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PLASMA MEMBRANES FROM INTESTINAL MICROVILLI AND ERYTHROCYTES CONTAIN CYTOCHROMES b_5 AND *P*-420

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Summary

The presence of cytochromes b_5, *P*-450 and *P*-420 and activities of NADH- and NADPH-cytochrome *c* reductases were determined in plasma membranes isolated from microvilli of the chick and rat intestinal epithelium and erythrocyte membranes from chick, rat and man. The results are compared with the amounts of these components found in microsomal fractions from intestinal epithelium and in nuclear membranes from chick erythrocytes. Plasma membranes from intestinal microvilli and from erythrocytes contained significant amounts of NADH-cytochrome *c* reductase activity and of a pigment spectrophotometrically indistinguishable from rat liver microsomal cytochrome b_5. In addition, cytochrome b_5 fragments were prepared from the membranes by limited trypsin digestion and consisted of two to four components with M_r values in the range 10 000—13 500. In low-temperature difference spectra, the presence of a second cytochrome was noted which was similar to cytochrome *P*-420. Cytochrome *P*-450 and NADPH-cytochrome *c* reductase activities were not detected in plasma membrane fractions in significant concentrations but were present in the corresponding endomembrane fractions. These findings in highly purified, well defined plasma membrane fractions, in which contamination by endomembranes is minimal, strengthen the evidence for the existence of cytochrome-containing redox systems in plasma membranes of various cells and suggest that such redox components are general components of the cell surface. Possible functions and origins of these redox components in plasma membranes are discussed.

Introduction

Components of the 'microsomal' electron transport system, i.e., cytochromes b_5 and *P*-450 and their associated NADH- and NADPH-dependent flavoproteins, are not restricted to the rough and smooth endoplasmic reticulum but also occur in nuclear membranes [1—4], outer mitochondrial membranes [5—7] and membranes of Golgi apparatus [8—12]. Reports on the presence of such proteins in plasma membranes, which have been studied in detail only from mammalian liver, are conflicting (Refs. 4, 6, 12—14; for further reports see Refs. 12 and 15). We have shown that cytochrome b_5 as well as cytochrome *P*-420, which is usually regarded as a degradation product of cytochrome *P*-450, are present in milk fat globule membranes, i.e., membranes derived from the apical surface of lactating mammary gland epithelial cells [16,17]. In addition, evidence has been presented that plasma membranes from rat liver contain these cytochromes and that they are associated with other components of the drug-metabolising enzyme system [12,18]. In the present study we extend these observations and show that such redox components are present in other well defined and highly purified preparations of plasma membranes in which cross-contamination by endomembranes is minimal, if not completely excluded. Here we describe the occurrence of two cytochromes in surface membranes of chick, rat and human erythrocytes and in microvillar membranes from chick and rat intestinal epithelium.

Methods

Microsomes and brush border plasma membranes were prepared from the small intestine epithelium of 6-month-old White Leghorn hens and of Sprague-Dawley rats (300 g body wt.). The animals were killed by decapitation and bled. The intestine was removed and rinsed several times with buffered saline solution (0.9% NaCl, 0.3 mM NaN_3, 10 mM Tris-HCl buffer, pH 7.4). A suspension of epithelial cells was prepared and microvilli isolated and purified as described [19]. Microsomal fractions were prepared as described for rat liver material [12]. All isolation media were supplemented with 0.1 mM dithioerythritol and 0.1 mM phenylmethylsulfonyl fluoride. Microsomal and microvillar membranes were suspended and resedimented for 30 min at 110 000 × *g* in 1.5 M KCl (10 mM Tris-HCl buffer, pH 7.4) and finally resuspended in 0.25 M sucrose containing 5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl buffer, pH 7.4. Alternatively, membranes were directly suspended in this medium without treatment with high salt concentrations. Control experiments were performed using 20% (v/v) glycerol, trypsin inhibitor and heparin in all isolation media (cf. Ref. 20) to minimize degradation of cytochrome *P*-450. This modification, however, had no significant effect on enzyme activities and cytochrome contents. For comparison, total microsomes, rough endoplasmic reticulum and plasma membranes were isolated from rat liver as described [12]. Cytochrome b_5 was extracted and purified from these membranes by limited trypsin digestion (Ref. 21; for characterization of the preparation see Ref. 12).

Nucleated erythrocytes were isolated from blood collected from the carotid artery of 6-month-old White Leghorn hens. The blood was immediately mixed

with 10 vols. of a medium containing 0.4 M sucrose, 2% gum arabic, 50 mM KCl, 1% heparin and 10 mM Tris-HCl buffer (pH 7.4), and was layered on top of a 1 M sucrose solution containing 2% gum arabic, 50 mM KCl and 10 mM Tris-HCl buffer (pH 7.4). Erythrocytes were sedimented for 2 h under gravity. The upper third of the sediment was discarded to remove white blood cells and immature red cells. Plasma membranes and nuclear membranes were isolated and purified from the mature erythrocytes as described [22].

Blood from Sprague-Dawley rats (300 g body wt.) was collected in 0.172 M Tris-HCl buffer (pH 7.6) which had been supplemented with 1% heparin, and erythrocytes were sedimented through 1 M sucrose solution as described for chick erythrocytes. Erythrocyte membranes were isolated and purified according to the method of Hanahan and Ekholm [23]. Samples of concentrated human erythrocytes in 0.125 M glucose solution containing 75 mM trisodium citrate and 40 mM citric acid were supplied by the University Hospital Blood Bank, Heidelberg. Red cells were sedimented through 1 M sucrose solution and further processed as described above. All membrane fractions from erythrocytes were finally washed twice by suspension in 0.1 M potassium pyrophosphate buffer (pH 7.4) and sedimentation for 15 min at 60 000 $\times g$. Membranes were treated with buffered 1 M KCl solution or were directly resuspended in buffered 0.25 M sucrose solution as described above. All preparation steps were performed at 0—4°C.

