Subcellular Distribution of Cytochrome *c* in Rat Liver

METHODS FOR ITS EXTRACTION AND PURIFICATION

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(Received 16 February 1967)

1. A method for the extraction and purification of cytochrome *c* from rat liver is described. The method depends on multiple chromatography on Amberlite IRC-50 with elution with ammonium phosphate buffers of differing ionic composition and pH, interspersed with gel filtration with Sephadex G-25. Conditions leading to denaturation are avoided and the product is chromatographically pure. 2. The method may be used for the quantitative analysis of cytochrome *c* either in unfractionated liver or in subcellular fractions. 3. Two pools of cytochrome *c* were detected, one extractable at pH 4.0 with distilled water and the other extracted from the residues of the first extraction with 0.15M-sodium chloride. 4 For subcellular distribution studies the liver was homogenized in 0.3M-sucrose and a nuclear fraction (washed thoroughly to remove trapped mitochondria), a mitochondrial fraction, a heavy microsomal fraction, a standard microsomal fraction and the cell sap were isolated. The mitochondrial fraction was subfractionated further by density-gradient centrifugation. Each fraction was analysed for protein, RNA, DNA, succinate-neotetrazolium oxidoreductase and glucose 6-phosphatase. 5. A total of 123 μ g. of cytochrome *c* was obtained/g. wet wt. of rat liver. 6. Values for the percentage subcellular distribution of cytochrome *c* are: nuclear fraction, 24.4; mitochondrial fraction, 57.2; heavy microsomal fraction, 5.2; standard microsomal fraction, 10.6; cell sap, 2.7. 7. Three out of the eight mitochondrial subfractions separated by gradient centrifugation contained 76% of the cytochrome *c* and 85% of the succinate-neotetrazolium oxidoreductase present in the mitochondrial fraction. 8. In unfractionated liver 94% of the cytochrome *c* was extracted at pH 4.0 with water whereas in most of the subcellular fractions the corresponding value was approx. 75–80%.

A determination of the distribution of cytochrome *c* among the morphological constituents of the liver cell is not only important for an understanding of the physiological role of this protein, but is also an essential preliminary for studies on the site of biosynthesis of cytochrome *c*. Previous authors (Schneider & Hogeboom, 1950; Beinert, 1951; Dianzani & Viti, 1955) are agreed that only about half of the total cytochrome *c* is located in the mitochondrial fraction from rat liver homogenate. The values obtained for the total amounts of cytochrome *c* in the various subcellular fractions and hence the percentage distribution in the extra-mitochondrial fractions were, however, rather variable. These discrepancies extended to the total concentration of cytochrome *c* in unfractionated rat liver so that the values ranged from 213 μ g./g. (Schneider & Hogeboom, 1950) to 70 μ g./g. of fresh adult liver (Dallman & Schwartz, 1964). In these reports it was usual to interpret the extra-

mitochondrial location of cytochrome *c* in terms of an artifact resulting from a redistribution of the protein during homogenization and subcellular fractionation (e.g. Schneider & Hogeboom, 1950; Beinert, 1951).

The methods used in these former studies may be criticized on two general grounds. The first concerns the conditions for the extraction of cytochrome *c* from the subcellular fractions. In the main these were based on the methods of Rosenthal & Drabkin (1943). This method involves an extraction at a very low pH followed by a purification derived from the original method of Keilin & Hartree (1937) in which the cytochrome *c* is precipitated with ammonium sulphate and trichloroacetic acid. It has since been shown (Margoliash, 1954*a*; Margoliash & Lustgarten, 1962*a,b*) that under these conditions cytochrome *c* is at least partially denatured and this could lower the recovery of the protein. Moreover, the methods

applied to liver were adaptations of the Keilin & Hartree (1937) procedure for heart. The value of the method for liver subcellular fractions was not checked. Thus in the Rosenthal & Drabkin (1943) procedure no spectrophotometric evaluation of the purity of the resulting cytochrome *c* was reported.

The second objection to the previous work concerns the subcellular fractionation itself. The main points of criticism are the poor separation of the microsomal fraction from the cell sap at the low centrifugal forces used and the absence of analytical data defining the degree of mutual contamination of each subcellular fraction.

The introduction of chromatographic procedures for the purification of horse heart cytochrome *c* (Paléus & Neilands, 1950; Boardman & Partridge, 1953; Margoliash, 1954*a,b*; Paléus, 1960) and their adaptation to mitochondrial cytochrome *c* from rat liver (Jacobs & Sanadi, 1960) meant that conditions involving the risk of denaturing the protein could be avoided and at the same time a much better purification achieved.

In the present paper a method is described for the extraction, estimation and purification of cytochrome *c* from rat liver and its subcellular fractions. Chromatographically homogeneous cytochrome *c* is recovered in a high yield. By a modification of the now classical procedures of subcellular fractionation and by analysing the fractions so obtained, it has been possible to determine the cytochrome *c* content of each fraction and to correlate it with some of the morphological constituents of the fractions. The existence of two pools of cytochrome *c* in the fractions is demonstrated, most of the cytochrome *c* being extractable with water at pH 4.0, and a further quantity being more tightly bound to the fractions and only being extracted with 0.15 M-sodium chloride.

A preliminary report of this work has appeared (González-Cadauid & Campbell, 1967*a*).

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and were of AnalaR grade wherever possible. Horse heart cytochrome *c* (grade III) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Glucose 6-phosphate (sodium salt) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Neotetrazolium chloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All reagents were prepared and all procedures were performed with twice-glass-distilled water.

Materials for chromatography and gel filtration

The cation-exchange resin Amberlite IRC-50 was obtained as the CG-50 chromatographic grade, type II (200–400 mesh). It was washed and equilibrated either at pH 7.0

(with 0.25 M-NH₄⁺ buffer) or at pH 9.0 (with 0.1 M-NH₄⁺ buffer) by the method of Hagihara, Morikawa, Sekuzu & Okunuki (1958*a*). Excess of buffer was removed from the columns containing the resin by washing with distilled water. The NH₄⁺ buffers were made by using (NH₄)₂HPO₄ (for pH 7.0) or (NH₄)₃PO₄·3H₂O (for pH 9.0) and adding H₃PO₄ to adjust the pH. All measurements of pH were with a glass electrode at 2–4°, or at room temperature for the enzymic analysis.

Sephadex G-25 (fine grade) was obtained from Pharmacia Ltd., Uppsala, Sweden. It was washed with distilled water and equilibrated with 0.02 M-NH₄⁺ buffer, pH 7.0. After use the Sephadex was removed from the columns and washed with successive treatments of distilled water, 0.5 N-NaOH, distilled water, 1.5 N-acetic acid, acetone, distilled water and 0.5 M-NH₄⁺ buffer, pH 7.0. Columns of Sephadex were equilibrated with 0.02 M-NH₄⁺ buffer, pH 7.0.

Animals

Male Wistar albino rats (150 ± 10 g. body wt.) from a closed colony bred at this Institute were used throughout the subcellular distribution studies. For the preparation of larger amounts of highly purified cytochrome *c*, rats of body wt. 250–300 g. were used. In all cases the rats were starved overnight before being killed to deplete liver glycogen and facilitate subfractionation.

