Biochem. J. (1969) **115**, 849
Printed in Great Britain

Some Properties of Cytochrome b_5 from Liver Microsomes of Man, Monkey, Pig and Chicken

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(*Received* 22 *May* 1969)

1. Cytochrome b_5 was released from liver microsomes of man, monkey, pig and chicken by incubation with a crude lipase preparation. 2. By using DEAE-cellulose chromatography, ammonium sulphate fractionation, Sephadex-gel filtration and a final gradient elution on DEAE-Sephadex A-50, cytochromes b_5 were obtained from the four species studied, all possessing similar spectral properties. 3. Stokes radii of the cytochromes were measured by gel filtration. 4. *N*-Terminal amino acids for the different cytochromes were serine for man and monkey, alanine for pig and glycine for chicken. 5. Amino acid analyses of the cytochromes are presented. 6. Peptide 'fingerprint' patterns of tryptic digests of the different cytochromes are discussed and clearly show increasing similarity for more closely related species.

Cytochrome b_5, a haemoprotein present in microsomes of a wide variety of tissues (Strittmatter, 1963), appears to be an intrinsic component of the endoplasmic reticulum of the cell. Raw & Mahler (1959) identified a pigment similar to cytochrome b_5 in pig mitochondrial fraction. Sottocasa, Kuilenstierna, Ernster & Bergstrand (1967) localized this cytochrome b_5-like pigment in the outer membrane of mitochondria. The cytochrome b_5 from liver microsomes was isolated in a pure state from the calf (Strittmatter & Velick 1956; Strittmatter & Ozols, 1966), the rabbit (Kajihara & Hagihara, 1967) and the rat (Sargent & Vadlamudi, 1968).

The cytochrome b_5 from pig liver was isolated by a number of workers (Garfinkel, 1957*a*,*b*; Raw, Molinari, Amaral & Mahler, 1958; Poltoratsky-Bois & Chaix, 1964) without a more complete evaluation of the purity of the protein so obtained. Interest then shifted to the determination of the primary structure of this cytochrome, and Ozols & Strittmatter (1968*a*) did this with the calf liver protein. In addition the same authors attempted to compare this structure with that of other cytochromes (Strittmatter & Ozols, 1967).

In the present work a method was devised for the purification of cytochrome b_5 from liver microsomes of man, monkey, pig and chicken.

Some properties of the purified cytochromes were studied and their 'fingerprints', *N*-terminal amino acids and amino acid composition were determined.

MATERIALS AND METHODS

Purification procedure. Livers. Large pieces of human livers were collected in ice at the autopsy room, Department of Pathology, Faculty of Medicine, São Paulo. Only healthy and relatively recent samples (7–12hr. after death) were collected and frozen at −20°. Monkeys (*Alouatta fusca*) were hunted at Horto Florestal Reserve, São Paulo. Their livers were immediately chilled at 0° and stored at −20°. Pig and chicken livers were obtained from the slaughterhouse, chilled immediately and processed in the same day.

Step 1: isolation of microsomes. All operations were carried out at 4° unless otherwise stated. Livers were ground in a meat grinder. The resulting paste was homogenized in batches (each with 2vol. of 0·25M-sucrose–1mM-EDTA, pH7·4) in a Waring Blendor at full speed for 1min. The suspension was filtered through four thicknesses of gauze and centrifuged at $9000g_{av.}$ for 5min. and the resulting supernatant was adjusted to pH5·3 with 2M-acetic acid added dropwise with constant stirring. Microsomes were collected by centrifuging this suspension for 10min. at $15000g_{av.}$ and resuspended in 0·01M-tris–HCl buffer, pH8·5, to give a protein concentration of 20–50mg./ml. With human livers, after the precipitation of nuclei and mitochondria, the resulting suspension was directly used as microsomal preparation. EDTA was added to a final concentration of 2mM and the pH was adjusted to 7·6 with M-NaOH.

Step 2: solubilization of cytochrome b_5 from microsomes. Crude lipase was added to the microsomal suspension (100mg./g. of microsomal protein). Pig and chicken preparations were kept at 30° for 1hr., then chilled rapidly to 4–5° and left overnight in the cold-room. Man and monkey preparations after addition of lipase were left overnight

(14–15hr.) at 4° in the cold-room. Next day the suspension was adjusted to pH5·3 with 5M-HCl and centrifuged for 15min. at 14000$g_{av.}$. The supernatant was filtered through coarse paper and adjusted to pH7·5 with 6M-NaOH. Chicken preparations were treated with Celite (5g./l.) before acidification. This procedure eliminates turbidity of the remaining supernatant. The supernatants from human preparations before filtration were sometimes shaken with toluene (10%, v/v), if cloudy and contaminated with lipid-rich floating pellets.