Protein was measured by using the method of Lowry et al. [24]. Lipids were extracted and lipid phosphorus was determined as described [25]. Cytochromes were examined at room temperature and at low temperature (—196°C) as described [16,26]. Carbon monoxide difference spectra were performed as described [26] and according to the method of Matsubara et al. [27]. Prior to spectral estimation, all membrane fractions were suspended in a medium containing 50% glycerol and 0.5 M potassium phosphate buffer (pH 7.4). NADH-cytochrome *c* reductase, in the presence of rotenone or antimycin, and NADPH-cytochrome *c* reductase activities were determined as described [26]. NADH-cytochrome *c* reductase activity was also measured in the presence of atebrin and sodium azide [28]. Activities of glucose-6-phosphatase, alkaline phosphatase (with *p*-nitrophenylphosphate as substrate) and 5′-nucleotidase were determined as described [29].

For gel electrophoresis, membrane samples were treated with buffer containing 5% sodium dodecyl sulphate as described [30] and were examined in slab gels containing 8 or 18% acrylamide [31].

Samples of membrane fractions were fixed for electron microscopy either in suspension or as pellets, and were processed for ultra-thin sectioning as described [12]. Morphometric determinations and classification of membrane profiles were performed as described [32].

NADH, NADPH, antimycin, cytochrome *c* and reference proteins for gel electrophoresis were obtained from Boehringer (Mannheim, F.R.G.). Rotenone which was post-purified by recrystallization from trichloroethylene, and dextran were from Sigma (St. Louis, MO, U.S.A.). Atebrin was obtained from Bayer (Leverkusen, F.R.G.) and heparin from Riker Pharma (Borken, Westphalia, F.R.G.). Gum arabic was purified as described [1]. Other chemicals were analytical grade reagents from Merck (Darmstadt, F.R.G.) or Serva (Heidelberg, F.R.G.).

Results

Morphology and purity of fractions

Both plasma membrane fractions examined were of high purity. Fig. 1 shows a representative electron micrograph of a section of the purified microvillar membranes from rat intestinal brush borders as seen after treatment with high-salt buffer. This fraction, before the high-salt treatment, was similar in morphology and purity to the microvilli fraction isolated from chick intestinal brush borders described previously [19]. After treatment with buffers containing high-salt concentrations, most of the glycocalyx and core microfilament material (cf. Ref. 19) was removed (Fig.1). Microvillar membrane fractions consisted mostly of closed vesicles of various shapes and sizes. In morphometric determinations of randomly chosen ultra-thin sections of pelleted microvillar fractions that had not been treated with high-salt buffers, consistently more than 80% (rat) and 85% (chick) of the total membrane profile lengths were clearly identified as brush border plasma membrane (Table I). The remaining 10—20% of membrane profiles consisted of smooth-surfaced vesicles and sheets, most of which were probably also of plasma membrane origin, and of rough endoplasmic reticulum vesicles. Nuclear and mitochondrial contaminants were

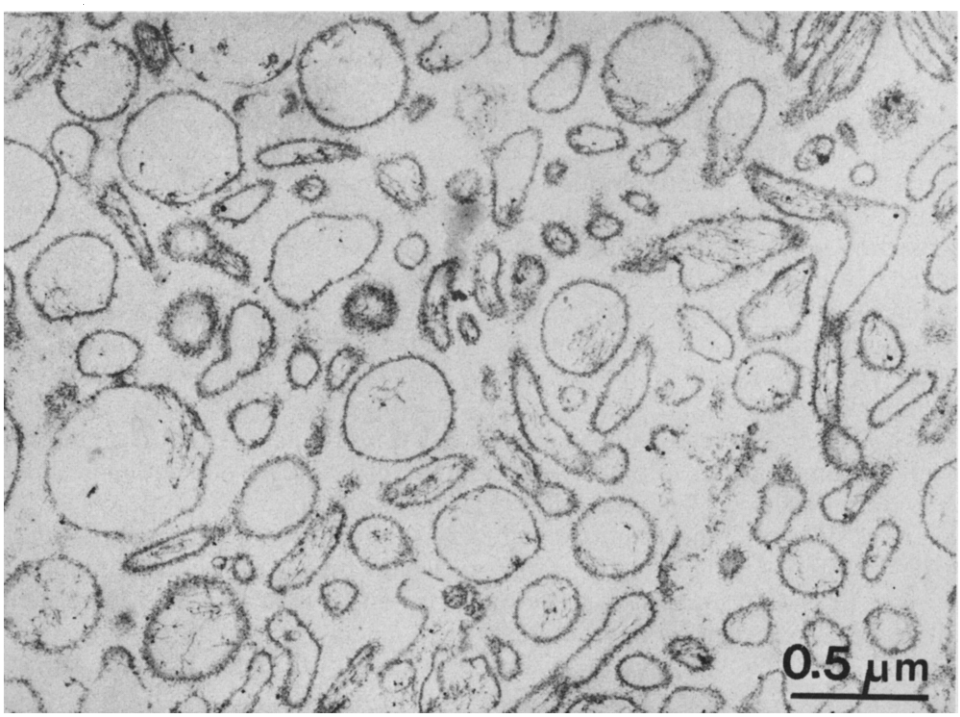


Fig. 1. Electron micrograph of a thin section through a fraction of purified microvillar membranes isolated from rat intestinal brush borders. The fraction, which had been treated with buffer containing 1.5 M KCl (see Methods), consists of irregularly formed vesicles. The central core material and the microfilaments have been largely extracted. Non-microvillar components such as terminal web, junctions and tonofilaments which are characteristic of intact brush borders have also been removed. Magnification, X36 000.

TABLE I

PURITY OF MICROVILLAR AND MICROSOMAL FRACTIONS FROM CHICK AND RAT INTESTINE AS DETERMINED BY ELECTRON MICROSCOPIC MORPHOMETRY AND MARKER ENZYME ACTIVITIES

Data represent mean values and standard deviations determined from four different preparations. Cytochrome aa_3 contents of crude mitochondrial fractions from chick and rat intestine were approx. 0.15 and 0.12 nmol/mg protein, respectively.