Subcellular fractionation

The method generally used was based on the procedures of Gear (1965) and of C. de Duve (personal communication) and was as follows. (An outline of the procedure appears in Scheme 1.) Rats were stunned by a blow on the head and killed by decapitation. The livers were removed rapidly, weighed (5.0–5.5 g.) and rinsed twice in ice-chilled 0.3 M-sucrose. The livers were then forced through a tissue press (purchased from Climpex Ltd., London, N.W. 7; made to the design of Porterfield, 1960). All operations thereafter were performed at 2–4°. The mince from each liver was suspended in 3 vol. of 0.3 M-sucrose and separately homogenized in a Potter-Elvehjem homogenizer with a tight polytetrafluoroethylene (Teflon) pestle (diam. 19 mm.) with 0.10–0.15 mm. clearance from the glass mortar. Three up-and-down strokes were used over a total period of 90 sec. with a constant rheostat setting such that the pestle rotated at about 2000 rev./min. at the beginning of cell disruption and 4000 rev./min. at the end. The homogenates from six livers were pooled and made up to 135 ml. with 0.3 M-sucrose and centrifuged in rotor SS-34 of the Servall Superspeed RC-2 centrifuge at 2700 rev./min. (600g) for 10 min. (Throughout the fractionation the centrifugal forces quoted are average.) The supernatant was decanted until the sediment reached the mouth of the tube. Each pellet was rehomogenized with 1.5 vol. of 0.3 M-sucrose/g. of original liver under the same conditions as before. The homogenates were pooled, made up to 135 ml. with 0.3 M-sucrose and centrifuged at 2000 rev./min. (340g) for 10 min. The supernatants were decanted as before and the procedure was repeated. The loose pellets were centrifuged finally at 8000 rev./min. (7700g) for 10 min. The remaining supernatants were decanted. The pellets were designated the 'nuclear fraction'. The supernatants resulting from washing the pellet in this step, as in all subsequent steps, were pooled for the preparation of the following fractions. The pooled supernatants were centrifuged in rotor 30 of

Scheme 1. Subcellular fractionation of rat liver. Groups of six rats were killed and their livers removed and homogenized. The homogenates were pooled and submitted to the subcellular fractionation summarized above and described in the text.

the Spinco L.1 preparative ultracentrifuge at 9400 rev./min. (8000g) for 10 min. The supernatant, together with most of the fluffy layer, was removed by suction. The loose pellet was gently rehomogenized in 0.3 M-sucrose with the Potter homogenizer, made up to 90 ml. and the 'mitochondrial pellet' collected by centrifugation under the previous conditions. The remaining liquid was removed from the loose pellet by centrifugation at 10500 rev./min. (10000g) for 20 min. and decantation. The pooled supernatants were centrifuged at 10500 rev./min. (10000g) for 20 min. and the liquid was decanted, leaving only the firm portion of the sediment. The pellet was designated the 'heavy microsomal fraction'. The supernatant was centrifuged at 40000 rev./min. (105000g) for 60 min. in rotor 40 of the Spinco centri-

fuge. The pellet was designated the 'standard microsomal fraction' and the supernatant the 'cell sap'.

Mitochondrial subfractionation

This was performed by centrifugation of the mitochondrial fraction through a discontinuous sucrose density gradient in rotor SW 25.1 of the Spinco centrifuge. Gradients were prepared by layering five 4.5 ml. samples of aq. sucrose solutions (densities 1.161, 1.176, 1.192, 1.208 and 1.224) on a cushion of 3.0 ml. of 2.2 M-sucrose in each of the three centrifuge tubes. The gradients were allowed to stand for 6-8 hr. before centrifugation. The mitochondrial pellet previously obtained was immediately resuspended in

0.3 M-sucrose by gentle homogenization and made up to 18 ml. The suspension was divided into three portions and layered on top of the sucrose gradients. Centrifugation was performed at 25 000 rev./min. (53 000 g) for 150 min., the rotor being allowed to come to rest without the use of the brake. Seven subfractions were removed from bottom to top by controlled gentle siphoning with a stainless-steel needle attached to a two-way valve connected to an all-glass syringe. The subfractions were numbered 1 to 7 from bottom to top and corresponded in volume to the original layers. Subfractions from different centrifuge tubes but with similar numbers were pooled and made up to 30 ml. with 0.3 M-sucrose. Pellets of each fraction were obtained by centrifuging in rotor 30 of the Spinco centrifuge at 20 000 rev./min. (35 000 g) for 60 min. The supernatants were decanted, together with a small fluffy layer present mainly in subfractions 4, 5 and 6, and pooled to give subfraction 8. Because of the small amount of protein and cytochrome c in the pellets from layers 1 and 7 they were combined with pellets 2 and 6 respectively. The new subfractions were designated 1-2 and 6-7.

In one subfraction a part of the mitochondrial suspension in 0.3 M-sucrose was separated into a 'heavy' subfraction and a 'light' subfraction by centrifuging at 600 g for 10 min. and siphoning off the supernatant. Both the loose pellet and the supernatant were diluted with 0.3 M-sucrose and sedimented at 100 000 g for 20 min.

All fractions were kept at -10° for analysis.

Analytical procedures

The subcellular fractions were thawed and the pellets resuspended by homogenization in twice-distilled water as described under the procedure for cytochrome c extraction. About 2-5% of each fraction was taken for analysis and the rest retained for the extraction of cytochrome c.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. The SNase* (Slater & Planterose, 1960a; Slater, 1963) was assayed by the method of Slater & Planterose (1960b). The subfractionation of the mitochondrial fraction by sucrose-density-gradient centrifugation requires several hours of centrifugation in sucrose solutions of high concentration, which leads to a partial loss of the enzyme activity. Therefore the total number of units of activity recovered in the sum of the mitochondrial subfraction was corrected to the activity of the total mitochondrial fraction determined in a sample assayed before subfractionation. G6Pase assays were performed by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The P_i released during the G6Pase assay was determined by the method of King, Abul-Fadl & Walker (1951), but the volumes were halved and the colour was read at 750 m μ .

For nucleic acid analysis a portion of the suspended

fraction was brought to a final concentration of 10% (w/v) trichloroacetic acid and the precipitate washed three times with cold 10% trichloroacetic acid. Nucleic acid was extracted and hydrolysed by heating the precipitates with 5% trichloroacetic acid for 20 min. at 90° . RNA was estimated by the orcinol method of Albaum & Umbreit (1947), and DNA by the method of Burton (1956). Hydrolysates from thymus DNA and yeast RNA were used as standards. The colour contribution of DNA in the RNA reaction was 20% of a similar amount of RNA; appropriate corrections were therefore applied, these being particularly necessary for the nuclear fraction.

Cytochrome c extraction, purification and estimation

(A) *Extraction and purification from unfractionated liver.* The general scheme was based on extraction at pH 4.0 first with water to give cytochrome c (W) and then with 0.15 M-NaCl to give cytochrome c (NaCl). Cytochrome c was purified from the extracts with multiple treatments with Amberlite CG-50 interspersed with gel filtration on Sephadex G-25. Some of the steps were based on the procedures applied to horse heart by Hagihara *et al.* (1958a), Hagihara, Morikawa, Tagawa & Okunuki (1958b), Paléus (1960) and Flatmark (1964b).

(a) Procedure for cytochrome c (W). (1) Extraction. The minced liver from each rat obtained as described for subcellular fractionation was homogenized thoroughly in 1 vol. of ice-cold distilled water with a tight-fitting Potter-type homogenizer. The homogenate was then diluted with 2 vol. of distilled water. For preparative purposes pooled rat liver was used and cell disruption was carried out in 3 vol. of distilled water with a Waring Blender.

All operations until the last step of the purification were carried out at $2-4^{\circ}$.

The pH of the homogenate was adjusted to 4.0 by adding 2.5 N- H_2SO_4 dropwise with continuous stirring. (About 0.05 ml. of acid was required/g. of liver.) The suspension was left for 1 hr. with occasional stirring and was then centrifuged in the MSE centrifuge at 13 000 rev./min. (20 000 g) for 15 min. The clear supernatant was decanted and the pellet rehomogenized with 1 vol. of distilled water. After 15 min. standing the suspension was centrifuged at the same speed for 30 min. The opalescent supernatant was decanted, pooled with the first extract and the pellet stored at -10° . The extract was brought to pH 6.5 by adding 3 N- NH_3 dropwise with continuous stirring. (About 0.03 ml. was required/g. of liver.) The precipitate formed was removed by centrifuging at 13 000 rev./min. (20 000 g) for 30 min. and the clear supernatant decanted. For convenience the pellet was stored at -10° .

(2) Adsorption on Amberlite at pH 7.0. $K_3Fe(CN)_6$ (0.1 M) was added to the supernatant to a final concentration of 0.5 mM in order to oxidize the cytochrome c. Any turbidity was removed by centrifuging at 13 000 rev./min. (20 000 g) for 30 min. The solution was then passed through a 5.0 cm.-high column of Amberlite CG-50, equilibrated with 0.25 M- NH_4^+ buffer, pH 7.0. The volume of the column was one-fourth that of the extract and the flow rate was 2-3 column vol./hr. Most of the reddish-brown pigments were adsorbed on top of the column. A large amount of other proteins and part of the red colour was eliminated by washing the column with 8 column vol. of 0.125 M- NH_4^+ -

* Abbreviations: SNase, succinate-neotetrazolium oxidoreductase system; G6Pase, glucose 6-phosphatase (EC 3.1.3.9); cytochrome c (W), cytochrome c extracted from tissue homogenates in distilled water brought to pH 4.0; cytochrome c (NaCl), cytochrome c extracted from the residues after extraction with distilled water, with 0.15 M-NaCl.