Step 3: isolation of cytochrome b_5 from supernatants after lipase treatment. The supernatants were chromatographed on DEAE-cellulose, 4l. being applied to 4·5cm. × 30cm. columns equilibrated with 0·05M-tris–HCl buffer, pH7·5. Haemoglobin and some contaminants were immediately separated from the cytochrome, which remains adsorbed on the column. The adsorbent was washed with 2vol. of the same buffer and then with 2vol. of this buffer containing 100mM-NaCl, eluting several contaminants. Cytochrome b_5 was eluted with 4 column volumes of the starting buffer made 150mM with respect of NaCl.

Step 4: first $(NH_4)_2SO_4$ fractionation. The cytochrome solution was brought to 60% saturation with $(NH_4)_2SO_4$ at pH8·0 for the human and monkey preparations. Chicken and pig cytochrome eluates were adjusted to 70% saturation with $(NH_4)_2SO_4$ at pH8·0. The resulting suspension was centrifuged at 15000$g_{av.}$ for 10min. and the precipitate was discarded. Supernatants were 100% saturated with $(NH_4)_2SO_4$ and the pH was adjusted to 4·2. The suspension was centrifuged as above and the pellet, containing cytochrome b_5, was suspended in the minimum volume of 0·1M-tris–HCl buffer, pH9·0, and dialysed against several changes of water adjusted to pH7–8 with NH_4HCO_3. Dialysis tubing was boiled for 30min. in 0·1M-NH_4HCO_3 before use.

Step 5: gel filtration on Sephadex G-50. The concentrated cytochrome solution (20–35ml.) was passed through a column (3·6cm. × 62cm.) of Sephadex G-50 equilibrated with 0·02M-sodium phosphate buffer, pH7·5, at room temperature. Cytochrome b_5 was eluted as a red band after a fast-moving yellow-brown band, which was discarded. The cytochrome b_5 content of the eluate was measured and pure trypsin, equal to 5% (w/w) of total cytochrome and dissolved in the same phosphate buffer containing 1mM-$CaCl_2$, was added. The solution was kept at 25° for 2hr. as described by Strittmatter & Ozols (1966) to obtain from the native cytochrome preparations only the trypsin-resistant portion.

Step 6: second $(NH_4)_2SO_4$ fractionation. The cytochrome solution was cooled to 0° and enough $(NH_4)_2SO_4$ added to obtain 80%, 80%, 75% and 80% saturation with the cytochromes from man, monkey, pig and chicken respectively. The pH was adjusted to 8·0 and the precipitate collected by centrifuging for 10min. at 15000$g_{av.}$ was discarded. The resulting suspension was brought to 95% saturation with the same salt and the pH was adjusted to 4·2. The precipitate was collected as described above and suspended in the minimum volume of 0·1M-tris–HCl buffer, pH9·0, and dialysed under the same conditions as stated above.

Step 7: gel filtration on Sephadex G-75. A 5–10ml. portion of the product of step 6 was applied to a column (3cm. × 45cm.) of Sephadex G-75 equilibrated with 0·05M-tris–HCl buffer, pH7·7, containing 0·5M-NaCl, and developed with the same buffer at room temperature.

Step 8: DEAE-Sephadex chromatography. The solution containing the eluted cytochrome band from step 7 was diluted to 0·09M-NaCl with 0·05M-tris–HCl buffer, pH7·7, and applied to a DEAE-Sephadex A-50 column (2·5cm. × 30cm.) previously equilibrated with the same buffer containing 0·09M-NaCl. The column was developed with a shallow concave gradient obtained by mixing the above tris buffer containing 0·09M-NaCl with the same buffer containing 0·25M-NaCl. The concentrated-buffer reservoir was an Erlenmeyer flask containing 1350ml. of buffer and the mixing chamber a balloon with 940ml. of the tris buffer containing 0·09M-NaCl. Reservoirs were connected by a siphon and the cytochrome was eluted at room temperature. Fractions (5–6ml.) were collected at 80–83ml./hr. The fractions containing the cytochrome were concentrated by $(NH_4)_2SO_4$ precipitation and dialysed against water or filtered through a Sephadex G-25 column to remove salt and stored at −20° in solution or after freeze-drying.