Proportion of total membrane area (%) identified as *	Chick intestine		Rat intestine	
	Microvillar membranes	Microsomal membranes	Microvillar membranes	Microsomal membranes
Plasma membranes	85 ± 7	20 ± 4	82 ± 6	22 ± 4
Endomembranes (rough endoplasmic reticulum, nuclear membrane, coated vesicles, smooth vesicles)	10 ± 2	75 ± 8	15 ± 3	68 ± 8
Mitochondrial membranes	1.5 ± 0.5	4 ± 2	1.5 ± 0.5	4 ± 2
Enzyme activities (nmol/min per mg protein)				
Glucose-6-phosphatase	<3 *	26 ± 2	<3 *	8.5 ± 0.5
Alkaline phosphatase	260 ± 22	81 ± 11	380 ± 27	98 ± 10
5′-Nucleotidase	1.7 ± 0.6	2.7 ± 0.8	0.8 ± 0.2	1.2 ± 0.4
Cytochrome aa_3 contents (nmol/mg protein)	<0.01 *	0.02 ± 0.01	<0.01 *	0.02 ± 0.01

* For criteria of classification see Ref. 32.
** Limit of detection.

very rarely detected. The virtual absence of mitochondrial membranes in microvillar fractions was also confirmed by the extremely low cytochrome aa_3 contents (Table I). The activities of glucose-6-phosphatase in microvillar membrane fractions were at the limit of detection, in general agreement with the morphometrically determined purity of these fractions. The relatively low glucose-6-phosphatase activity in rat intestine compared to chick intestine (cf. the activities in the microsomal fractions; Table I) has been explained by other authors by the sensitivity of this enzyme towards the high phospholipase A activity in rat intestinal cell membranes [33]. Alkaline phosphatase, an enzyme characteristic of the apical cell surface of intestinal epithelium [34], was about 4-fold higher in activity in microvillar than in microsomal fractions, whereas 5′-nucleotidase, an enzyme of the basolateral plasma membrane domain [35], had relatively low activities in both microvillar and microsomal fractions (Table I).

Microsomal fractions from chick and rat intestinal cells consisted of vesicles with attached ribosomes representing approx. 60% of the total membrane profiles identified in ultra-thin sections. The fraction also included 5—15% smooth-surfaced vesicles, 3—5% coated vesicles and about 15—20% of right-side-out vesicles derived from brush border microvilli (identified by the characteristic periodical surface coat and internal core structures). Mitochondrial contamination in microsomal fractions was minimal (less than 5% of membrane profiles), and the contents of a marker protein of inner mitochondrial membranes, cytochrome aa_3, was at the limit of detection (approx. 20 pmol/mg protein; see Table I).

Membrane preparations from mammalian (human and rat) erythrocytes which had been repeatedly washed with buffers containing 0.1 M pyrophosphate and 1.5 M KCl, respectively, contained open membrane sheets as well as small vesicles (Fig. 2). The fractions were essentially free of filamentous and fuzzy electron-dense material attached to the inner membrane surface (Fig. 2), indicative of the removal of most of the membrane-attached cytoskeletal elements (in agreement with the observed absence of spectrin and actin in gel electrophoretic analyses of such fractions; data not shown here). Haemoglobin and methaemoglobin were routinely determined by difference spectrophotometry using dithionite, carbon monoxide and cyanide, respectively (cf. Ref. 36). In this study, only preparations were used in which the haemoglobin and/or methaemoglobin concentration was less than 20 pmol/mg protein.

The morphology and purity of plasma membranes and nuclear membranes isolated from chick erythrocytes have been documented elsewhere [22].

Rat liver endoplasmic reticulum and plasma membranes, which were isolated and examined for comparison, have been described.

Spectral characterization

When difference spectra (using NADH as reducing agent) of purified microvillar membranes from either chick or rat small intestine were recorded at low temperature, a typical cytochrome b_5 spectrum, indistinguishable from that of hepatic microsomes and clearly distinguished from spectra of mitochondrial

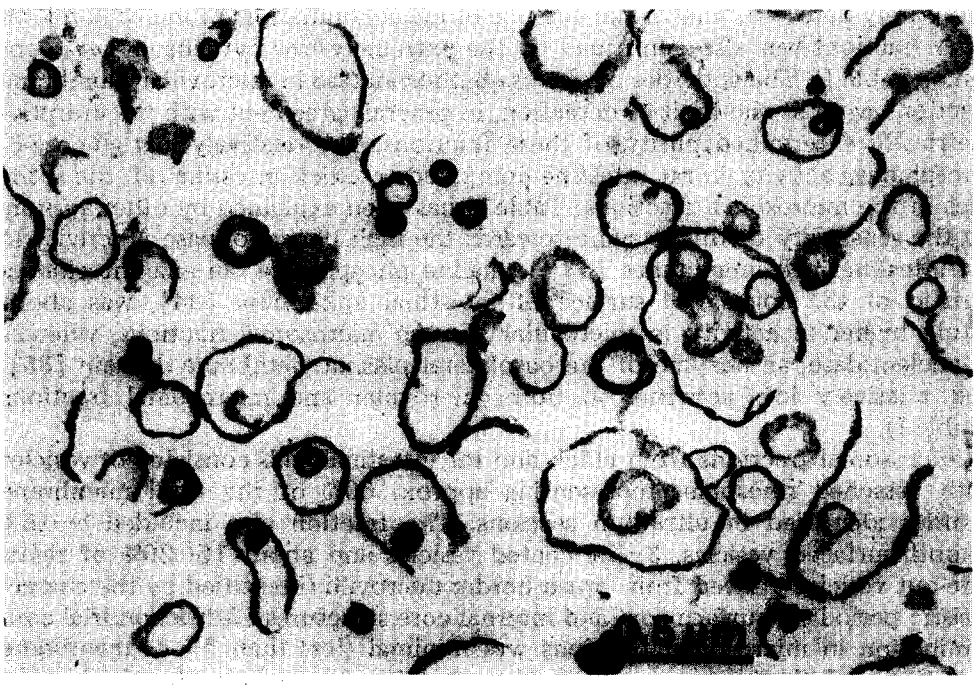


Fig. 2. Electron micrograph of a thin section through a fraction of purified fragments of human erythrocyte plasma membranes. The fraction, which had been washed twice with 0.1 M pyrophosphate buffer (see Methods), consists of vesicles and open membrane sheets of varying sizes. Magnification, X36 000.

