as possible gives a clear separation between nuclei, mitochondria, microsomes and cell sap without at the same time causing any leaching of the mitochondrial proteins from these organelles.

The choice of the Krebs II mouse ascites-tumour cell rather than liver for further investigation was dictated by two considerations: first, there seemed little hope of studying kinetics of synthesis uncomplicated by other factors unless a uniform suspension of cells could be used; secondly, there was already available in this Laboratory a considerable background of experience on protein synthesis in these cells (Martin, Malec, Coote & Work, 1961*b*; Kerr, Martin, Hamilton & Work, 1962).

In the present paper, methods for disruption of Krebs II mouse ascites-tumour cells and for the isolation of their mitochondria are examined. Particular attention was paid to the distribution of malate dehydrogenase and cytochrome *c*. These proteins are readily soluble and can be leached from the mitochondria without any obvious morphological damage; they are therefore a good index of

the effective preservation of mitochondria in their normal state. The ascites cell has a membrane resistant to disruption in sucrose with a Potter-Elvehjem-type homogenizer. Martin *et al.* (1961*b*) found that the Krebs II ascites cell was especially resistant to disruption, and methods successful with Ehrlich ascites cells were not applicable to the Krebs cell. Previous methods of disruption have depended on osmotic shock or high shearing forces (Martin *et al.* 1961*b*; McComb & Yushok, 1959). Some of these methods have been examined and a new one was developed. Both osmotic shock and high shearing forces after washing the cells in sucrose were effective in breaking the cell membrane, but they lead to mitochondria with different properties.

METHODS

Cells. Krebs II mouse ascites-tumour cells were used. Propagation, collection, washing and counting were done by the methods of Martin, Malec, Sved & Work (1961*a*). Cell disruption was followed microscopically by the method of Martin *et al.* (1961*b*).

Cell disruption

Osmotic shock. The double-osmotic shock method with a Potter–Elvehjem homogenizer as described by Martin *et al.* (1961*b*) was used. The first pellet of nuclei and undisrupted cells was washed with sucrose (0·25 M)–potassium chloride (0·10 M) instead of sucrose (0·25 M)–EDTA (40 mM).

High shearing forces (Dounce homogenizer). The homogenizer was made of glass according to the instructions of Dounce (1955). It had a clearance of 0·03 mm. between the ball and the 1·2 cm. diam. precision-bore tubing. This method of disruption is referred to below as the Dounce homogenizer method. The method is similar to that of Sauer, Martin & Stotz (1962).

Ascites cells were washed free of erythrocytes in phosphate-buffered saline and then once in calcium- and magnesium-free phosphate-buffered saline and once in sucrose (0·30 M)–EDTA (2 mM)–nicotinamide (30 mM), pH 7·2 (medium 1). This medium was used by Roodyn, Reis & Work (1961*a*) for the isolation of rat-liver mitochondria.

From 2×10^8 to 8×10^8 cells were homogenized in 20 ml. of medium 1 with 60–100 strokes to give 70–80% disruption. Potassium chloride (1·0 M) was added to give a 0·1 M final concentration, and nuclei and undisrupted cells were spun down at 750 *g* for 10 min. The pellet was homogenized with a further 20 strokes in sucrose (0·25 M)–potassium chloride (0·10 M), and nuclei were spun down as before. Usually, 80% disruption was achieved and further homogenizing led to greater nuclear damage.

Mitochondria were separated from the combined nuclear supernatants by centrifugation at 9200 *g* for 15 min. and washed once in medium 1.

Ultra-Turrax disintegrator. The Ultra-Turrax disintegrator used was made by Johne und Kunkel A.-G. Staufen. It has a maximum speed of 24000 rev./min., but for cellular disruption was run at lower speeds through a Variac transformer, the voltage being controlled by reference to a voltmeter.

Washed ascites cells were suspended in calcium- and magnesium-free phosphate-buffered saline and layered on top of 0·3 M-sucrose. After centrifugation, the cells were either washed once more in 0·3 M-sucrose or disrupted directly in 9 ml. of this medium in a 40 ml. round-bottomed plastic centrifuge tube in an ice bath. The best disruptions were obtained with from 1×10^8 to 4×10^8 cells.

The disintegrator was run for 45 sec. at 49 v and then turned off for 15 sec. No significant rise in temperature was noted. Homogenization was continued until 100% free nuclei were obtained. At higher voltages there was faster disruption but greater nuclear damage.