Determination of cytochrome b_5 *and protein.* Cytochrome b_5 was determined quantitatively from the spectrum of the dithionite-reduced compound recorded in a Cary 14 spectrophotometer. Ratios of the extinction value at 556nm. (reduced) to that at 280nm. (oxidized) were also determined from spectra recorded in the Cary 14 instrument. To study the u.v. region of the spectra of reduced cytochrome b_5, $NaBH_4$ was used as the reducing agent at pH5·0. An ϵ_{mM} value of 25·6cm.$^{-1}$ for the α-peak at 556nm. was used (Strittmatter & Velick, 1956). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

Crystallization. A pure cytochrome b_5 solution in water (150–400μM) was placed in a dialysis bag and equilibrated against 0·05M-potassium phosphate buffer, pH7·0, 50% saturated with $(NH_4)_2SO_4$ at 4°. Solid $(NH_4)_2SO_4$ was added in 5%-saturation increments daily. The pH was checked at the same time and maintained at 7·0. Between 75% and 80% saturation with $(NH_4)_2SO_4$ pig liver cytochrome b_5 began to crystallize (as hexagonal plates).

Determination of protohaem and preparation of apocytochrome b_5. Protohaem content was determined as the pyridine haemochromogen by the method of Appleby & Morton (1959). Removal of haem followed the procedure of Teale (1959), with M-HCl being used to adjust a solution of cytochrome b_5 in water to pH2·0 at 0°. The resulting solution was extracted four times with 2vol. of butan-2-one, the organic phase containing the haem. The aqueous phase was either freeze-dried or dialysed to eliminate the residual solvent.

Molar extinction coefficients. ϵ values given for cytochromes refer to the extinction coefficients for molar concentration of protein-bound haem determined as outlined in the previous section. Spectra were recorded in a Cary 14 automatic spectrophotometer, previously checked as described by Morton (1962). Samples were suitably diluted with 0·1M-potassium phosphate buffer, pH7·0, filtered through a Millipore GS filter (0·22μm. pore size).

Electrophoresis of cytochrome b_5. Electrophoresis was carried out on 2·5cm. × 9cm. strips of cellulose acetate (Carl Schleicher and Schuell Co., N.H., U.S.A.) at 20° for 1–2hr. at 1·25mA/strip. Buffers were 0·025M-potassium phosphate pH7·0 and I 0·10, 0·1M-sodium acetate, pH5·0 and I 0·10, or 0·05M-sodium veronal, pH8·6 and I 0·05.

Protein was detected with Amido Black by the procedure of Vesselinovitch (1958).

Stability to heat. Stability of the different cytochrome

preparations was investigated with cytochrome solutions (10μM) in 0·1M-potassium phosphate buffer, pH 7·0. Solutions were incubated for 15min. at 40°, 50°, 60°, 70° and 80° successively. Between incubations solutions were brought to 20°, centrifuged to eliminate denatured protein and spectra were recorded in a Cary spectrophotometer.

Autoxidation. Cytochrome b_5 (5μM) samples in 0·1M-potassium phosphate buffer, pH 7·0, were reduced with a slight excess of sodium dithionite at 25° in a 1ml. quartz cuvette of 1cm. light-path. Autoxidation of cytochrome samples by air was followed every minute at 424nm. in a Beckman DU spectrophotometer. Each cuvette was inverted for mixing 0·3min. after a spectrophotometer reading and again 0·3min. before the next reading. Some trials were made with cytochrome samples passed through a Sephadex G-25 column to separate the cytochrome from the excess of reagent, but the samples were oxidized by air when collected.

Salting-out of the cytochromes. A 0·5mg. portion of the cytochrome was dissolved in 1ml. of 50%-saturated $(NH_4)_2SO_4$, pH 5·0, at 0°. Solid $(NH_4)_2SO_4$ was added to give successive 5%-saturation increments. Each time after 10min. the samples were centrifuged at 20000$g_{av.}$ for 10min. and cytochrome b_5 was assayed in the supernatant by using the expanded scale of a Cary 14 spectrophotometer. Determinations were made with 10μl. of the supernatant in 0·9ml. of 0·1M-phosphate buffer, pH 7·0, at 424nm., with dithionite as the reducing agent. Corrections were made for the dilution due to addition of salt and for the volume change due to removal of samples.

Enzymic reduction of cytochromes. Reduction of the cytochrome was effected by using the cytochrome b_5 reductase (EC 1.6.2.2) from pig liver purified by the method of Mahler, Raw, Molinari & Amaral (1958). Reduction was effected with the enzyme plus 320μmoles of NADH in 0·1M-potassium phosphate buffer, pH 7·0, at 25°.

Determination of Stokes radii and molecular weights. Beaded Sephadex G-75 particles (size 10–40μm.) were swollen in 0·02M-sodium phosphate buffer, pH 7·0, containing 0·1mM-EDTA and 0·3M-NaCl. The gel was packed into a column of silicone-treated glass (1·3cm. × 75cm.). Buffer was passed through this column until a constant bed height was attained (75cm.). The flow rate was controlled by the height of the buffer reservoir and was 8–9ml./hr. Bovine serum albumin, chymotrypsinogen and horse heart cytochrome *c* were standard protein solutions (5mg./ml.). Samples (0·1ml.) were layered on top of the bed with a needle and a piece of polythene capillary tubing placed 1cm. above the gel. Concentrated sucrose solution (20μl.) was added to each sample to increase its density. Fractions (0·40–0·50ml.) were collected and after dilution of the samples with an equal volume of buffer the extinctions at 230nm. and 421nm. were measured in a Beckman DU spectrophotometer. The results were processed as described by Andrews (1965) for molecular-weight determination and Stokes radii were evaluated by the procedure of Laurent & Killander (1964).