b-type and *c*-type cytochromes, was obtained. The Soret band had its maximum at 423.5 nm, and the α- and β-bands were each split into two peaks at 552 and 557 nm and at 526 and 530 nm, respectively. NADH-difference spectra with the same characteristics were also recorded using intestinal microsomal fractions. Dithionite-difference spectra of microvillar membranes, however, showed a Soret band maximum at 427 nm and a third peak at 561 nm in the α-region, in addition to the maxima at 552 and 557 nm (Fig. 3). In previous publications we have described similar spectra from plasma membrane fractions of lactating mammary gland [17] and liver [12] and have suggested that such spectra were derived from interference of the absorbances of reduced cytochrome b_5 (the only component visible in the NADH-difference spectra) and reduced cytochrome *P*-420. The presence of cytochrome *P*-420 in microvillar membranes was also demonstrated by the peak at 421 nm in the carbon monoxide-difference spectra (Fig. 4). Freshly prepared intestinal microsomes isolated from the same homogenate, on the other hand, contained little if any cytochrome *P*-420 but were rich in cytochrome *P*-450 (Fig. 4). Upon storage in suspension for several hours at 4°C the spectrophotometrically detectable cytochrome *P*-450 content drastically decreased and a peak about 420 nm became apparent, indicating some conversion of cytochrome *P*-450 into *P*-420 [37]. In microvillar membranes and in 'aged' microsomes, not only cytochrome *P*-450 but also cytochrome *P*-420 were very labile. After 3 h of storage at 4°C less than 30% of cytochromes *P*-450 and *P*-420 were recovered as cytochrome

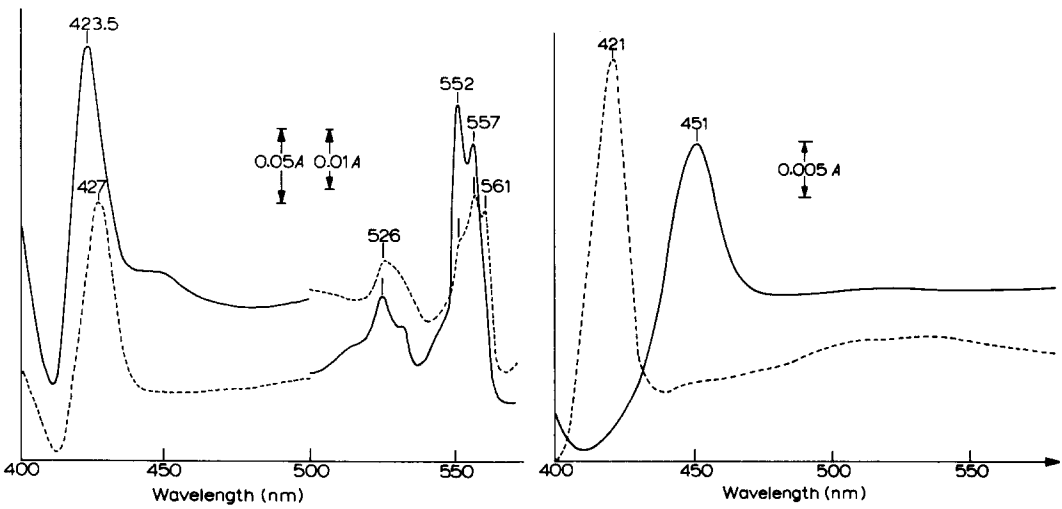


Fig. 3. Dithonite-reduced versus oxidized difference spectra of chick intestinal microsomal (———) and brush border microvillar membranes (- - - - - -) at −196°C. Twin cuvettes of 2 mm optical pathlength were used; the spectral bandwidth was 1 nm; the absorbance is given by the specific *A* scales. Protein concentrations were 1 mg/ml for microsomes and 5 mg/ml for microvilli. Other conditions were as described [17]. Note the additional peak at 561 nm and the location of the Soret band at longer wavelengths in the spectrum of microvillar membranes as compared to that of microsomes.

Fig. 4. CO-difference spectra of freshly prepared fractions of chick intestinal microsomes (———) and microvillar membranes (- - - - - -) at 8°C. The optical pathlength was 10 mm and the spectral bandwidth 3 nm; the absorbance is given by the specific *A* scale. Protein concentrations were as given for Fig. 3, other conditions were as described [17]. Note the complete absence of cytochrome *P*-420 in the microsomes and the absence of cytochrome *P*-450 in the microvilli.

P-420, identified by CO-difference spectra. The 561 nm peak in the dithionite-difference spectra at low temperature, however, was observed even when the 420 nm band in the CO-difference spectra was no longer detected. We interpret this observation as resulting from the loss of CO-binding in the reduced cytochrome *P*-420 [17]. Consequently, we calculated the relative contents of cytochrome *P*-420 from dithionite-difference spectra at −196°C by subtraction of the spectral contribution of cytochrome b_5 using the absorbance at 552 nm as reference. At this wavelength the absorbance of reduced cytochrome *P*-420 was negligible (Fig. 5). An estimate of the molar ratios of the cytochromes b_5 and *P*-420 present in chick microvillar membranes from the specific forms of the α-band and the location of its absorbance maxima in dithionite-difference spectra at −196°C (Figs. 3 and 5) indicated a value of 1.3 (cf. Table II). We also tried to separate the two pigments, cytochrome b_5 and *P*-420, by limited trypsin digestion of chick intestinal microvillar membranes (cf. Ref. 21). This treatment resulted in the solubilization of cytochrome b_5 fragments spectrophotometrically indistinguishable from those of liver and mammary gland endoplasmic reticulum (see below). Cytochrome *P*-420, however, was no longer detectable in either the soluble trypsin extract or the insoluble membrane residue.