After disruption, 1·0 M-potassium chloride was added to give a 0·1 M final concentration. Nuclei were spun down at 750 *g* for 10 min. and washed once in sucrose (0·27 M)–potassium chloride (0·10 M).

Mitochondria were spun down at 9200 *g* for 15 min., and after a washing in 0·3 M-sucrose the microsomal fraction was separated from the combined supernatant solutions by centrifugation at 105000 *g* for 1 hr.

In some experiments the nuclear fraction was washed further in sucrose (0·30 M)–EDTA (10 mM)–nicotinamide (30 mM) (medium 2). The supernatant solution was used to resuspend the mitochondrial pellet and mitochondria were separated by centrifugation. The supernatant solution was kept separate from the previous mitochondrial supernatant solutions.

When only mitochondria were required, medium 1 instead of 0·3 M-sucrose was used as the suspending medium during disruption.

The mitochondrial fraction was freed from ribosomes by washing three times in 10 ml. of medium 2 or by suspending them in medium 2 diluted 1·2-fold with water, layering over medium 2 and centrifuging at the usual speed.

Density-gradient centrifugation

The mitochondrial fraction was further characterized by isopycnic centrifugation in sucrose solutions. A density gradient of 4·3 ml. from 1·88 M- to 0·88 M-sucrose in 1 mM-EDTA was prepared in a Spinco SW39 tube by allowing five layers of sucrose, equally spaced between 1·88 M and 0·88 M, to diffuse for 24 hr. According to Charlwood (1963) this produces an essentially linear gradient.

The mitochondrial fraction from 2×10^8 cells was suspended in 1·0 ml. of sucrose (0·88 M)–EDTA (1 mM), layered on the gradient and spun for 2 hr. on a Spinco SW39 head at 39000 rev./min. (cf. Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959; Beaufay, 1962). The mitochondrial layer, clearly visible as a band about half-way down the tube, was recovered with a Pasteur pipette after removal of the overlying solution.

When sampling the whole gradient a Spinco SW25 head was used and centrifugation was carried out for 5 hr. at 25000 rev./min. Fractions were collected by puncturing the bottom of the tube.

Measurement of enzyme activities and cytochrome c

All spectrophotometric measurements were on a Unicam SP.700 spectrophotometer and the enzyme activities were measured at room temperature.

Malate dehydrogenase. Total enzyme activity was measured by following the reduction of NAD^+ by malate, reduction being measured spectrophotometrically at 340 mμ according to the method of Roodyn *et al.* (1962). The enzyme fractions were solubilized in water and Triton X-100 diluted to 0·8%. A unit of activity is the amount of enzyme giving an increase in E_{340} of 1·0/min. in a 1·0 cm. cell containing 2·7 ml. of fluid.

Delbrück, Schimassek, Bartsch & Bücher (1959) have shown that cells of many species of animals contain two malate dehydrogenases, one in the mitochondria and one in the cell sap. Only the mitochondrial enzyme is inhibited by high concentrations of oxaloacetate, and this was used to distinguish between the two enzymes. Enzyme activity was followed by measuring the oxidation of NADH at two concentrations of oxaloacetate (1 mM and 0·1 M) by the method of Siegel & Englard (1961). Oxaloacetate was prepared fresh each day.

Cytochrome oxidase. Cytochrome-oxidase activity was measured by following the oxidation of reduced cytochrome *c* at 550 mμ by the methods of Cooperstein & Lazarow (1951). A solution of 17 μM-cytochrome *c* in 0·03 M-phosphate buffer, pH 7·4, was reduced with a minimum of sodium dithionite. The solution was shaken until a decrease in the extinction at 550 mμ indicated that all the dithionite was oxidized. Horse-heart cytochrome *c* was obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany. It was partly autoxidizable, and corrections for this were applied when necessary. A unit of activity is defined in the same way as the unit of activity of malate deyhydrogenase.

Succinoxidase. Succinoxidase was measured by the method of Schneider & Potter (1943).

Cytochrome c. Cytochrome *c* was isolated from cells or subcellular fractions by a combination of the methods of Rosenthal & Drabkin (1943) and Keilin & Hartree (1937). It was measured by the method of Paul (1955).