Table 1. *Volumes used for the extraction and preliminary purification of cytochrome c from unfractionated rat liver and its subcellular fractions*

Livers (about 30 g.) were obtained from groups of six rats, pooled and cytochrome *c* was extracted either before or after subcellular fractionation. In the latter case subcellular fractions were obtained from homogenates in 0.3 M-sucrose according to the procedure described in the text. The mitochondrial fraction was separated into several subfractions by sucrose-density-gradient centrifugation.

Fraction	Extracts with distilled water					Extracts with 0.15 M-NaCl			
	Vol. of distilled water for first extraction (ml.)	Vol. of 0.25 N-H ₂ SO ₄ (ml.)	Approx. concn. of protein (mg./ml.)	Vol. of distilled water for second extraction (ml.)	Vol. of 0.3 N-NH ₃ (ml.)	Vol. of 5 cm.-high Amberlite CG-50 column (ml.)		Vol. of 0.15 M-NaCl for extraction (ml.)	Vol. of 5 cm.-high Amberlite CG-50 column (ml.) (pH 7.0)
						(pH 7.0)	(pH 9.0)		
Nuclear	30	2.3†	15	15	1.0†	10	5	10	5
Mitochondrial 1-2	5	0.09	15	3	0.12	3.5	—	3	3.5
Mitochondrial 3	8	0.25	20	4	0.30	3.5	—	5	3.5
Mitochondrial 4	8	0.55	35	4	0.60	3.5	—	5	3.5
Mitochondrial 5	8	0.60	35	4	0.70	3.5	—	5	3.5
Mitochondrial 6-7	5	0.15	15	3	0.20	3.5	—	3	3.5
Mitochondrial 8	220*	0.70†	0.7	10	1.2†	10	—	5	3.5
Heavy microsomal	8	0.90†	25	4	0.5†	3.5	—	3	3.5
Standard microsomal	18	2.5†	35	10	0.7†	5	3.5	5	3.5
Cell sap	330*	8.0†	5	10	0.9†	20	5	5	3.5
Unfractionated liver	90	15.5†	40	30	10.5†	30	15	30	10

* Final volumes of the sucrose-containing supernatants.

† In these cases the solutions were ten times the usual concentration, but volumes are referred to 0.25 N-H₂SO₄ and 0.3 N-NH₃.

0.5 mM- $K_3Fe(CN)_6$ buffer, pH 7.0. The $K_3Fe(CN)_6$ was displaced from the column by 2 column vol. of distilled water and the crude cytochrome *c* fraction eluted with $M-NH_4^+$ buffer, pH 7.0. The eluate was collected only when the reddish-brown bands reached the bottom of the column and the volume collected was 60–80% of the column volume.

Spectrophotometric determinations were made as described below and showed the presence of large amounts of proteins absorbing at 280 m μ and pigments absorbing in the Soret band.

(3) Adsorption on Amberlite at pH 9.0. The NH_4^+ concentration of the eluate was lowered to 0.02 M by gel filtration through a column of Sephadex G-25 equilibrated with 0.02 M- NH_4^+ buffer, pH 7.0 (see below). The eluate containing cytochrome *c* was oxidized with $K_3Fe(CN)_6$ (final concn. 0.5 mM) and passed through a 5.0 cm.-high column of Amberlite CG-50 equilibrated with 0.1 M- NH_4^+ buffer, pH 9.0. Its total volume was about one-half of the column previously used. The reddish-brown bands that ran slightly ahead of and could not be separated from cytochrome *c* at pH 7.0 were now displaced by using approx. 30 column vol. of 0.1 M- NH_4^+ –0.5 mM- $K_3Fe(CN)_6$ buffer, pH 9.0. Elution, spectrophotometric readings, gel filtration and oxidation were done as before, except that elution was performed with $M-NH_4^+$ buffer, pH 9.0.

(4) Chromatography on Amberlite at pH 7.0. The cytochrome *c* was adsorbed on to a 20 cm.-high column of Amberlite CG-50 equilibrated with 0.25 M- NH_4^+ –0.1 mM- $K_3Fe(CN)_6$ buffer, pH 7.0. The total column volume was usually 2–3 ml. for each mg. of cytochrome *c* with a minimum of 7 ml. (0.65 cm. column diam.). Elution was performed with the equilibration buffer at a flow rate of about 1 column vol. every 2 hr. The volume of each fraction collected was about 20% of the column volume and the run was usually completed after 10 column vol. had been eluted (about 20 hr.). Spectrophotometric determinations of the fractions were then performed. The fractions containing the peak of cytochrome *c* were pooled and the total amount and purity

of cytochrome *c* were estimated by spectrophotometry. The solutions were kept either as such at -10° or after reducing their volumes as in step (5).

(5) Final concentration. The NH_4^+ ion concentration was lowered to 0.02 M by gel filtration on a column of Sephadex G-25 and the cytochrome *c* adsorbed on a 1 cm.-high column of Amberlite CG-50 equilibrated with 0.25 M- NH_4^+ buffer, pH 7.0. The column was washed with several column volumes of distilled water and the cytochrome *c* eluted with a small volume of M-ammonium acetate. Elution with this volatile buffer allows the samples to be freeze-dried.

(b) Procedure for cytochrome *c* (NaCl). The residue remaining after extraction with distilled water was pooled with the corresponding precipitate obtained during neutralization of the extracts. The material was resuspended in 1 vol. of ice-cold 0.15 M-NaCl and left standing for 20 min. with occasional stirring. After centrifugation in the MSE centrifuge at 13000 rev./min. (20000g) for 30 min. the supernatant was decanted and diluted sixfold with distilled water, to bring the final Na^+ ion concentration to 0.025 M. Thereafter, the procedure for saline-extracted cytochrome *c* was similar to the one applied for cytochrome *c* (W) except that the volume of the Amberlite column (pH 7.0) was one-third of that used previously and that the step with Amberlite at pH 9.0 was omitted.

(B) *Extraction and purification from subcellular fractions.* Cytochrome *c* (W) and cytochrome *c* (NaCl) were prepared from subcellular fractions derived from a pool representing six livers in the same general way as for unfractionated liver. The precise conditions for each fraction are defined in Table 1. The step with Amberlite at pH 9.0 was omitted except for the preparations from the nuclear fraction and cell sap and, usually, from the microsomal fraction.

(C) *General technique for gel filtration on columns of Sephadex G-25.* The total volume of the column was always four times that of the solution to be desalted, a height/diameter ratio greater than 10 being maintained and the

Table 2. *Reversible adsorption of micro amounts of oxidized cytochrome c on Sephadex G-25 columns*

Sephadex G-25 was washed in batch as described in the Materials and Methods section, except in Expt. 9, where previously unused Sephadex G-25 was washed only with 0.5 M-ammonium acetate before use. Columns were equilibrated and elution was done with ammonium acetate solutions, except in Expt. 7, where aq. NH_3 was used. Chromatographically homogeneous cytochrome *c* from horse heart was used as a standard. Gel filtrations were done with samples containing either $K_3Fe(CN)_6$ or $Na_2S_2O_4$ as described in the text (see Fig. 2). 'Delayed' cytochrome *c* is defined as the small fraction being eluted after the normal completion of elution of proteins (NE_p) but before the start of elution of salts (S_s), being therefore recovered free of salts. Under the present conditions NE_p is approx. $V_0 + 1.25 V_s$, V_0 being the void volume of the Sephadex column and V_s the volume of sample. S_s is approx. $0.85 V_t$, V_t being the total volume of the Sephadex column. 'Reversibly adsorbed' cytochrome *c* is defined as the small fraction being eluted with the salts (after S_s and before $1.50 V_t$).