Similar spectra as described above for brush border plasma membranes were also recorded for plasma membrane fractions from human erythrocytes (Fig. 6) as well as from rat and chick erythrocytes. An NADH-difference spectrum with absorbance maxima typical of cytochrome b_5 was observed only with freshly prepared erythrocyte membranes. Treatment of the membranes with dithionite

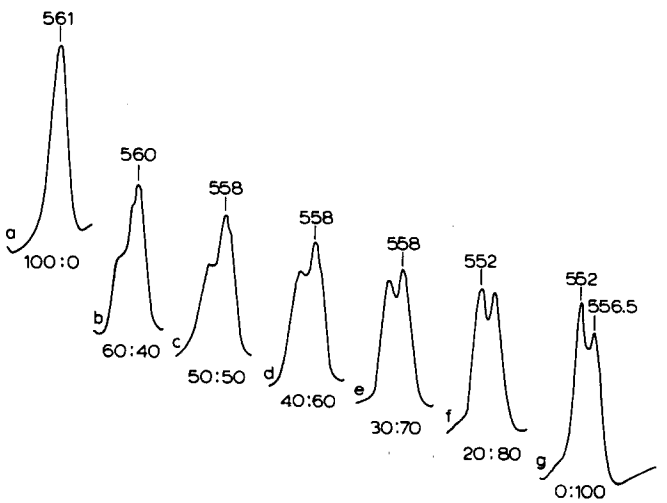


Fig. 5. Forms of the α-bands and locations of the absorbance maxima at different molar ratios of cytochromes b_5 and *P*-420 as revealed in dithionite versus oxidized difference spectra at −196°C. Curve a represents the α-band of cytochrome *P*-420 and curve g that of cytochrome b_5. The other curves (b—f) show the calculated forms of the α-band region at defined concentration ratios of these cytochromes (numbers below the curves), assuming similar absorbance coefficients of both components. Such intermediate forms of the α-band and different locations of the absorbance maxima (numbers above the curves) were experimentally observed in chick microvillar fractions that had been stored for prolonged times (6—36 h) at 4°C, indicating a selective degradation of cytochrome *P*-420. In freshly prepared microvillar membranes, α-bands similar to curves b or c (see Fig. 3) were usually observed in low-temperature spectra.

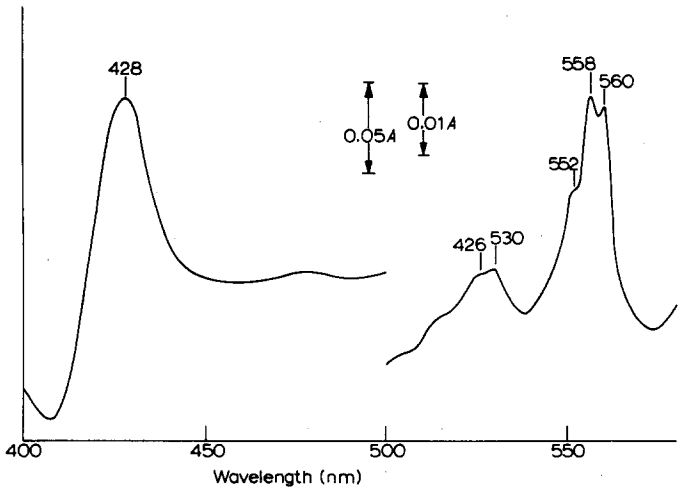


Fig. 6. Dithionite-reduced versus oxidized difference spectrum of purified human erythrocyte plasma membranes recorded at −196°C. Membranes had been washed with pyrophosphate buffer and were suspended in glycerol-containing phosphate buffer (see Methods) to a protein concentration of 2.5 mg/ml. The absorbance is given by the specific *A* scales. Other conditions were as described for Fig. 3. The presence of cytochrome b_5 is indicated by the maximum at 552 nm and the presence of cytochrome *P*-420 by the maximum at 560 nm.

resulted in a shift of the Soret band towards longer wavelengths and in the appearance of a third peak at 560 nm in the α-band region (Fig. 6; cf. Fig. 3), indicating the presence of cytochrome *P*-420. A significant spectral contribution by contaminating haemoglobin was excluded by the specific characteristics of the α-band as described and by the analyses of the effects of cyanide and CO (see above; for a detailed discussion of haemoglobin spectra see Ref. 36). In isolated nuclear membranes from chick erythrocytes, which essentially represent the only endomembranes present in these cells (cf. Ref. 22), small amounts of cytochrome *P*-450 were found in CO-difference spectra besides relatively high concentrations of cytochrome *P*-420.

Cytochrome contents

The concentrations of cytochromes in the various plasma membrane and endomembrane fractions are given in Table II. Data for plasma membranes and endoplasmic reticulum from rat liver are shown for comparison. We have expressed the cytochrome concentrations on a phospholipid basis to allow a direct comparison of membrane fractions with different amounts of non-integral membrane proteins. The data presented in Table II were determined in fractions repeatedly washed with large volumes of buffer (see Methods). The cytochrome b_5 concentration in microvillar membranes was about 29% (chick) and 38% (rat) of that of the corresponding intestinal microsomal fractions. These relative figures are about twice as high as would be expected from the maximum possible contamination by endomembranes (Table I) if one assumed that cytochrome b_5 was an exclusive component of endomembranes. The relative cytochrome b_5 contents in microvillar membranes also agree well with those previously determined for rat liver plasma membranes and endoplasmic reticulum (Table II; cf. Ref. 12). When cytochromes *P*-450 and *P*-420 were

TABLE II

CYTOCHROME CONTENTS IN PLASMA MEMBRANES OF INTESTINAL EPITHELIAL CELLS AND ERYTHROCYTES, IN COMPARISON WITH THOSE OF ENDOMEMBRANES