Extraction and estimation of protein and nucleic acids

A modification of the method of Simkin & Work (1957) was used. The fraction was precipitated with an equal volume of cold 10% (w/v) trichloroacetic acid and washed six times in ice-cold 5% (w/v) trichloroacetic acid before the DNA and RNA were extracted in 5% trichloroacetic acid for 15 min. at 90°. Protein was obtained by washing the insoluble residue once with 5% trichloroacetic acid and twice each with acetone, ethanol–ether (3:1, v/v) at 60° and ether.

Protein was estimated with a micro-Kjeldahl procedure or, for fractions along the density gradient, by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

The RNA was measured by the orcinol method of Mejbaum (1939) with a heating time of 40 min. Adenosine was used as the standard.

The DNA was estimated by the method of Burton (1956). A preparation of calf-thymus DNA containing 7·31% of phosphorus was used as a standard.

RESULTS

Cell disruption

In Tables 1 and 2 are presented the analyses of the subcellular fractions obtained in the two main methods of disruption. In all cases there was nearly 100% release of free nuclei. As reported by Martin *et al.* (1961*b*), cells disrupted with osmotic shock give the cleanest separation of subcellular constituents.

When disruption was carried out with the Ultra-Turrax disintegrator, more nuclear damage occurred in medium 1 than in 0·3 M-sucrose, as judged by the DNA content of the mitochondrial fraction, but there was a greater recovery of protein and enzyme activity in the mitochondrial, microsomal and cell-sap fractions. This greater release of protein was most marked in the mitochondrial fraction and less in the microsomal fraction. With medium 1, there was a low recovery of RNA in the microsomal fraction, probably because of ribosomal disruption by EDTA. For this reason, 0·3 M-sucrose was preferred as the medium when microsomes were required.

The cytochrome oxidase was mainly in the mitochondrial and nuclear fractions, but the activity in the nuclear fraction is lower than the true value because of nuclear aggregation. The reason for the low recovery of malate dehydrogenase is not clear.

The main factor affecting disruption was the presence of ions. After centrifugation once through 0·3 M-sucrose, the percentage of free nuclei with 1, 2 and 3 min. of homogenization in the Ultra-Turrax disintegrator was 32, 85 and 95%. After a second wash in 0·3 M-sucrose, the cells swelled and were disrupted to yield 100% free nuclei in 45 sec. with the Ultra-Turrax disintegrator. If the

Table 1. *Distribution of protein, ribonucleic acid and deoxyribonucleic acid in subcellular fractions obtained from Krebs II ascites-tumour cells by osmotic shock*

Washed cells were disrupted with osmotic shock and a Potter homogenizer and fractions were analysed as described in the Methods section.

Fraction	Protein (mg./10^8 cells)	RNA (μg. of P/10^8 cells)	DNA (μg. of P/10^8 cells)
Lysate	16·14	228	215
Nuclei	3·53	38	148
Mitochondria	2·87	46	7
Microsomes	1·68	87	3
Cell sap	5·0	42	4
Total recovery ...	13·63	213	162
Percentage recovery ...	84·5	93·5	75·3

Table 2. *Distribution of protein, ribonucleic acid, deoxyribonucleic acid, cytochrome oxidase and malate dehydrogenase in subcellular fractions obtained from Krebs II ascites-tumour cells by disruption with the Ultra-Turrax disintegrator*

Cells were washed and disrupted in either 0·3 M-sucrose or sucrose (0·3 M)–EDTA (2 mM)–nicotinamide (30 mM) (medium 1). Mitochondria were washed in the sucrose solution used for disruption.

Fraction	Protein (mg./10^8 cells)		RNA (μg. of P/10^8 cells)		DNA (μg. of P/10^8 cells)		Cytochrome oxidase (units/10^9 cells)		Malate dehydrogenase (units/10^9 cells)	
Medium...	0·3 M-Sucrose	Medium 1	0·3 M-Sucrose	Medium 1	0·3 M-Sucrose	Medium 1	0·3 M-Sucrose	Medium 1	0·3 M-Sucrose	Medium 1
Cell lysate	16·2	17·2	265	253	234	232	107	81	202	192
Nuclei	5·15	3·35	49·2	21	183	182	42	15	48	20
Mitochondria	0·98	2·32	10·3	16·3	5·2	27·5	67	121	29	66
Microsomes	2·14	3·06	132	117·6	0·7	1·4	2	2	0	0
Cell sap	6·71	8·04	37·5	69·1	0·2	1·0	7	2	12·6	13·3
Total recovery ...	14·98	16·77	229	224	189·1	211·9	118	140	89·4	99·5
Percentage recovery ...	91·5	97·5	86·5	88·5	80·7	91·3	110	173	44	52

sucrose solution contained potassium chloride (0·1 M) or magnesium chloride (0·01 M), no cell breakage occurred.