Digestion of apoprotein with trypsin and separation of tryptic peptides. Apoprotein was digested essentially as detailed by Sargent & Vadlamudi (1968). Apocytochrome b_5 samples prepared as above were dissolved in 0·5M-NH_4HCO_3 to a final concentration of 1mg./ml. and pure trypsin, equal to 1% (w/w) of the amount of apoprotein, dissolved in water, was added. After incubation at 37° for 2hr. a further equal quantity of trypsin was added and digestion continued for 2hr. The peptide solution was freeze-dried and resuspended in pyridine–acetic acid–water (100:4:900, by vol.), pH 6·7, to a concn. of approx. 50μg./μl. To silica gel G layers (250μm. thick) coated on 20cm. × 20cm. glass plates, evenly sprayed with pyridine–acetate buffer, 5μl. samples of the peptide suspension were applied 9cm. from the anode end of the layers and approximately in the middle. Contact was effected between the layer and buffer tanks by sheets of Whatman no. 3MM paper soaked in buffer. Electrophoresis was carried out at 15mA/plate for 3hr. No special cooling precautions were taken and no appreciable drying of the layers was observed. At the end of the electrophoresis the layers were air-dried and placed in an oven at 110° for 15min. Ascending chromatography was carried out in butan-1-ol–acetic acid–water (5:2:2, by vol.). The layers were air-dried again and stained with the cadmium–ninhydrin reagent described by Heilman, Barollier & Watzke (1957).

Determination of N*-terminal amino acid of the apoproteins.* A 1–2mg. portion of the freeze-dried apoprotein plus an equal weight of $NaHCO_3$ were dissolved in 0·15ml. of water, then 0·4ml. of 5% (v/v) 1-fluoro-2,4-dinitrobenzene in ethanol was added and the mixture was left for 3hr. in the dark with continuous agitation at room temperature. Excess of reagent was extracted with peroxide-free ether after acidification and the DNP-protein was washed four times with water adjusted to pH 2–3 with HCl. The freeze-dried protein was hydrolysed with 0·5ml. of constant-boiling HCl in a sealed glass tube under vacuum at 110° for 16hr. The ether-soluble DNP-amino acids of the hydrolysate were examined by t.l.c. on silica gel G in the solvent systems described by Randerath (1966). The aqueous phase was examined with the butan-1-ol–acetic acid–water (4:1:5, by vol.) solvent described by Fraenkel-Conrat, Harris & Levy (1961).

Amino acid analysis. Apoprotein samples were dried and weighed before hydrolysis by the method of Subramanian & Kalnitsky (1964) and then hydrolysed with three times-distilled constant-boiling HCl under vacuum at 110° for periods of 20 and 70hr. and prepared for the amino acid analyser as described by Moore & Stein (1963). Analysis was carried out in a Beckman 120C instrument. Tyrosine and tryptophan were determined spectrophotometrically in the apoproteins as described by Bencze & Schmid (1957) and Goodwin & Morton (1946). Tryptophan was also determined by the method of Barman & Koshland (1967), by using 2-hydroxy-5-nitrobenzyl bromide to label tryptophan residues of purified cytochrome b_5 apoprotein.

Enzymes. Lipase used for solubilization of cytochrome b_5 from microsomes was a crude preparation from pig pancreas. Trypsin (pure, from bovine pancreas, three times crystallized) was used to obtain from native cytochrome b_5 the trypsin-resistant portion and for 'fingerprints'. Bovine plasma albumin (fraction V) and cytochrome *c* (from horse heart), together with the above-mentioned enzymes, were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Chymotrypsinogen was a product from Chouay (Paris, France).

Chemicals. Tris was a product from Sigma Chemical Co.; 1-fluoro-2,4-dinitrobenzene was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.); 2-hydroxy-5-nitrobenzyl bromide was synthesized by the method of Buchler, Kirchner & Deebel (1940). DEAE-cellulose (reagent grade)

Table 1. *Purification of cytochrome* b_5

The results obtained with a pig liver preparation (6·5 kg. of liver tissue) are shown. Experimental details are given in the Materials and Methods section.