Expt. no. ...	1	2	3	4	5	6	7	8	9
Sample volume (V_s)	3.1	3.1	10.4	3.1	4.0	3.1	3.1	3.0	3.1
Column volume (V_t)	12.0	12.0	40.0	12.0	39.5	12.0	12.0	11.7	12.0
V_s/V_t ratio	0.26	0.26	0.26	0.27	0.10	0.26	0.26	0.26	0.26
State of oxidation of cytochrome <i>c</i>	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.	Red.	Ox.
Concn. of NH_4^+ in eluent (M)	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02
pH of eluent	7.0	9.3	7.0	7.0	7.0	7.0	11.7	7.0	7.0
Cytochrome <i>c</i> input (μ g.)	143	143	143	1073	171	171	171	150	150
Cytochrome <i>c</i> delayed (μ g.)	11	11	10	34	43	7	8	16	10
Cytochrome <i>c</i> adsorbed (μ g.)	34	35	44	38	29	25	31	7	13

Fig. 1. Typical curve for reversible adsorption of micro amounts of oxidized cytochrome *c* on Sephadex G-25 columns. The values obtained with oxidized cytochrome *c* are shown in Expt. 1 (●) and with reduced cytochrome *c* in Expt. 8 (○), both appearing in Table 2. The theoretical elution predicted for cytochrome *c* in both experiments is represented by □. Each fraction corresponds to 0.92 ml. For other details and definition of NE_p and S_a see Table 2.

minimum dead space being employed. Columns were equilibrated and elutions performed with 0.02 M- NH_4^+ buffer, pH 7.0. Protein was collected in a volume eluting between 40 and 85% of the total column volume (maximum theoretical permissible volume for elution of salt-free protein). Even with these precautions small losses of cytochrome *c* were found that could markedly affect the results for fractions containing only small amounts of the protein. For this reason the behaviour of oxidized and reduced horse heart cytochrome *c* dissolved in M- NH_4^+ buffer, pH 7.0, was studied on columns of Sephadex G-25. The results are shown in Table 2 and Fig. 1. It was clear that to keep the losses of cytochrome *c* to a minimum it was necessary to reduce the protein by the addition of $Na_2S_2O_4$ immediately before gel filtration.

(D) *Spectrophotometric determinations.* Spectrophotometry was performed in micro cells. The exact position of the peaks of the α - and γ -bands in the reduced state was established in each series of determinations by using a solution of horse heart cytochrome *c*. The extinction of samples containing cytochrome *c* was measured at 280 m μ in the oxidized state and at the α - and γ -bands in the reduced state. Readings in the α -band were omitted when they were too low and in general in most of the chromatographic runs. In some eluates from the adsorption on short Amberlite columns the samples were not absolutely clear and so the extinction was determined at 600 m μ in the reduced state, and any excess increasing the value of 0.014 for the $E_{600}(\text{red.})/E_{550}(\text{red.})$ ratio was subtracted from the α - and γ -bands. The extinction coefficient applied for the α - and γ -bands as well as the spectrophotometric ratios were those of pure horse heart cytochrome *c* (Margoliash & Frohwirt, 1959; Margoliash & Lustgarten, 1962*a,b*). Assuming mol.wt. 12363, as determined by Margoliash (1962*a*) from the primary structure, the extinction coefficients for 1 mg./ml. were calculated as 2260 and 10540 respectively, the ratio being 0.215.

(E) *Correction factors in the estimation of cytochrome c.* The concentration of cytochrome *c* was estimated from the spectrophotometric readings on the eluates from Amberlite

treatments [see steps (A)(a)(2) and (A)(a)(3)] at pH 7.0 (or at pH 9.0 when applicable) and in the peaks obtained by chromatography [see step (A)(a)(4)]. The latter was always lower than the former so that the recovery of small amounts (100–150 μ g.) and larger amounts (1 mg.) of horse heart cytochrome *c* during the isolation procedure was determined. The results showed that about 30 μ g. of cytochrome *c* was lost during the isolation procedure irrespective of the amount of protein. In addition it was estimated that about 5% was lost through manipulative procedures. Therefore, for the study of the distribution of cytochrome *c*, values obtained after chromatography were corrected by adding a uniform amount of 30 μ g. plus a further 5% of the total amount thus calculated.

(F) *Study of some protein impurities in crude extracts of cytochrome c.* In certain cases cytochrome *c* from total liver and from nuclear and cell-sap fractions was extracted with distilled water as described above and purified by the $(NH_4)_2SO_4$ -trichloroacetic acid procedure of Keilin & Hartree (1937) instead of adsorption on Amberlite. The red precipitate obtained was partially dissolved in 0.02 M- NH_4^+ buffer, pH 7.0, clarified by centrifuging at 10000*g* for 20 min. and passed through a Sephadex G-25 column under the usual conditions. The solution was then subjected to chromatography on 20 cm.-high columns of Amberlite, as described above. The reddish-brown impurities were separated from cytochrome *c* as a forerunning peak that turned yellow on reduction.

A similar peak was obtained in the chromatography when the Amberlite treatment at pH 9.0 was omitted from the standard procedure. The fractions containing those pigments were pooled and then treated by the $(NH_4)_2SO_4$ -trichloroacetic acid procedure of Keilin & Hartree (1937). Under these conditions an appreciable proportion of the pigments exhibited the same behaviour as cytochrome *c*.

RESULTS

Subcellular fractionation

(A) *General considerations.* The basic requirements of a method to be used in the present study are: (a) good separation of the chosen subcellular components; (b) satisfactory recovery of all the constituents of the disrupted liver cells among the different subcellular fractions; (c) reasonable preservation of the mitochondria; (d) necessity of subfractionation of the mitochondrial fraction. It is unfortunate that methods designed to produce a pellet containing only one subcellular component are in general not suitable for distribution studies. This is because of the poor recovery of the component concerned and the necessity of using separate tissue samples and different procedures for the preparation of each subcellular component. For this reason it was necessary to employ modifications of the now classical fractionation system for the preparation of the four main fractions. Since some degree of cross-examination of each fraction cannot be avoided the composition of each fraction had to be analysed.

As a marker for mitochondria SNase (Slater,

1963) was chosen, since it is a convenient method technically and the distribution of its activity is very similar to that of cytochrome oxidase (Slater & Planterose, 1960a). The activity measured can be identified with succinate dehydrogenase (EC 1.3.99.1) (Slater, 1963). For the membranes of the endoplasmic reticulum G6Pase was chosen as a marker (de Duve *et al.* 1955).

In choosing the medium for cell disruption we were aware that for the preservation of mitochondria it has been usual to employ EDTA (Roodyn, Suttie & Work, 1962). Preliminary experiments confirmed that EDTA had a marked effect on the components of the microsomal fraction (Palade & Siekevitz, 1956), as shown by the low RNA recovery in this fraction and the high concentration of RNA in the cell sap. For these reasons EDTA was excluded. In selecting the concentration of the sucrose, 0.88 M is favoured for the preparation of pure intact mitochondria but would have made difficult the isolation of the other components. Scheider (1959) considered that for the preservation of mitochondria 0.3 M-sucrose was comparable with 0.25 M-sucrose in the presence of EDTA, so that 0.3 M was chosen. According to the same author there is no advantage in buffering the sucrose medium for the fractionation of liver homogenates. Moreover, since the addition of salts was likely to affect the binding of cytochrome *c* to the subcellular components, buffer was omitted from the medium.

For the best preservation of the cell organelles cell disruption under mild conditions, i.e. homogenization with a loose-fitting pestle, is desirable. However, for distribution studies it was essential to effect the disruption of virtually all the cells and in

our hands the use of a homogenizer with a clearance of 0.24–0.36 mm., for 1 min., left a substantial proportion of the cells unbroken. Therefore a tight-fitting pestle with a clearance of 0.10–0.15 mm., for 1½ min., was used. Under these conditions over 90% of the cells were disrupted, on the basis of the distribution of SNRase and G6Pase activity (see Table 3). Even with this treatment an appreciable proportion of the mitochondria and fragments of the endoplasmic reticulum remained in the nuclear fraction. Therefore rehomogenization of the nuclear fraction under the conditions for cell disruption was an essential part of the procedure.

In the separation of the mitochondria from the nuclei in the resuspended nuclear fraction a lower centrifugal force was required than in the original separation, as indicated by Gear (1965) and C. de Duve (personal communication).

For our projected studies on the biosynthesis of cytochrome *c* it was particularly important to obtain a mitochondrial fraction containing but a minimal amount of endoplasmic reticulum and a microsomal fraction virtually free of mitochondria. For this reason an intermediate 'heavy microsomal' fraction was separated.