Density-gradient centrifugation

In Fig. 1 are shown the results of a density-gradient centrifugation. The mitochondria come to equilibrium at an average value of 1·3 M-sucrose (sp.gr. 1·17). Rat-liver mitochondria in a similar gradient come to equilibrium at sp.gr. 1·19 (Beaufay, 1962).

It is apparent that the mitochondrial fraction, i.e. the 9500 *g* pellet, is nearly homogeneous as judged by the correspondence of enzyme activity and protein, but there is a non-mitochondrial component present. Turbidity follows enzyme activity more closely than it follows protein. A small peak of lighter material was always detected in isopycnic separation, but its nature is not known. Acid-phosphatase activity was present in these fractions (lysosomes), but also in the main peak to a greater extent. Any nuclei form a pellet at the bottom of the tube.

The RNA and DNA contents of the mitochondrial fraction at various stages of purification are shown in Table 3. The mitochondria contain very little DNA but a surprisingly large amount of RNA. The washing with sucrose containing nicotinamide and EDTA removes a considerable amount of RNA,

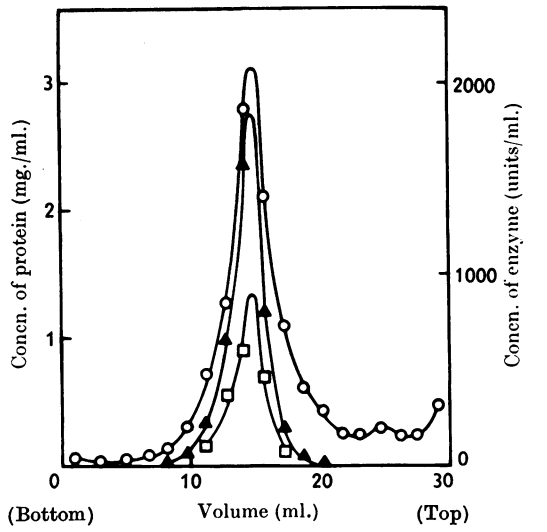

Fig. 1. Isopycnic centrifugation of the mitochondrial fraction in sucrose. The methods are described in the text. ○, Concn. of protein; ▲, malate dehydrogenase; □, cytochrome oxidase.

Table 3. *Amounts of ribonucleic acid and deoxyribonucleic acid in mitochondrial fractions purified by washing with sucrose–EDTA and centrifuging to density equilibrium*

Washed cells (5×10^8) were disrupted with osmotic shock or the Dounce homogenizer. The mitochondrial fractions were washed after spinning down three times in sucrose (0·3 M)–EDTA (10 mM)–nicotinamide (30 mM) (medium 2) and then spun on a sucrose gradient to density equilibrium as described in the Methods section.

Stage of mitochondrial purification	Osmotic shock		Dounce homogenizer	
	RNA (μg. of RNA P/mg. of protein)	DNA (μg. of DNA P/mg. of protein)	RNA (μg. of RNA P/mg. of protein)	DNA (μg. of DNA P/mg. of protein)
Original pellet	12·5	0·86	12·1	4·6
Thrice washed in medium 2	9·35	0·76	6·8	6·4
Density gradient	8·9	0·26	5·4	0·93

Table 4. *Distribution of cytochrome c and cytochrome oxidase between cell fractions after disruption of Krebs cells by shear in sucrose solution*

Cells were disrupted in 0·3 M-sucrose with the Ultra-Turrax disintegrator as described in the Methods section. In one experiment the nuclear pellet was washed with medium 2 and the supernatant from this wash used to wash the mitochondrial pellet; in the other experiment the nuclear pellet was washed with 0·3 M-sucrose only. —, Not determined.