Fraction	Vol. (ml.)	Total cytochrome b_5 (μmoles)	E_{556}(reduced)/ E_{280}(oxidized)	Purification	Yield (%)
1. Microsomal suspension before lipase treatment	6000	58·0	—	—	—
2. Lipase supernatant after acid precipitation of microsomes	4500	31·5	—	—	—
3. DEAE-cellulose effluent	820	22·4	0·05	1	100
4. First $(NH_4)_2SO_4$ fractionation	35	19·9	0·27	5	89
5. Sephadex G-50 effluent	88	19·0	0·96	19	85
6. Second $(NH_4)_2SO_4$ fractionation	7	18·4	1·06	21	82
7. Sephadex G-75 effluent	57	17·3	1·32	26	77
8. DEAE-Sephadex A-50 chromatography	25	10·8	1·42	28	48

was Selecta-Cell brand (0·87 m-equiv./l.) from Brown Co. (Corvallis, Oreg., U.S.A.). Sephadex G-75 and G-50 (medium grade) were from Pharmacia Fine Chemicals (Uppsala, Sweden). Sephadex G-75 (40 μm. bead form) and DEAE-Sephadex A-50 (3·5 m-equiv./l, particle size 40–120 μm.) were obtained from Sigma Chemical Co. Silica gel G was a product from E. Merck A.-G. (Darmstadt, Germany). All the other chemicals used were reagent-grade products.

The pH values of all the buffers used were recorded at room temperature.

RESULTS

Table 1 shows the summary of the purification procedure for cytochrome b_5 from pig liver, and Table 2 gives the yields of cytochrome b_5 from liver of the four species studied. Solubilization of the cytochrome from microsomes was subject to large fluctuations in yield.

Further, cytochrome b_5 assay in steps 1 and 2 of the purification procedure, by using differential spectrophotometry and reduction by means of NADH, is frequently difficult or, in human preparations, impossible (probably owing to autolysis). Relative effectiveness in extracting the cytochrome from the same weight of fresh liver tissue, together with the recovery of the cytochrome from steps 3–8, is presented, allowing comparison between the species studied throughout the purification procedure. The cytochromes subjected to chromatography in step 8, as detailed in the Materials and Methods section, are usually eluted as a single, symmetrical peak at 220–250 m-equiv. of Cl^-/l. Minor cytochrome b_5 fractions, if present, were resolved from the main peak by the gradient and discarded.

Several attempts were made to crystallize the pure cytochrome from the different sources, with negative results. Only cytochrome b_5 from the pig was successfully crystallized (Fig. 1). There was no improvement in purification of pig cytochrome b_5 after one or several successive crystallizations.

Table 2. *Yields of cytochrome* b_5 *from different species*

Experimental details are given in the Materials and Methods section. The final column gives the yield as the ratio of the amount of cytochrome b_5 in step 8 to that in step 3.

Liver source	Yield of cytochrome b_5 (μmoles/kg. fresh wt. of liver) In step 3	In step 8	Yield (%)
Human	2·6	0·6	23
Monkey	2·0	0·5	25
Pig	3·5	1·6	48
Chicken	0·6	0·2	33

Formation of the characteristic pyridine haemochromogen as outlined in the Materials and Methods section and verification of the conditions established by the Enzyme Commission on Cytochrome Nomenclature and Classification showed that iron protoporphyrin IX was the haem prosthetic group of the cytochromes studied.

Table 3 presents the extinction coefficients and the localizations of the principal spectral features of the purified cytochromes.

Electrophoresis of the cytochromes revealed a single band pattern for species studied.

Resistance to heat-denaturation and autoxidation of the cytochromes, were studied as detailed in the Materials and Methods section. All the cytochromes exhibit a similar resistance towards heat-denaturation and the curves relating assay temperature against denaturation showed a sharp break after 60°. The velocities of autoxidation were almost equal and oxidation of 50% of the reduced cytochrome takes about 6–7 min. for all.

Salting-out of the cytochromes followed the classical curves when expressed as ammonium

sulphate saturation against percentage of cytochrome remaining in solution. Under the condition described in the Materials and Methods section 50% of the cytochromes were precipitated at the following ammonium sulphate saturations: 63% for chicken, 75% for monkey and human, and 76% for pig cytochrome b_5.

All the cytochromes are reduced by the system cytochrome b_5 reductase (EC 1.6.2.2)+NADH, under the conditions detailed in the Materials and Methods section.

Stokes radii and molecular weights determined by Sephadex gel filtration are presented in Table 4 and compared with the respective molecular weights estimated from the amino acid composition of the cytochromes.

Determinations of *N*-terminal amino acids were performed in standard conditions (see the Materials and Methods section). In the aqueous phase of the hydrolysates of the DNP-apoproteins only ε-DNP-lysine, *O*-DNP-tyrosine and *Im*-DNP-histidine were found. The thin-layer chromatograms of the ether phase of the hydrolysates contained, besides dinitrophenol and dinitroaniline, only one more spot representing the *N*-terminal amino acid; this was found to be DNP-serine for human and monkey apocytochromes, DNP-alanine for pig and DNP-glycine for chicken.