(B) *Analysis of the major subcellular fractions.* The results of the analysis of the subcellular fractions obtained are given in Table 3.

The estimated contamination of each fraction is expressed as a percentage of the total protein of this fraction being contributed either by mitochondrial protein (on the basis of SNRase estimation) or by microsomal protein (on the basis of G6Pase estimation). The ratios considered were 0.18 unit of SNRase/mg. of mitochondrial protein and 0.50 unit

Table 3. *Analysis of subcellular fractions from rat liver*

Subcellular fractions were obtained from a pool of six liver homogenates in 0.3 M-sucrose, fractionated according to the procedure described in the text and outlined in Scheme 1. Values are averages of duplicate determinations on each of the four pools studied except for SNRase, where only two pools were analysed. The results are expressed for the individual fractions, as ratios or percentage distribution of the total recovered, and for the sum of all fractions as mg. or enzyme units per g. of liver. G6Pase units: μ moles of P_i released/min./g. of liver; SNRase units: E_{510} /min./g. of liver.

Fraction	Protein (%)	RNA (%)	DNA (%)	G6Pase (%)	SNRase (%)	RNA/ protein ratio	DNA/ RNA ratio	Estimated contamination (%)	
								By mito- chondria	By micro- somes
Nuclear	15.6	5.6	80.4	3.3	9.5	0.02	4.0	9	7
Mitochondrial	22.3	10.0	8.9	15.6	80.5	0.02	0.25	—	22
Heavy microsomal	6.6	18.0	2.6	17.5	3.3	0.14	0.04	7	—
Standard microsomal	20.9	55.0	5.1	62.0	5.7	0.13	0.03	4	—
Cell sap	34.7	11.4	3.0	1.4	1.0	0.02	0.07	<1	1
Sum of fractions	167.2 mg.	8.3 mg.	2.4 mg.	26.3 units	4.2 units	0.05	0.28	—	—

of G6Pase/mg. of microsomal protein. They were obtained from the ratios for mitochondrial subfraction 4 (corrected by the estimated contamination by other fractions on the basis of cytochrome *c* values) and the 'standard' microsomal fraction respectively. On the basis of the SNase and G6Pase activity the yield of the mitochondrial and microsomal (heavy + standard) fractions was adequate. Each fraction contained 80% of the total activity of these enzyme markers and the protein content of the fractions was satisfactorily high.

The separation of mitochondria from the nuclear fraction was reasonable since only 10% of the total SNase activity sedimented in the heavier fraction. The high DNA/RNA ratio and the low G6Pase activity of the nuclear fraction shows that most of the fragments of the endoplasmic reticulum were removed from the pellet. As judged by DNA content the mitochondrial fractions contained very few nuclei. This fraction had an appreciable G6Pase activity, but it was calculated that not more than 20% of the protein in the mitochondrial fraction is due to contamination with fragments of the endoplasmic reticulum. Since the mitochondrial pellet was sedimented at 8000*g* for 10 min. and the loose portion of the brown pellet was not removed it almost certainly contains lysosomes and microbodies. The postmitochondrial fractions had a little SNase activity so that they are likely to contain some material of mitochondrial origin.

(C) *Analysis of mitochondrial subfractions.* Bearing in mind the possible heterogeneity of the mitochondria in terms of their biogenesis it was desirable

to attempt their subfractionation. This was particularly attractive in view of our projected studies on the biosynthesis of cytochrome *c*. The difference in age of the mitochondria might well be reflected in differences in their physical properties and for this reason the mitochondrial fraction was subjected to sucrose-density-gradient centrifugation. The results shown in Table 4 indicate that the three central layers (original densities 1.176–1.208) were the richest in mitochondria, containing 73% of the protein and 85% of the SNase activity of the mitochondrial fraction. These three subfractions were also the least contaminated in terms of microsomal protein as indicated by G6Pase activity; the endoplasmic reticulum representing about 16–18% of the protein present. In contrast, microsomal contamination increased in the fractions towards the top of the gradient. Subfraction 8, which resembles a 'fluffy layer' on top of the low-density layers, had a high RNA/protein ratio, indicating substantial microsomal contamination, as confirmed by the high G6Pase activity found in this fraction.

Cytochrome c extraction and purification from rat liver

A method intended for studies on the biosynthesis of cytochrome *c* has three main requirements: (1) purity, to effect the removal of the impurities likely to incorporate radioactive amino acids; (2) quantitative extraction and recovery, to establish the true distribution of radioactivity among the subcellular fractions; (3) suitability for very small

Table 4. *Analysis of mitochondrial subfractions from rat liver*

Mitochondrial subfractions were obtained from the 'crude' mitochondrial fraction either by differential centrifugation in 0.3*M*-sucrose ('heavy' and 'light' subfractions) or by sucrose-density-gradient centrifugations (subfractions 1–8) as described in the text. Values for the 'heavy' subfraction and the 'light' subfraction are duplicates from one subfractionation and in the other cases are averages of duplicate determinations on each of the four pools studied, except for SNase, where only two pools were analysed. The densities expressed with each subfraction are the ones corresponding to the original sucrose layers before equilibration of the gradient. For other references see Table 3.

Differential centrifugation	Protein (%)	RNA (%)	G6Pase (%)	SNase (%)	RNA/protein ratio	Estimated contamination by microsomes (%)
'Heavy'	9.6	11	2	8	0.03	4
'Light'	90.4	89	98	92	0.02	20
Sum of subfractions	36.8 mg.	0.86 mg.	3.4 mg.	3.4 units	0.02	18%
Gradient centrifugation						
1–2 ($\rho=1.281$ and 1.224)	6.1	3.1	6.3	3.2	0.01	21
3 ($\rho=1.208$)	13.4	8.3	11.3	10.3	0.01	18
4 ($\rho=1.192$)	28.8	15.9	21.2	40.2	0.01	16
5 ($\rho=1.176$)	29.2	19.5	22.4	34.2	0.02	17
6–7 ($\rho=1.161$ and 1.038)	8.5	9.4	12.9	12.1	0.02	33
8 (fluffy layer)	13.9	43.8	25.6	—	0.07	41
Sum of subfractions	37.2 mg.	0.83 mg.	4.1 mg.	3.4 units	0.02	22%

amounts of cytochrome *c*, the recovery of which may not be important in terms of quantity but which may have a significant effect on calculations of specific radioactivity.

To satisfy the requirement of purity it is necessary to use a chromatographic procedure for the final purification of cytochrome *c* from the tissue extracts (see review by Margoliash & Schejter, 1966). Besides, for a quantitative recovery of cytochrome *c* from tissue extracts a procedure involving ion-exchange adsorption (Hagihara *et al.* 1958*a,b*; Paléus, 1960; Flatmark, 1964*a*) is preferred to the usual methods based on a salt precipitation. The use of these improved methods of purification meant that denaturation of the protein had to be avoided because the latter would lead to chromatographic heterogeneity. Denaturation may be produced by trichloroacetic acid and low pH, two steps commonly used in the older procedures. Thus the trichloroacetic acid used in the original method of Keilin & Hartree (1937) has been shown by Margoliash (1954*b*) and Margoliash & Lustgarten (1962*a,b*) to result in the formation of polymers that bind tightly to ion-exchange resins. The low pH values, such as those used in the procedure of Rosenthal & Drabkin (1943), cause deamidation (Margoliash, 1962*b*; Margoliash & Schejter, 1966), which decreases the firmness of binding to cation-exchange resins and may even prevent the adsorption of cytochrome *c*.

(1) *Extraction*. The above criteria for purification would have been met by extracting the tissue with sodium chloride solutions at neutral pH, since this procedure yields native cytochrome *c* (see Okunuki, 1961). However, even when the salt

extraction is preceded by hypo-osmotic treatment (Jacobs & Sanadi, 1960) a quantitative recovery of cytochrome *c* from some mitochondrial particles is not possible (Widmer & Crane, 1958; Lenaz & MacLennan, 1966), so that the method was not suitable in the present case. Resort had to be made to extraction at an acid pH that, though low enough to effect quantitative extraction from tissues, was not so low as to cause denaturation. Flatmark (1964*a,b*) has shown that pH 4.0 is suitable for this purpose. That the recovery of cytochrome *c* from liver treated with distilled water at pH 4.0 is satisfactory is shown in Table 5. Only a further 6% of cytochrome *c* was extracted from the residue by 0.15 M-sodium chloride at pH 4.0. This distribution between distilled water at pH 4.0 and 0.15 M-sodium chloride at pH 4.0 does not hold for extraction from subcellular fractions (see below).