Medium used for washing nuclei	Medium 2		0·3 M-Sucrose	
Fraction	Cytochrome oxidase (units/10^9 cells)	Cytochrome *c* (μg./10^9 cells)	Cytochrome oxidase (units/10^9 cells)	Cytochrome *c* (μg./10^9 cells)
Homogenate	57·6	153	57·6	153
Nuclei	8·1	16*	14·1	85
Mitochondria	42·8	102	17·3	51
Mitochondrial wash fluid	1·2	0	—	—
Microsomes	1·2	0	4·1	15*
Cell sap	—	—		

* Because of the low concentrations of cytochrome *c* the accuracy of these values is low.

presumably by disruption of contaminating ribosomes (cf. Schweet *et al.* 1961), but the residual RNA, which appears to be firmly attached to the mitochondria, is still six to seven times that found in rat-liver mitochondria (Roodyn *et al.* 1961*a*, 1962). The amount of DNA found in Krebs-cell mitochondria is about the same as that found in rat-liver mitochondria (Roodyn, Freeman & Tata, 1965).

Release of soluble proteins from the mitochondria

The main difference between mitochondria isolated by osmotic shock and mitochondria isolated by shear rupture in sucrose solution seems to reside in the almost complete absence of cytochrome *c* from mitochondria obtained by osmotic rupture of Krebs cells. In contrast, mitochondria from cells disrupted in 0·3 M-sucrose either with the Dounce homogenizer or with the Ultra-Turrax disintegrator contained nearly all the cytochrome *c* of the cell. The results of one such experiment with the Ultra-Turrax disintegrator are shown in Table 4. It is evident also from Table 4 that washing the nuclei with the sucrose–nicotinamide–EDTA mixture is an essential step in effective separation of the subcellular components, since in the absence of this step much of the cytoplasmic material can stick to the nuclei. The loss of cytochrome *c* from the mitochondria of osmotically disrupted cells is accompanied by a similar loss of malate dehydrogenase, but as shown in Table 5 the loss of this enzyme is incomplete. The presence of the mitochondrial enzyme in the cell-sap fraction can be readily detected since this enzyme is inhibited at higher oxaloacetate concentrations. This effect is shown in the last column of Table 5, where it is evident that the two methods of disruption produce cell sap of different enzymic character.

Some idea of the quantitative extent of the loss of protein from mitochondria produced during osmotic rupture of Krebs cells may be gained by submitting mitochondria obtained by rupture in 0·3 M-sucrose to a single brief cycle of osmotic shock. It was

Table 5. *Malate dehydrogenase of mitochondrial and cell-sap fractions prepared from cells disrupted with the Dounce homogenizer or by osmotic shock and with the Potter homogenizer*

Cells were disrupted with the Potter homogenizer after osmotic shock or with the Dounce homogenizer in 0·3 M-sucrose, and the subcellular fractions were isolated as described in the Methods section.

Disruption procedure	Fraction	Malate dehydrogenase (units/5×10^8 cells)	Ratio of activities in oxaloacetate (0·1 mM/1 mM)
Osmotic shock	Mitochondria	16·76	1·58
	Cell sap	34·4	1·1
Dounce homogenizer	Mitochondria	42·6	1·85
	Cell sap	12·1	0·64

Table 6. *Succinoxidase activity of mitochondria prepared from cells disrupted by osmotic shock or with the Dounce homogenizer*

Succinoxidase activity of mitochondria prepared by osmotic shock and Dounce procedures was determined by the method of Schneider & Potter (1943). The mitochondria after sedimentation were washed in 0·3 M-sucrose. Mitochondria from $4·5 \times 10^7$ cells were used for each determination. When sucrose was added the final concentration was 0·3 M. If it was not added the concentration was 0·03 M.

Disruption procedure	Cytochrome *c*	Sucrose	Succinoxidase (μl. of O_2/hr./mg. of mitochondrial protein)
Dounce	+	−	409
Osmotic shock	+	−	294
Dounce	+	+	371
Osmotic shock	+	+	226
Dounce	−	−	131
Osmotic shock	−	−	22
Dounce	−	+	168
Osmotic shock	−	+	30

found that 12·8% of the total protein was solubilized by this treatment.

Succinoxidase activity. Because of the differences observed in the cytochrome *c* content of mitochondria prepared by different methods, a comparison was made of their succinoxidase activities in the presence and absence of added cytochrome *c* and in the presence of 0·3 M- or 0·03 M-sucrose. In Table 6 it is shown that the succinoxidase activity in the absence of added cytochrome *c* is high only when the mitochondria contain cytochrome *c* (Dounce homogenization). Adding cytochrome *c* does increase the activity of mitochondria prepared in both procedures. Keeping the medium 0·3 M with respect to sucrose increased the activity with no added cytochrome *c* but decreased it if cytochrome *c* was added.