Amino acid analyses of the apoproteins are presented in Table 5, which also gives the results of the determinations of tyrosine and tryptophan.

'Fingerprint' patterns from the tryptic hydrolysates of the apoprotein from the species studied are shown in Fig. 2.

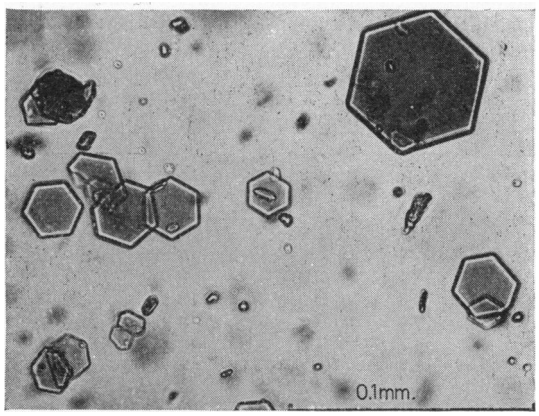

Fig. 1. Photograph of crystals of oxidized cytochrome b_5 from pig liver (× 650).

DISCUSSION

The heterogeneity of the cytochrome b_5 solubilized from pig liver was early verified in this laboratory (Raw *et al.* 1958) and by Strittmatter & Ozols (1966). They showed that two forms of calf liver cytochrome b_5 (I and II) were converted by trypsin treatment into a unique form. In the present work it was necessary to purify and characterize a comparable portion of this cytochrome from the four species studied. To achieve this, the reported resistance of the native protein to tryptic hydrolysis makes it possible to obtain a homogeneous protein by trypsin treatment. After step 5

Table 3. *Comparative extinction coefficients of the more significant spectral features of cytochromes* b_5

Experimental procedure is given in the Materials and Methods section. Wavelengths are given in nm.; the extinction coefficients (ε) in each case are given for a molar concentration of protein-bound haem.

		Human		Monkey		Pig		Chicken	
		Oxidized	Reduced	Oxidized	Reduced	Oxidized	Reduced	Oxidized	Reduced
α-band	λ	556	556	556	556	556	556	555	555
	$10^{-3}\varepsilon$	8·3	26·6	8·3	26·2	8·3	26·6	8·3	26·5
Minimum	λ	—	541	—	541	—	541	—	541
	$10^{-3}\varepsilon$	—	7·4	—	7·3	—	7·6	—	7·7
β-band	λ	527·5	527·5	527·5	527·5	527·5	527·5	527·5	527·5
	$10^{-3}\varepsilon$	9·9	14·2	10·1	13·6	9·9	14·0	10·2	14·2
Isobestic point	λ	438·5		438·5		438·5		438·5	
	$10^{-3}\varepsilon$	17·6		18·1		18·8		18·3	
γ-band	λ	413	423·5	413	423	413	423	413	423
	$10^{-3}\varepsilon$	121·5	182·3	117·1	181·0	119·0	180·4	120·9	186·9
δ-band	λ	360	328	360	—	360	327	360	327
	$10^{-3}\varepsilon$	23·9	~34·2	22·5	—	23·8	~33·7	24·6	~32·6
E_{556}(reduced)/ E_{280}(oxidized)		1·39–1·42		1·27–1·30		1·41–1·44		1·39–1·42	

Table 4. *Stokes radii and molecular weights of the cytochromes* b_5

Stokes radii and molecular weights were determined by Sephadex gel filtration as outlined in the Materials and Methods section. The results were expressed as means ± S.E.M. with the numbers of determinations given in parentheses. The values for the molecular weights calculated by gel filtration were compared with those estimated from the amino acid composition of the cytochromes.

Source of cytochrome b_5	Stokes radius (Å) by gel filtration	Mol.wt. by gel filtration	Mol.wt. by amino acid composition
Man	17·6 ± 0·02 (4)	13700 ± 300 (4)	10600
Monkey	17·2 ± 0·02 (3)	13700 ± 300 (3)	11400
Pig	17·4 ± 0·00 (4)	12500 ± 200 (4)	10400
Chicken	15·0 ± 0·05 (4)	11400 ± 400 (4)	10800

Table 5. *Amino acid analyses of the apocytochromes* b_5

Amino acid analyses were performed as detailed in the Materials and Methods section. Results for each cytochrome were based on hydrolysis for 20 hr. and 70 hr. Results for threonine and serine were corrected for destruction assuming first-order kinetics. Numbers of residues/mol. were calculated to the nearest integer after assuming four alanine residues/mol. for all the cytochromes. —, Not determined. Tyrosine and tryptophan determinations made separately from the amino acid analyses are given at the bottom of the table.