(2) *Adsorption of cytochrome c on Amberlite at pH 7.0*. For the adsorption of cytochrome *c* on this resin to be efficient the cation concentration of the eluate must be less than 0.03 M at pH 7.0 (Margoliash, 1954*a*) or 0.3 M at pH 6.3 (Hagihara *et al.* 1958*b*), and in all cases the cytochrome *c* must be fully oxidized (Hagihara *et al.* 1958*b*). For these reasons the tissue extracts in 0.15 M-sodium chloride, pH 4.0, had to be diluted, and both the sodium chloride and water extracts had to be neutralized and oxidized with potassium ferricyanide. The optimum pH, both for eluate and resin, for a quantitative adsorption of cytochrome *c* with a minimal contamination of pigments and impurities was studied. As a result the resin was equilibrated at pH 7.0 and the eluate adjusted to pH 6.5. Under these conditions adsorption of pigments (including haemoglobin) is de-

Table 5. *Distribution of cytochrome c among rat liver subcellular fractions*

Cytochrome *c* was extracted from the homogenates of the total liver and subcellular fractions brought to pH 4.0, first with water [cytochrome *c* (W)] and then with 0.15 M-NaCl [cytochrome *c* (NaCl)]. Values are the averages of independent determinations on four pools of six rat livers each (subcellular fractions) or, separately, on five different livers (unfractionated liver). Estimations were done either before (*B*) or after (*A*) chromatography and corrected as described in the text. The *A* values were used to calculate the percentage of total, percentage of sum of fractions and $\mu\text{g./mg.}$ of protein, except in some cases where the estimations after chromatography were not done and had to be replaced by the *B* values (*).

	Cytochrome <i>c</i> (W)		Cytochrome <i>c</i> (NaCl)			Total cytochrome <i>c</i> (W + NaCl)						
	$(\mu\text{g./g. of liver})$		$\mu\text{g./g. of liver}$		(% of total)	$(\mu\text{g./g. of liver})$		(% of sum of fractions)	$(\mu\text{g./mg. of protein})$			
	(B)	(A)	(B)	(A)		(B)	(A)					
Nuclear	28.2	25.6	2.5	2.3	8	30.7	27.9	24.4	1.07			
Mitochondrial	52.6	48.5	17.7	17.0	26	70.3	65.5	57.2	1.76			
Heavy microsomal	6.3	5.2	0.7	0.7*	12	7.0	5.9*	5.2	0.54			
Standard microsomal	10.9	8.6	3.3	3.5	29	14.2	12.1	10.6	0.35			
Cell sap	3.4	3.1	0.6	—	—	4.0	3.1	2.7	0.05			
Sum of fractions	101.4	91.0	24.8	23.5	21	126.2	114.5	100.0	0.68			
Unfractionated liver	123.8	115.8	7.5	7.5*	6	130.5	123.3*	—	0.73			

creased (as compared with the resin at pH 6.5) and the lower pH of the eluate is a precaution against the high protein concentration causing a release of NH_4^+ , leading to a rise in pH.

When, after adsorption, 0.125 M- NH_4^+ -0.5 mM-potassium ferricyanide buffer, pH 7.0, was passed through the Amberlite column most of the red pigment was displaced. A reddish-brown band separated partly from the band containing cytochrome *c*, but the two bands were eluted together when the column was treated with M- NH_4^+ buffer, pH 7.0. The presence of these pigments prevented the accurate estimation of the amount of cytochrome *c* in the eluate because of the high absorption of the impurities at 416 and 550 m μ .

(3) *Gel filtration with Sephadex G-25.* With fully oxidized cytochrome *c* it was observed that as a result of gel filtration there was a constant loss of protein amounting to about 40 $\mu\text{g.}$ of cytochrome *c*. Various efforts were made to overcome this loss, which would be serious in the extraction of small amounts of cytochrome *c* subjected to several gel filtrations. Changes involving ionic strength, pH and column size failed, but on reduction of the cytochrome *c* with sodium dithionite the losses were decreased to 5 $\mu\text{g.}$ (Table 2 and Fig. 1). The loss with oxidized protein may be due partially to a change in the properties of Sephadex under the rather drastic conditions employed for its washing (compare Expts. 7, 8 and 9 in Table 2). The change in the adsorption properties of cytochrome *c* on reduction may be explained as an effect resulting from the change of protein conformation that occurs during the transition from the oxidized to the reduced state (see Margoliash & Schejter, 1966). The effect of 'burying' the ferroporphyrin ring and the decrease in the net positive charge of the protein on reduction diminish the adsorption effects attributable to the interaction of ionic groups and heterocyclic rings with Sephadex (Gelotte, 1960).

(4) *Adsorption of cytochrome c on Amberlite at pH 9.0 and chromatography at pH 7.0.* When the eluates from Amberlite columns at pH 7.0 were chromatographed on 20 cm. columns of Amberlite equilibrated at pH 7.0 with 0.25 M- NH_4^+ buffer as eluent, a substantial peak of reddish-brown pigments appeared in the first fractions collected. The elution of cytochrome *c* itself was greatly advanced in comparison with samples either of pure rat liver or horse heart cytochrome *c* (Fig. 2). This effect may be ascribed to the presence of other proteins, which decreased the availability of the free carboxyl groups of the resin for cytochrome *c*. As a result the peak containing cytochrome *c* was not always completely separated from the protein impurities so that the $E_{550}(\text{red.})/E_{280}(\text{ox.})$ ratio was often below the minimum acceptable value of 1.18.

The $E_{280}(\text{ox.})/E_{416}(\text{red.})$ ratio for pure rat liver

Fig. 2. Chromatography of cytochrome *c*-containing fractions extracted from unfractionated liver at pH 4.0 with distilled water. Cytochrome *c* (W) was obtained from two livers (total wt. 10.0 g.) homogenized in distilled water and brought to pH 4.0. The isolation and preliminary purification of cytochrome *c* was done as described in the text, except that the eluate obtained from the Amberlite column at pH 7.0 was divided into two equal volumes. One (a) was used directly for chromatography, the adsorption on an Amberlite column at pH 9.0 being omitted, and the other (b) was purified including this step before chromatography. The chromatographic run was done as described in the text on a column (20 cm. $\times$ 0.67 cm.; 7.2 ml. total volume) of Amberlite CG-50, equilibrated at pH 7.0. Elution was performed with 0.25 M- NH_4^+ -0.1 mM- $\text{K}_3\text{Fe}(\text{CN})_6$ buffer, pH 7.0, and 1.3 ml. fractions were collected. Spectrophotometric determinations were done, after chromatography, with micro cells of 1 cm. light-path at 280 m μ in the oxidized state (○) and at 416 m μ in the reduced state (●).

cytochrome *c* [chromatographed and then treated with ammonium sulphate and trichloroacetic acid as in the Keilin & Hartree (1937) procedure] was established independently to be 0.165. In sample (b) the ratio obtained was 0.168 and the amount calculated from the extinctions at 416 m μ was 540 $\mu\text{g.}$, equivalent to 114 $\mu\text{g./g.}$ of fresh liver. In sample (a) the ratio obtained was much higher (0.278) and the amount of cytochrome *c* calculated from the extinction at 416 m μ was abnormally high (total of 658 $\mu\text{g.}$, equivalent to 138 $\mu\text{g./g.}$ of fresh liver) owing to the incomplete removal of other pigments absorbing at 416 m μ . The advance of the elution of cytochrome *c* in Fig. 2(a) as compared with Fig. 2(b) may be noted.