Microsomal fraction. Martin *et al.* (1961*b*) used the capacity of the microsomal fraction to incorporate amino acids into protein as a useful criterion of damage caused by cellular disruption. The incorporation of L-[^{14}C]phenylalanine into microsomes from cells disrupted with the Ultra-Turrax disintegrator with 0·3 M-sucrose and with the Dounce homogenizer in 0·01 M-magnesium chloride (method of Martin *et al.* 1961*b*) were 690 and 460 mμc/g. of microsomal protein respectively. Ribosomes obtained from the microsomal fraction prepared in the latter procedure had an incorporation of 2130 mμc/g. of ribosomal protein. The values show that microsomes prepared with the Ultra-Turrax disintegrator are undamaged by this criterion.

DISCUSSION

The cell membrane of ascites cells, especially the Krebs II cell, is resistant to disruption in 0·3 M-sucrose with the Potter–Elvehjem-type homogenizer (Martin *et al.* 1961*b*). McComb & Yushok (1959) found it necessary to use 75 strokes of a Potter homogenizer to disrupt Krebs II ascites cells in 0·25 M-sucrose containing EDTA. In comparison, rat-liver-cell membranes can be broken with only a single stroke (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

This difficulty can be overcome with high shearing forces or osmotic shock, either alone or together (see Martin *et al.* 1961*b*).

The mitochondria isolated from Ehrlich cells have often been characterized by the P:O ratio. Both Chance & Hess (1959) and Sauer, Martin & Stotz (1960) showed the presence of cytochrome *c* in their mitochondrial preparations. Chance & Hess (1959) found a constant ratio of cytochrome *c* to cytochrome oxidase in cells and mitochondria and, by assuming that all the cytochrome oxidase was in mitochondria, concluded that all the cytochrome *c* was also there. The present results indicate that cytochrome *c* is also confined to the mitochondria of Krebs cells provided that they are undamaged during isolation. In the present study, the cells could be disrupted either by osmotic shock or by washing thoroughly with sucrose and disruption with the Ultra-Turrax disintegrator. The former method gave a cleaner separation of subcellular components but led to loss of soluble mitochondrial proteins, especially cytochrome *c*.

The success of the method with the Ultra-Turrax disintegrator depends on the efficient removal of ions from the medium by washing the cells in sucrose. The cells then swell, as do nuclei in these conditions (Anderson & Wilbur, 1952), and can be readily disrupted with the Ultra-Turrax disintegrator. Although there was a 100% release of nuclei, a wash with a sucrose–EDTA solution was necessary to free the nuclei from contamination with other subcellular fractions, especially mitochondria.

The loss of cytochrome *c* in the osmotic-shock procedure seems to depend on the preliminary wash and suspension of cells in water followed by the later addition of potassium chloride. Schneider, Claude & Hogeboom (1948) showed that cytochrome *c* of rat-liver mitochondria could be removed by washing first with water and then 0·9% sodium chloride solution, but not by washing with either water or sodium chloride solution alone. The mitochondria isolated in 0·3 M-sucrose contain most of the cellular cytochrome *c*.

The succinoxidase activity of mitochondria containing their own cytochrome *c* (Dounce preparation) was considerably higher than that of mitochondria prepared by osmotic shock. Schneider *et al.* (1948) and Siekevitz & Watson (1956) obtained results similar to those observed in the present work for succinoxidase. The succinoxidase activity of mitochondria in 'hypotonic' solutions compared with that in 'isotonic' solutions is higher when cytochrome *c* is added but lower when it is not. Siekevitz & Watson (1956) relate this to the swelling of mitochondria in 'hypotonic' solutions allowing for greater access of exogenous cytochrome *c* and a loss of endogenous cytochrome *c*.

By density-gradient centrifugation it has been found that the 9500 *g* pellet contains other components besides mitochondria. The additional protein on the lighter side of the mitochondria and the small second peak have not been identified. Lysosomes are probably present, since there is some acid-phosphatase activity.