	Man		Monkey		Pig		Chicken	
Mol.wt. (+haem)	10600		11400		10400		10800	
	Amino acid residues		Amino acid residues		Amino acid residues		Amino acid residues	
	(g./100g. of protein)	(residues/mol.)	(g./100g. of protein)	(residues/mol.)	(g./100g. of protein)	(residues/mol.)	(g./100g. of protein)	(residues/mol.)
Lys	7·5	6	7·2	6	6·7	5	3·4	3
His	10·7	8	11·7	9	11·7	8	10·5	8
Arg	4·2	3	4·6	3	5·1	3	9·8	7
Asp	10·4	9	10·1	10	10·8	9	12·1	10
Thr	6·5	6	5·9	6	7·4	7	6·2	6
Ser	4·2	5	6·3	8	3·4	4	3·6	4
Glu	18·4	14	18·2	15	17·8	14	17·7	14
Pro	3·3	3	3·2	4	2·1	2	2·9	3
Gly	3·4	6	3·2	6	3·4	6	3·9	7
Ala	2·9	4	2·6	4	2·8	4	2·9	4
Val	4·1	4	3·8	4	3·8	4	3·8	4
Cys	0·0	0	0·0	0	0·0	0	0·0	0
Met	1·1	1	0·0	0	0·0	0	0·0	0
Ile	4·6	4	4·2	4	4·5	4	6·5	6
Leu	8·1	7	8·7	8	9·5	8	5·7	5
Tyr	4·6	3	6·0	4	4·9	3	4·6	3
Phe	4·4	3	2·8	2	4·6	3	4·5	3
Trp	—	1	—	1	—	1	—	1
Total		87		94		85		88
Tyr*		2·73		4·10		3·14		2·90
Trp†		0·84		0·85		0·84		1·16
Trp*		0·97		0·90		1·01		1·20
Tyr/Trp ratio‡		2·9		3·7		2·9		2·7

* By the method of Goodwin & Morton (1946).
† By the method of Barman & Koshland (1967)
‡ By the method of Bencze & Schmid (1957).

of the purification procedure the cytochrome eluted from the Sephadex G-50 column was treated with trypsin as described by Strittmatter & Ozols (1966). Some minor contaminant still present in the preparation was eliminated in the last step of the purification (DEAE-Sephadex chromatography).

Spectral features shown in Table 3 are similar for all the cytochromes studied. Small differences in the shape of the reduced α-band were detected, but further analysis will require low-temperature spectral studies. Ratios of the extinction of the pure cytochrome solutions at 556nm. (reduced)