The material contained in the reddish-brown peak turned yellow on reduction. Under the conditions of the Keilin & Hartree (1937) procedure it resembled cytochrome *c* in that it remained soluble in 80%-saturated ammonium sulphate, was precipitated with trichloroacetic acid and partially dissolved in distilled water. In considering ways of eliminating the impurities before the cytochrome *c* extract was chromatographed on Amberlite at pH 7.0, the

ammonium sulphate-trichloroacetic acid procedure was therefore clearly unsuitable. For this reason a new step involving adsorption on Amberlite at pH 9.0 was introduced. Since direct washing of the column, previously equilibrated at pH 7.0, with 0.1 M-NH₄⁺ buffer, pH 9.0, was slow and inefficient a fresh column was used. The use of the column equilibrated at pH 9.0 and washed with the buffer of the same pH allowed cytochrome *c* to be eluted free from other pigments. The amount of cytochrome *c* in the eluent can be accurately determined by spectrophotometry even though there are still impurities absorbing at 280 mμ. Such impurities are removed by the subsequent chromatography on Amberlite at pH 7.0. The purity of the fractions containing cytochrome *c* is quite uniform and the $E_{550}(\text{red.})/E_{280}(\text{ox.})$ ratio is about 1.25–1.30. The average yield after chromatography was 116 μg. of cytochrome *c* (W)/g. of fresh liver (five experiments). For cytochrome *c* (NaCl) the yield obtained after adsorption on the short Amberlite column at pH 7.0 was an average of 7 μg./g. of fresh liver (five experiments). Since no other pigment was seen on the columns at pH 7.0 these are considered to be accurate determinations of the content of cytochrome *c* (NaCl). The total cytochrome *c* obtained was therefore an average of 123 μg./g. of fresh liver.

Cytochrome c extraction and purification from subcellular fractions

In the application of the method described for whole liver to subcellular fractions the volume of distilled water used in the first extraction of the pellets was such as to give a concentration of 15–35 mg. of total protein/ml. In this way similar conditions were ensured for extraction from all fractions.

The purification scheme just described was modified by omitting the adsorption step on Amberlite at pH 9.0 for all the mitochondrial subfractions, the

heavy microsomal fraction and in some cases the standard microsomal fraction. This was possible because the pigments affecting the chromatographic purification of cytochrome *c* from whole liver were only present in the nuclear fraction, the cell sap and often in the standard microsomal fraction.

In the chromatographic fractionation on Amberlite columns at pH 7.0 the elution volume of cytochrome *c* was essentially uniform in all cases and was about seven times the total volume of the column. The only exception was the nuclear fraction [for both cytochrome *c* (W) and cytochrome *c* (NaCl)], where the peak started at a volume of about 70–80% of the usual elution volume. When the cytochrome *c* was rechromatographed it behaved normally, so that this increase in mobility must be attributed to the presence of the impurities in the crude fraction. The results obtained appear in Tables 5 and 6, in which the cytochrome *c* estimations are given before and after chromatography.

DISCUSSION

It is proposed to confine this discussion to two main points: first, the extent to which the method described fulfils the aims as expressed in the introduction; secondly, the significance of the determined distribution of cytochrome *c* among the subcellular fractions.

The aim of the method was that it should provide satisfactory purity, a quantitative recovery and applicability to small quantities. The purity of the cytochrome *c* obtained from whole liver in our procedure can only be compared with preparations from horse heart or other mammalian organs, since no data are available for pure cytochrome *c* from rat tissues. So far as the $E_{550}(\text{red.})/E_{280}(\text{ox.})$ ratio is concerned, the determined values of 1.25–1.30 are higher than those reported by Margoliash &

Table 6. *Distribution of cytochrome c among mitochondrial subfractions from rat liver*

For details see Tables 4 and 5.

Fraction	Cytochrome <i>c</i> (W)		Cytochrome <i>c</i> (NaCl)			Total cytochrome <i>c</i> (W + NaCl)			
	(μg./g. of liver)		(μg./g. of liver)		(% of total)	(μg./g. of liver)		(% of sum of fractions)	(μg./mg. of protein)
	(B)	(A)	(B)	(A)		(B)	(A)		
Mitochondrial 1–2	3.3	3.4*	1.3	1.3*	28	4.6	4.7*	7.2	2.06
Mitochondrial 3	8.2	7.4	1.8	1.6	18	10.0	9.0	13.7	1.80
Mitochondrial 4	16.6	15.0	5.1	4.9	25	21.7	19.9	30.4	1.85
Mitochondrial 5	17.2	15.7	5.4	5.0	24	22.6	20.7	31.6	1.91
Mitochondrial 6–7	5.8	5.5	2.3	2.4*	30	8.1	7.9*	12.1	2.49
Mitochondrial 8	1.5	1.5*	1.8	1.8*	55	3.3	3.3*	5.0	0.96
Sum of subfractions	52.6	48.5	17.7	17.0	26	70.3	65.5	100.0	1.76

Frohwirt (1959) for horse heart cytochrome *c* and are comparable with the highest in the literature for any mammalian cytochrome *c* (see Margoliash & Schejter, 1966).

With reference to quantitative recovery two objectives were before us. First, to ensure that the sample of cytochrome *c* handled shall be truly representative of the total pool of this protein in the tissue extract, it was necessary for the initial extraction to be virtually quantitative. We are satisfied that the procedure, whereby the extraction is first done with distilled water at pH 4.0 and is then followed by 0.15M-sodium chloride at pH 4.0, fulfils the objective. The attachment of cytochrome *c* to tissue constituents is essentially ionic, so that even an association of this protein with phospholipid is disrupted by sodium chloride at a concentration higher than 0.1M (Das, Hiratsuka, Machinist & Crane, 1962; Das & Crane, 1964). Secondly, the procedure for the purification of cytochrome *c* from the extracts should be highly reproducible. This is because it was realized that such a method could never be absolutely quantitative for the minor fractions, so that it was necessary to apply correction factors if the true distribution of cytochrome *c* among the subcellular fractions was to be established. Control experiments with samples of pure cytochrome *c* and the repeated application of the procedure to tissue extracts have satisfied us on this point.

The cytochrome *c* content of unfractionated rat liver obtained by the present procedure (123 $\mu\text{g./g.}$ of fresh liver) is higher than that (113 $\mu\text{g./g.}$ of fresh liver) obtained by Dianzani & Viti (1955), who used enzymic determinations with cytochrome oxidase. Since these authors have demonstrated that techniques involving the precipitation of cytochrome *c* with ammonium sulphate at 56° result in substantial losses of cytochrome *c* it seems pointless to compare our results with those obtained by such methods (Prader & Gonella, 1947; Rosenthal & Drabkin, 1943).

The adaptation of our method to the extraction and purification of cytochrome *c* from subcellular fractions demonstrates its flexibility and suitability for extracts containing only small quantities of cytochrome *c*. The first conclusion to be drawn from the results is the existence of two pools of cytochrome *c* differing in their firmness of binding to the subcellular fractions, i.e. cytochrome *c* (W) and cytochrome *c* (NaCl). Since cytochrome *c* is a protein with a strong net positive charge at physiological pH, it is assumed that it is bound ionically to the structures within the cell. The effect of lowering the pH to 4.0 on its extraction with distilled water is probably twofold; first, there is the decrease of the ionization of the acidic groups in the subcellular components binding cytochrome *c*;

secondly, there is the osmotic 'shock' suffered by the particles homogenized in a medium of low ionic strength. The latter may play a role in rendering these sites accessible to the action of the low pH and allowing the free passage of the released cytochrome *c*. The existence of a small pool of cytochrome *c* (NaCl) may be ascribed to the linkage of the protein to different groups that are still active at pH 4.0 and from which the cytochrome *c* is only displaced by metal cations. In unfractionated liver cytochrome *c* (NaCl) accounted for only 6% of the total, whereas in most of the subcellular extracts the comparable value was 20–25%. This is probably due to the presence of a higher concentration of cations in the cell sap of the total liver homogenate.

An alternative explanation might be that cytochrome *c* (W) and cytochrome *c* (NaCl) differ in their conformation. It would only be necessary for the difference in conformation to resemble that between the reduced and oxidized protein for the degree of binding to acidic groups to be affected. Since cytochrome *c* (W) and cytochrome *c* (NaCl) as isolated had identical chromatographic properties this theory would only be possible if the conformational changes were reversible. Other than this we have no evidence on this point.