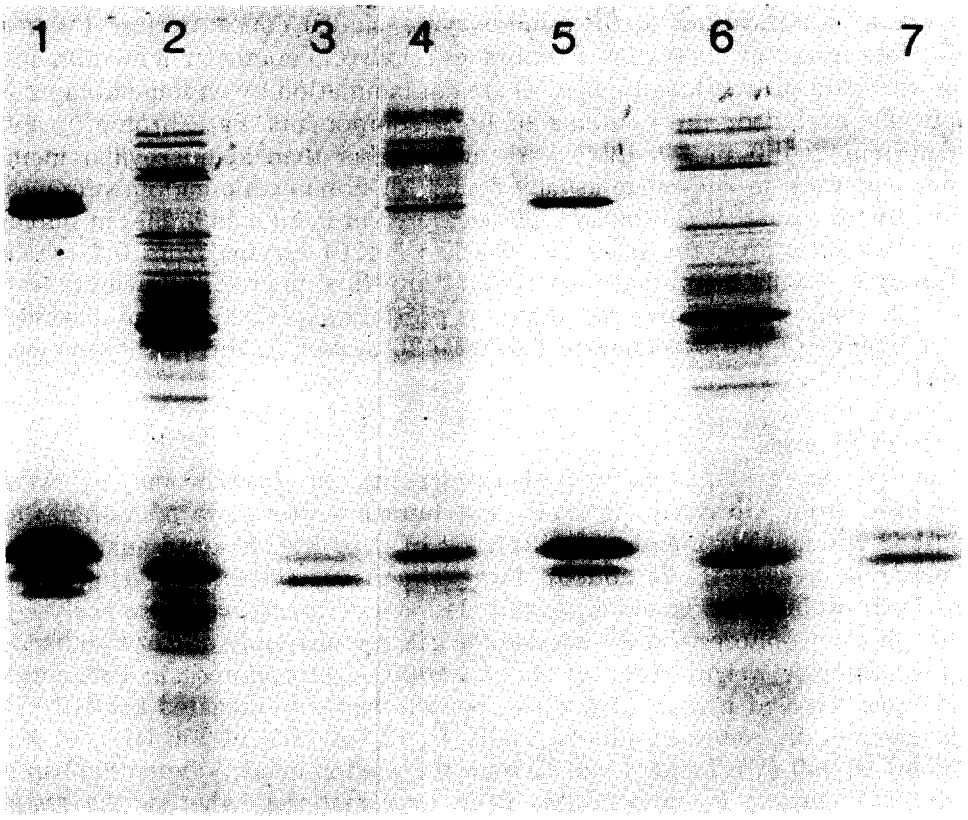
Data represent mean values and standard deviations determined from at least five different preparations. Cytochrome *P*-420 was determined from dithionite-difference spectra at −196°C as described for Fig. 5.

Plasma membrane fraction	Cytochrome contents (pmol/mg phospholipids)			Endo-membrane fraction	Cytochrome contents (pmol/mg phospholipid)		
	b_5	*P*-450	*P*-420		b_5	*P*-450	*P*-420
Chick intestine microvillar membranes	200 ± 17	20 ± 4	150 ± 21	Chick intestine microsomal membranes	695 ± 24	720 ± 35	25 ± 9
Rat intestine microvillar membranes	119 ± 19	9 ± 5	76 ± 15	Rat intestine microsomal membranes	314 ± 30	186 ± 26	31 ± 12
Chick erythrocyte plasma membranes	442 ± 22	<5 **	766 ± 35	Chick erythrocyte nuclear membranes	785 ± 68	33 ± 12	1112 ± 120
Rat erythrocyte plasma membranes	390 ± 20	< 5 **	350 ± 22				
Human erythrocyte plasma membranes	520 ± 31	<5 **	536 ± 28				
Rat liver plasma membranes *	450 ± 30	280 ± 35	260 ± 28	Rat liver endoplasmic reticulum	1550 ± 170	1750 ± 220	270 ± 43

* Data taken from Ref. 12.

** Limit of detection.

Fig. 7. Comparison of major polypeptides of cytochrome b_5-containing fractions solubilized by limited trypsin treatment from human erythrocytes, rat intestinal microvilli and rat liver membranes. Polypeptides were separated by dodecyl sulphate-polyacrylamide gel electrophoresis using 18% acrylamide gels. (a) Slots 1 and 5: partially purified cytochrome b_5 fragments from rat liver endoplasmic reticulum, shown for comparison. Slots 2 and 6: total material solubilized by trypsin treatment from human erythrocyte membranes. Slots 3 and 7: highly purified cytochrome b_5 fragments from rat liver endoplasmic reticulum. Slot 4: material solubilized by trypsin treatment from purified rat liver plasma membranes. (b) Slots 1, 3 and 7: purified cytochrome b_5 fragments solubilized from rat liver total microsomes. Slots 2, 4 and 6: fractions solubilized by trypsin treatment from rat intestinal microvillar membranes. Slot 5: fraction enriched in cytochrome b_5 fragments solubilized from rat erythrocyte membranes and purified according to the method of Omura and Takesue [21]. Slot 8: reference polypeptides (from top to bottom: phosphorylase *a* from rabbit muscle; bovine serum albumin; actin from rabbit skeletal muscle; chymotrypsinogen A from bovine pancreas; horse heart cytochrome *c*). A total of five polypeptide bands (marked by arrows and designated bands I—V with apparent M_r values ranging from 10 000 to 13 500) are distinguishable in varying concentration ratios in the different preparations. Rat liver membranes were characterized by the predominance of the larger polypeptide bands I and II; the minor components (bands III and IV) were apparently lost during further purification. Whereas all five bands were identified in crude preparations of erythrocyte and brush border membranes, the purification treatment resulted in an enrichment of the smaller polypeptides (bands III—V). In addition, major bands were identified at apparent M_r values of 22 000 and 32 000 (microvillar and erythrocyte membranes, respectively) and at 49 000 (liver endoplasmic reticulum). Whether these components are oligomeric aggregates of cytochrome b_5 not dissociated after treatment with sodium dodecyl sulphate and mercaptoethanol or co-purified proteins is not known.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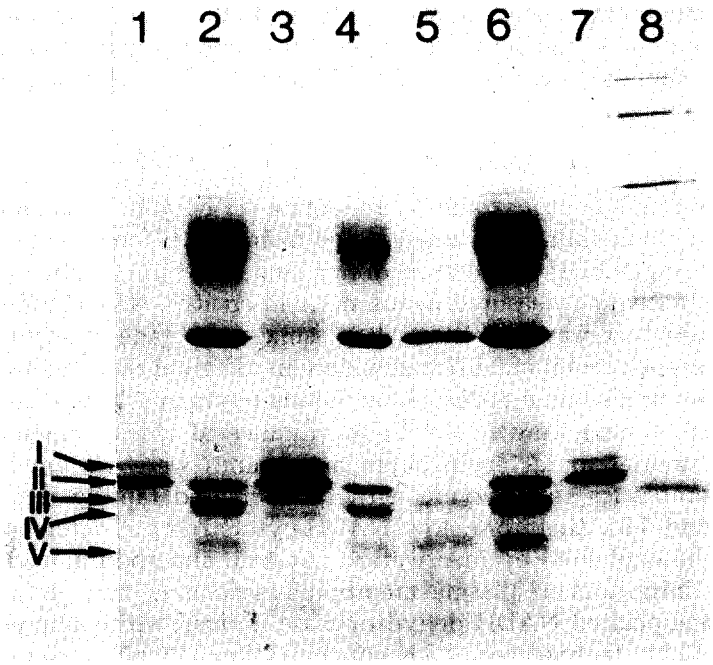
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regarded as modifications of the same enzyme, similar concentrations (23 and 39%) were found in microvillar membranes relative to microsomal membranes. In erythrocyte plasma membranes, where contamination by endomembranes is practically excluded, the contents of both components, cytochrome b_5 and cytochrome '*P*-450 plus *P*-420', were even higher than in microvillar membranes and were in the range usually found for non-hepatic endomembranes. Cytochromes b_5 and *P*-450 plus *P*-420 were present in all membranes in similar molar concentrations, the ratio b_5 : (*P*-450 + *P*-420) varying from 0.6 (chick erythrocyte plasma membranes) to 1.4 (rat intestinal microvillar membranes). The high value in the latter membrane is possibly explained by the observed higher lability of the cytochrome *P*-450/*P*-420 system in this membrane (see above).