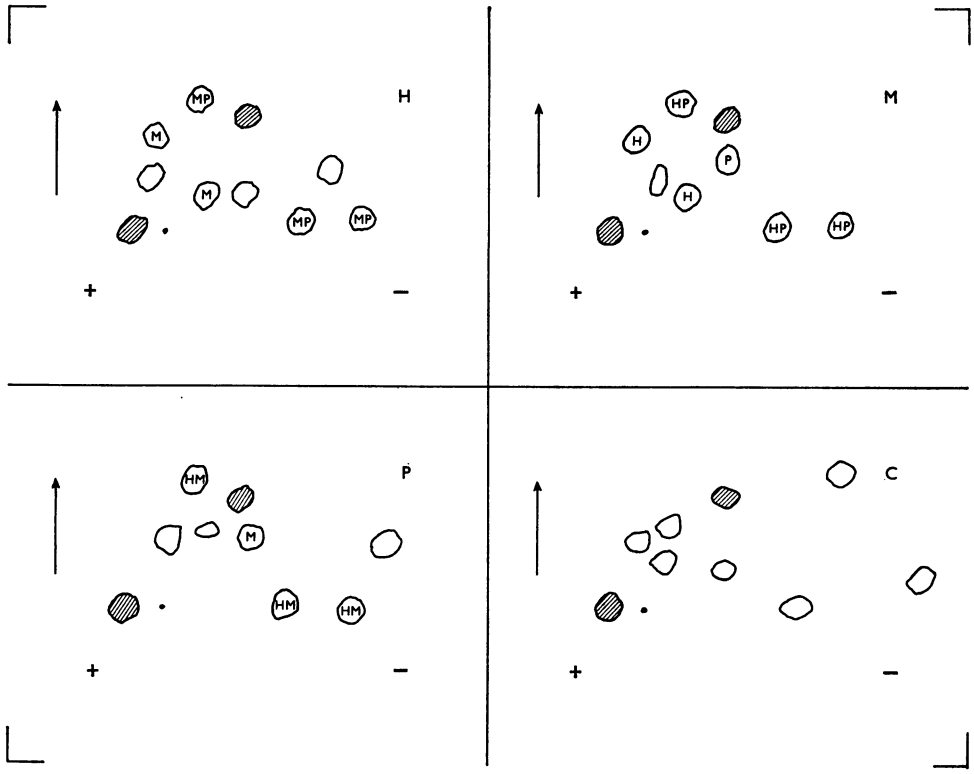

Fig. 2. Tryptic 'fingerprint' patterns of cytochrome b_5 of man (H), monkey (M), pig (P) and chicken (C). The apoproteins were digested with trypsin and the resulting peptides were separated on thin layers as detailed in the Materials and Methods section. The peptide zones shown stained positively with ninhydrin. The direction of chromatography is indicated by the arrows. Shaded zones indicate peptides common to the four species studied. Peptides common to two or three species are indicated by appropriate lettering.

and 280nm. (oxidized) were similar for the cytochromes obtained from man, pig and chicken. This ratio was less for the monkey cytochrome, and this result was shown to be correct by the results of tyrosine and tryptophan determinations.

The cytochromes were remarkably similar in the different properties studied. Human and monkey cytochromes have identical migrations in electrophoresis. Pig and chicken cytochromes have the same migration value, although lower than that for the above species. Resistance to heat-denaturation and autoxidation of the different cytochromes in the conditions studied were similar for all.

In salting-out experiments chicken cytochrome b_5 was the most easily precipitated and pig cytochrome the most soluble of all. Monkey and human cytochromes were 50% precipitated by the same ammonium sulphate concentration.

Sephadex gel filtration results were analysed by two procedures. According to Andrews (1965) the elution volume is directly proportional to the logarithm of the molecular weight. The results obtained by this method were displaced towards higher molecular weights than those estimated from the amino acid analyses. By following the Laurent & Killander (1964) method and using their parameter, $[-\log K_{av.}]^{1/2}$, for the determination of the Stokes radius of a molecule, it was found that the molecular size and shape of pig cytochrome b_5 were very similar to those of the cytochrome *c*, which agrees with the work of Ehrenberg & Poltoratsky-Bois (1967). The value found for the Stokes radius of the chicken cytochrome b_5 was lower, which could be a possible indication for a more compact or globular structure of this cytochrome.

Serine was the *N*-terminal amino acid for human and monkey cytochromes, the same as that found for the calf cytochrome (cytochrome b_5 I) by Strittmatter & Ozols (1966). Alanine was the *N*-terminal amino acid for pig cytochrome b_5, the same as for

the trypsin-resistant portion of the calf liver cytochrome b_5. Chicken cytochrome b_5 contains glycine as *N*-terminal amino acid, being thus different from other cytochromes b_5 already studied, including the rabbit liver protein (Tsugita, Kobayashi, Kajihara & Hagihara, 1968).

Although amino acid composition is only a very rough guide, homologous proteins are unlikely to be very different. As in the cytochromes b_5 previously studied, there were no sulphur-containing amino acids in the proteins purified from monkey, pig and chicken. Human cytochrome b_5 was found to have one methionine residue. The numbers of alanine and valine residues are quite invariant, comparing our results with the reported amino acid composition of cytochrome b_5 from rat (Sargent & Vadlamudi, 1968) and calf (Ozols & Strittmatter, 1968*b*). A noteworthy difference between chicken and the other species is the inversion in the ratio of lysine to arginine residues.

'Fingerprint' analysis of the tryptic hydrolysates of the different apoproteins yielded good information about structural similarities between species. Comparison of the peptide maps depicted in Fig. 2 shows that cytochromes b_5 of closely related species such as man and monkey have seven common peptides, whereas those of man and chicken show only two common spots. These two peptides are noteworthy in that they were obtained from cytochromes b_5 of all four species studied. Probably they play a role in haem binding in the cytochromes, carrying the ligands for iron or providing the appropriate hydrophobic 'pocket' for the haem. Human, monkey and pig cytochromes have only these two peptides in common with the avian cytochrome. The numbers of identical peptides were the following for the indicated pairs of 'fingerprints' compared: seven (man and monkey); five (man and pig); two (man and chicken); six (monkey and pig); two (monkey and chicken); two (pig and chicken). Thus the number of peptides common to the cytochromes of two species follows somewhat their taxonomic relationship.

We are grateful to Mr Antonio Cassalho for the help in obtaining monkey livers and to Mr Luiz Fernando Barrella for the synthesis of 2-hydroxy-5-nitrobenzyl bromide. We are also very grateful to Dr Alberto Carvalho da Silva for the amino acid analyser facilities and Mrs Natalina Takahashi for her technical assistance. The support of Fundação de Amparo à Pesquisa do Estado de São Paulo is acknowledged.

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