In the present study of the distribution of cytochrome *c* among the subcellular fractions, 57% of the total was found in the mitochondrial fraction. This is higher than the value reported by Dianzani & Viti (1955), but lower than that calculated from the results obtained by Beinert (1951). Table 7 is a summary of the results obtained by various authors. Our value of 1.8 $\mu\text{g.}$ of cytochrome *c*/mg. of total protein in the mitochondrial fraction (subfractions 1–8) is lower than that of other authors (about 2.6) working with a specially purified mitochondrial preparation (see Table 8). The difference in the values would mean that our mitochondrial fraction contained 30% of non-mitochondrial protein. (For the reason already given it was not possible for us to work with a homogeneous mitochondrial fraction.) From the G6Pase activity about 20% of the protein in the mitochondrial fraction is of microsomal origin, so that the other 10% of contamination may be due to lysosomes and microbodies. The cytochrome *c* concentration with respect to the total protein increases to a value of 2.5 $\mu\text{g./mg.}$ of total protein in one of the subfractions (6–7) when the mitochondria are subfractionated according to density. This is no doubt due to the removal of extramitochondrial proteins from the cytochrome *c*-rich fractions; it is noteworthy that there is a low cytochrome *c* content in subfraction 8.

There is a difference in the firmness of binding of cytochrome *c* among the subfractions. Thus in subfraction 8 the amount of cytochrome *c* (NaCl) was on average 55% of the total, this being twice

Table 7. *Comparison of some values in the literature for cytochrome c in total liver and its subcellular fractions from adult rats*

Reference	Reference nos. for methods		Cytochrome c in total liver ($\mu\text{g./g. of liver}$)		Cytochrome c in subcellular fractions [% of unfractionated homogenate (reference 2) or of total sum (other references)]			
	Sub-cellular fractionation	Cytochrome c estimation	Unfractionated liver	Sum of fractions	Nuclear	Mitochondrial	Microsomal	Cell sap
(1) Rosenthal & Drabkin (1943)	—	(1)	183*	—	—	—	—	—
(2) Schneider & Hogeboom (1950)	(2)†	(1)	213	137	10	52	2	?
(3) Beinert (1951)†	(2)†	(1)	133	125	9.9	65.1	6.3	18.7
(4) Dianzani & Viti (1955)	(7)§	(8)§	113	107.5	24.9	48.3	15.6	11.2
(5) Dallman & Schwartz (1964)	—	(1)	70	—	—	—	—	—
(6) Present paper	Present methods		123	114.5	24.4	57.2	15.8	2.7

* Wide variation of values, ranging from 80 to 261 in different rats.

† Calculated from the original data.

‡ With 0.25 M-sucrose in the fractionation.

§ Other references: (7) Schneider (1948); (8) Potter & Dubois (1942).

Table 8. *Comparison of some literature values for cytochrome c in specially purified mitochondrial fractions*

Values are expressed in $\mu\text{g. of cytochrome c/mg. of mitochondrial proteins}$. In the present paper the average of all the mitochondrial subfractions, except for subfraction 8 (grossly contaminated by endoplasmic reticulum), has been taken as a representative value for the total mitochondrial fraction (not specially purified). In the papers quoted values have been calculated from the original data. Cytochrome c is considered to have mol.wt. 12363 (Margoliash, 1962b), so that $1 \text{ m}\mu\text{mole} = 12.36 \mu\text{g.}$ LTS, Low-temperature spectroscopy on mitochondrial suspension or fractions; E, spectrophotometric determinations on cytochrome c extracts. TM, Total mitochondria; MS, mitochondrial subfraction; SMP, submitochondrial particles.

Reference	Technique for cytochrome c estimation	Total mitochondria	Maximum value obtained
(1) Jacobs & Sanadi (1960)	E	2.10	2.64 (TM)
(2) Estabrook & Holowinski (1961)	LTS	2.47	2.84 (TM)
(3) Roodyn <i>et al.</i> (1962)	E	1.89	3.10 (TM)
(4) Chance, Parson & Williams (1964)	LTS	2.60	4.33 (SMP)
(5) Gear (1965)	E	2.24	2.35 (MS)
(6) This paper	E	1.95	2.49 (MS)

the value for the sum of the subfractions. Since subfraction 8 consisted of the pooled 'fluffy layers' from each of the subfractions and had a low density, it probably consists of fragments of mitochondria and the endoplasmic reticulum in a rather variable proportion, since the assays performed gave different results in each subfraction. In one case where the amount of total protein was rather high the cytochrome c (NaCl) represented 90% of the total.

The fact that 40% of the cytochrome c in liver was found in fractions other than the mitochondrial

calls for a discussion of the physiological significance of this finding. The first point is to consider the degree of contamination of the fractions by intact or fragmented mitochondria. The distribution of SNase activity shows that 20% of the total was not sedimented in the mitochondrial fraction. This finding must be interpreted according to the particular fraction concerned. For the nuclear fraction SNase activity probably indicates the presence of trapped large or heavy mitochondria; for the heavy microsomal fraction it might be due to the presence of small or light mitochondria and

fragments of these; and in the standard microsomal fraction mitochondrial fragments are the most likely contaminant.

On the assumption that the cytochrome *c*/SNRase ratio remains constant in all the mitochondria-containing components (approx. 20 μ g. of cytochrome *c*/unit of SNRase) we calculate the contribution of mitochondrial cytochrome *c* to the nuclear fraction to be about 30% of the cytochrome *c* found in this fraction. Conover & Siebert (1965) have shown that purified nuclei contain about 0.35 μ g. of cytochrome *c*/mg. of nuclear protein. Since cytochromes *a*, *a*₃ and *b* were not present in the preparation the presence of cytochrome *c* could not have been due to mitochondrial contamination. We calculate from these results that the contribution of the true nuclear cytochrome *c* would represent about 3% of the total cytochrome *c* in the liver or 13% of the cytochrome *c* estimated in the nuclear fraction. No physiological role for this cytochrome *c* has yet been reported.

So far as the microsomal fraction is concerned the SNRase activity suggests that approx. 40% of the cytochrome *c* present in the two microsomal fractions might be accounted for by contamination with mitochondrial components.

Enzymic and other biochemical assays have shown the presence in the microsomal fraction of 'light' vesicles, named by some authors 'electron-transferring vesicles' (Imai, Ito & Sato, 1966), which contain enzymes and haemoproteins concerned with electron-transfer reactions (see also Dallner, Siekevitz & Palade, 1966*a,b*). Although a role for cytochrome *c* in these vesicles has not yet been postulated it might well intervene in electron-transfer reactions concerned with hydroxylation and other detoxicating activities of the rough endoplasmic reticulum. As shown in the following paper (González-Cadavid & Campbell, 1967*b*) a component of the microsomal fraction is also probably responsible for the synthesis of cytochrome *c*, so that this fraction would then also be expected to contain *in vivo* small quantities of the protein. Apart from the endogenous cytochrome *c* present in extramitochondrial sites and the fraction contributed by mitochondrial contamination, it is likely that a certain proportion arises from redistribution of the original mitochondrial cytochrome *c*.

Schneider & Hogeboom (1950) explained the presence of extramitochondrial cytochrome *c* on the basis that, being soluble, it was released from the mitochondria during homogenization and was then bound by the acidic groups present in the other subcellular fractions. Beinert (1951) put this explanation to the test by adding labelled cytochrome *c* to rat liver homogenates. We now realize that these experiments could be criticized on the basis that the cytochrome *c* was probably partially

denatured and impure and that the cell sap was not satisfactorily separated from the microsomal fraction. Further, we cannot utilize the observations of Schwartz & Petermann (1966) on the binding of cytochrome *c* by isolated ribosomes or the studies on the re-entry of cytochrome *c* from depleted mitochondria (Jacobs & Sanadi, 1960), since the ionic environment and availability of soluble cytochrome *c* differ markedly from our conditions.

An essential requirement for a redistribution is the leakage of cytochrome *c* from the mitochondria, for it is accepted that the subcellular organelles will bind a certain amount of the cytochrome *c* present in the cell sap. Moreover, even in this case the proportion of binding might be different within each extramitochondrial localization according to the sites available for cytochrome *c* linkage. Therefore cytochrome *c* (W) and cytochrome *c* (NaCl) will not necessarily contain the same proportion of cytochrome *c* derived from the mitochondria.

In the present studies the concentration of cytochrome *c* remaining in the cell sap was much less than that previously reported. Even so, it is not certain that this cytochrome *c* pool is truly soluble, since the cell sap is known to contain particulate components.

N.F.G.-C. acknowledges the receipt of research fellowships from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina and from the Wellcome Trust. The work was made possible by a generous grant to The Middlesex Hospital Medical School by the British Empire Cancer Campaign.

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