Gel electrophoresis

The trypsin-resistant polypeptide fragments of cytochrome b_5 were solubilized from intestinal microvilli and human erythrocyte membranes as described for rat liver membranes (Ref. 21; cf. also Ref. 12) and analysed by gel electrophoresis (Fig. 7a and b). For comparison, cytochrome b_5 preparations from rat liver endomembranes and plasma membranes were also analysed. Although electrophoresis in 8% acrylamide gels revealed only one or two bands in the molecular weight range of about 13 000, electrophoresis in 18% acrylamide gels revealed a total of five polypeptide bands (designated bands I—V) with apparent M_r values of about 13 500 (I), 12 700 (II), 11 200 (III), 10 700 (IV) and 10 000 (V). Bands I and II were the predominant polypeptide bands in the cytochrome b_5 preparations from liver material, whereas the faster migrating components II—V were more characteristic of preparations from brush border and erythrocyte plasma membranes (Fig. 7b). While in crude trypsin extracts from human erythrocytes all five polypeptide bands were visible, bands III and V were enriched after purification by chromatography on Sephadex G-100 and DEAE cellulose (compare slots 2 and 6 in Fig. 7a with slot 5 in Fig. 7b).

Enzyme activities

The presence of an NADH-cytochrome b_5 reductase activity in intestinal microvillar and erythrocyte membranes was suggested from the difference spectra of cytochrome b_5 using NADH as electron donor (see above). As shown in Table III, such enzyme activity (measured as rotenone-insensitive NADH-cytochrome *c* reductase activity) was found in all plasma membrane fractions studied although the absolute numbers differed widely in different membranes. Relatively high NADH-cytochrome *c* reductase activities (comparable to those of rat liver plasma membranes) were found in membranes of chick intestine microvilli, while the activities in rat erythrocyte membranes were extremely low. In all plasma membranes the enzyme activity decreased rapidly upon storage (cf. Refs. 12 and 16); therefore, low NADH-cytochrome *c* reductase activities might partly be explained by inactivation. Atebrin and sodium azide have been reported as inhibitors of plasma membrane-associated, but not of endoplasmic reticulum-associated NADH dehydrogenase in liver, white adipose and mammary tissue [15]. In relatively high concentrations (3 mM atebrin,

TABLE III

NADH- AND NADPH-CYTOCHROME *c* REDUCTASE ACTIVITIES IN PLASMA MEMBRANES OF INTESTINAL EPITHELIAL CELLS AND ERYTHROCYTES, IN COMPARISON WITH THOSE OF ENDOMEMBRANES

Data represent mean values and standard deviations determined from at least five different preparations. Values are expressed as nmol/min per mg phospholipid.

Plasma membrane fraction	NADH-cytochrome *c* reductase	NADPH-cytochrome *c* reductase	Endomembrane fraction	NADH-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
Chick intestine microvillar membranes	235 ± 18	13 ± 4	Chick intestine microsomal membranes	816 ± 95	135 ± 22
Rat intestine microvillar membranes	28 ± 6	9 ± 2	Rat intestine microsomal membranes	286 ± 15	94 ± 10
Chick erythrocyte plasma membranes	30 ± 5	<1 **	Chick erythrocyte nuclear membranes	585 ± 74	<1 **
Rat erythrocyte plasma membranes	5 ± 2	<1 **			
Human erythrocyte plasma membranes	38 ± 7	<1 **			
Rat liver plasma membranes *	380 ± 26	30 ± 5	Rat liver endoplasmic reticulum *	3190 ± 240	300 ± 56

* Data taken from Ref. 12.
** Limit of detection.

100 mM NaN_3), these reagents showed about 20% inhibition of NADH-cytochrome *c* reductase activity in rat and chick microvillar membranes and no significant inhibition in the corresponding microsomal fractions. In the various membrane fractions from erythrocytes, azide and atebrin had very little effect on the enzyme.

NADPH-cytochrome *c* reductase activities were present in both chick and rat intestinal microsomal fractions. In the corresponding microvillar membrane fractions only 10% or less of the microsomal activities were detected, i.e., figures that are too low to be considered as proof of localization. In all erythrocyte membranes studied (including chick nuclear membranes), NADPH-cytochrome *c* reductase activity was absent.

Discussion

Our finding that plasma membranes from intestinal microvilli and erythrocytes contain both cytochromes b_5 and *P*-420 as well as NADH-cytochrome *c* reductase is a clear demonstration that these redox components can occur in cell surface membranes and are not restricted to endomembranes. These results confirm and extend our previous observations in plasma membranes of milk fat

globules [16,17] and rat liver [12]. Alternative explanations of the finding of these components in plasma membranes from diverse cells would have to be based on the assumption of considerable contamination by endomembrane material; this, however, is very unlikely in the case of milk fat globule membranes and microvillar membranes and is excluded in the case of the mammalian erythrocyte ghost. Therefore, we conclude that these redox components are widespread constituents of cell surface membranes and suggest that they serve specific biological functions in these membranes.