Because turbidity followed enzyme activity more closely than it did protein, it was possible by visual examination to recover the mitochondria free of much of the contamination material. These mitochondria contain a little DNA and have a much higher RNA content than those of rat liver. Novikoff (1957) found that the esterase activity (microsomal enzyme) and the RNA content of liver mitochondria decreased in a parallel fashion during washing of the mitochondria, and concluded that most, if not all, of the RNA is from contamination with microsomes. The high RNA content of ascites-cell mitochondria suggests that this is not so, and may indicate that mitochondria of fully-differentiated non-dividing cells (liver) differ from those of rapidly dividing cells. My results would be consistent with the hypothesis presented by Roodyn, Reis & Work (1961*b*) that mitochondria are independent organelles that arise by budding from the nuclear membrane and undergo a process of maturation. The mitochondria of fully-differentiated non-dividing cells such as liver would be expected to have used up most of their supply of RNA in developing the specialized proteins characteristic of mature mitochondria, whereas the Krebs cell, which is not fully differentiated and which is harvested during its period of rapid growth, would contain many immature mitochondria still retaining a considerable proportion of their original endowment of RNA.

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REFERENCES

Anderson, N. G. & Wilbur, K. M. (1952). *J. gen. Physiol.* **35**, 781.
Beaufay, H. (1962). *Biochem. J.* **84**, 10P.
Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R. & de Duve, C. (1959). *Biochem. J.* **73**, 628.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Chance, B. & Hess, B. (1959). *J. biol. Chem.* **234**, 2404.
Charlwood, P. A. (1963). *Analyt. Biochem.* **5**, 226.
Cooperstein, S. J. & Lazarow, A. (1951). *J. biol. Chem.* **189**, 665.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Applemans, F. (1955). *Biochem. J.* **60**, 604.

Delbrück, A., Schimassek, H., Bartsch, K. & Bücher, Th. (1959). *Biochem. Z.* **331**, 297.
Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Hayano, K., Tsubone, T., Azuma, I. & Yamamura, Y. (1962). *J. Biochem., Tokyo*, **52**, 379.
Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.
Kerr, I. M., Martin, E. M., Hamilton, M. G. & Work, T. S. (1962). *Cold Spr. Harb. Symp. quant. Biol.* **27**, 259.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McComb, R. B. & Yushok, W. D. (1959). *Biochim. biophys. Acta*, **34**, 515.
Martin, E. M., Malec, J., Coote, J. L. & Work, T. S. (1961*b*). *Biochem. J.* **80**, 606.
Martin, E. M., Malec, J., Sved, S. & Work, T. S. (1961*a*). *Biochem. J.* **80**, 585.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Novikoff, A. B. (1957). *Symp. Soc. exp. Biol.* **10**, 92.
Paul, K. G. (1955). In *Methods in Enzymology*, vol. 2, p. 749. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Peters, T. (1962). *J. biol. Chem.* **237**, 1186.
Roodyn, D. B. (1962). *Biochem. J.* **85**, 177.
Roodyn, D. B., Freeman, K. B. & Tata, J. R. (1965). *Biochem. J.* (in the Press).
Roodyn, D. B., Reis, P. J. & Work, T. S. (1961*a*). *Biochem. J.* **80**, 9.
Roodyn, D. B., Reis, P. J. & Work, T. S. (1961*b*). In *Protein Biosynthesis*, p. 37. Ed. by Harris, R. J. C. London: Academic Press (Inc.) Ltd.
Roodyn, D. B., Suttie, J. W. & Work, T. S. (1962). *Biochem. J.* **83**, 29.
Rosenthal, D. & Drabkin, D. (1943). *J. biol. Chem.* **149**, 437.
Sauer, L. A., Martin, A. P. & Stotz, E. (1960). *Cancer Res.* **20**, 251.
Sauer, L. A., Martin, A. P. & Stotz, E. (1962). *Cancer Res.* **22**, 632.
Schneider, W. C., Claude, A. & Hogeboom, G. H. (1948). *J. biol. Chem.* **172**, 451.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
Schweet, R., Bishop, J. & Morris, A. (1961). *Lab. Invest.* **10**, 992.
Siegel, L. & Englard, S. (1961). *Biochim. biophys. Acta*, **54**, 67.
Siekevitz, P. & Palade, G. E. (1960). *J. biophys. biochem. Cytol.* **7**, 619.
Siekevitz, P. & Watson, M. L. (1956). *J. biophys. biochem. Cytol.* **2**, 653.
Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.