In previous studies on the cytochrome *P*-450-dependent drug-metabolizing enzyme system of the small intestinal mucosa, usually 'microsomal' fractions, i.e., particulate material not sedimentable at about 10 000 × *g* for 10 min, have been used (e.g., Refs. 20 and 38—40; such fractions also include light microsomal membranes described as 'plasma membranes', cf. Ref. 41). Such microsomes from intestinal epithelial cells are poorly defined fractions since they are only moderately enriched in endoplasmic reticulum-derived membranes and contain relatively high proportions of vesiculated fragments of microvilli and Golgi apparatus, secretory and endocytotic vesicles including coated vesicles, mitochondria and ribosomes. In contrast, pure fractions of brush border microvilli and microvillar membranes can be obtained from this tissue (e.g., Refs. 19, 35, 42, 43). Little information, however, is available on the presence of redox components in apical plasma membranes of the intestine. Fujita et al. [34] have found some NADH-cytochrome *c* reductase activity in isolated whole brush borders from mouse intestine, and Weiser et al. [44] have mentioned the absence of NADPH-cytochrome *c* reductase activity in rat microvillar membranes. These reports are essentially in agreement with the findings reported here.

We do not know whether cytochromes b_5 and *P*-420 and the NADH-cytochrome *c* reductase are ubiquitous components in microvillar membranes of other cells. In whole brush border membranes from rabbit kidney cortex, Ichikawa [45] reported the presence of both cytochromes b_5 and *P*-450 as well as NADH- and NADPH-cytochrome *c* reductase activities. Garcia et al. [46] have even described a complete mitochondria-like respiratory chain containing cytochromes *b*, *c* and *a* in their brush border preparation from rat kidney. However, adequate morphological and chemical characterization of the fractions was not included in these reports, and possible contamination was discussed solely on the basis of the absence of some enzyme activities (e.g., the absence of activities of succinate dehydrogenase, an enzyme known to be deactivated by a variety of factors; cf. Refs. 47, 48). Our finding that high concentrations of cytochrome *P*-420 (but not of *P*-450) are present in microvillar membranes, whereas microsomal membranes from the same tissue contain relatively high concentrations of cytochrome *P*-450 (but not of *P*-420), suggests that these components have different properties in different membranes. As cytochrome *P*-450 is known to be converted to cytochrome *P*-420 by activities of proteases and phospholipases [37], it is also conceivable that the observed differences in cytochromes are due to the action of hydrolytic enzymes in the specific membranes. Intestinal microvillar membranes are notoriously rich in hydrolytic enzyme activities (e.g., Refs. 33—35).

While cytochrome *P*-420 has already been reported from a particulate

('stroma') fraction of mammalian erythrocytes [49], cytochrome b_5 has been hitherto detected only as a soluble component, and not as a membrane-bound component, of the erythrocyte [50—52]. On the other hand, the occurrence of NADH-cytochrome *c* reductase and related enzymes in erythrocyte membranes has been repeatedly reported (e.g., Refs. 53—57). Slaughter and Hultquist [58] recently described the presence of cytochrome b_5 in a particulate fraction from erythroleukaemia cells and concluded that the soluble forms of cytochrome b_5 and NADH-cytochrome *c* reductase of mature erythrocytes were derived from membrane-bound forms resulting from proteolysis in endomembranes of immature erythroid cells [51—53, 58—60]. Our present demonstration that a firmly membrane-bound form of cytochrome b_5 exists in the mature mammalian erythrocyte ghost casts some doubts on the concept of an exclusive origin of the soluble cytochrome b_5 from proteolysis in endomembranes during earlier stages of erythropoesis. Rather, it seems likely that some soluble cytochrome b_5 and NADH-cytochrome b_5 reductase are derived from the plasma membrane of the reticulocyte and/or erythrocyte. The immunological similarity between the soluble erythrocyte redox components and liver microsomal cytochrome b_5 and NADH-cytochrome *c* reductase, which has been observed by various authors [52,53,58,59], is in accord with demonstrations that the plasma membrane-bound cytochrome b_5 of hepatocytes [12] and mammary epithelium [17] is also immunologically related to the microsomal cytochrome b_5 of liver. On the other hand, we cannot at present exclude that differences between cytochrome(s) b_5 of plasma membranes and endomembranes exist. Our observations of (i) different patterns of the cytochrome b_5 fragments obtained by trypsin treatment from the different membranes, and (ii) the abundance of cytochrome *P*-420 but not cytochrome *P*-450 in plasma membranes of a variety of different cells support the hypothesis that the plasma membrane redox components are different in properties from those of endomembranes. Differences of the properties in different membranes have also been observed for NADH-cytochrome *c* reductase which is more labile in isolated Golgi apparatus and plasma membranes, compared to endoplasmic reticulum and nuclear membranes [12,16,61—64].

It remains to be elucidated whether the plasma membrane-bound cytochrome b_5 and NADH-cytochrome *c* reductase serve different functions than the corresponding soluble components in the erythrocyte cytosol, where they are involved in methaemoglobin reduction (e.g., Refs, 52, 60, 65). In liver microsomes, both cytochrome b_5 and NADH-cytochrome b_5 reductase have been shown to be amphiphilic proteins, with their membrane-binding segment located at the carboxyl terminus [66,67], and it has been proposed that this segment of the molecule is inserted into the microsomal membrane after completion of the synthesis of the apoprotein on free ribosomes (Refs. 63, 68; for a contrasting view, see Ref. 69). Consequently, the demonstration of membrane-bound forms of cytochromes b_5 in Golgi apparatus and plasma membranes raises the question as to whether these proteins are directly inserted into the plasma membrane or whether they are inserted into endomembranes and subsequently translocated to the surface membrane by membrane flow processes [70]. In this context, the observed coincidental occurrence in surface membranes of cytochrome *P*-420, i.e., a protein probably derived from cyto-

chrome *P*-450 which is synthesized on membrane-bound polyribosomes and is directly inserted into the microsomal membrane (Ref. 71; Bar-Nun, S. and Sabatini, D.D., personal communication) would be indicative of contributions from endoplasmic reticulum and nuclear envelope (in avian erythrocytes) to the biogenesis of plasma membrane components.

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