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ELECTRON CARRIERS OF THE BOVINE ADRENAL CHROMAFFIN GRANULES

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SUMMARY

Chromaffin granules of bovine adrenal medulla essentially free of contamination from mitochondria, lysosomes and fragments of endoplasmic reticulum, have been lysed by hypo-osmotic shock. The membrane fraction has been shown to contain a *b*-type cytochrome (cytochrome b_{561}), flavoprotein(s) and NADH: (acceptor) oxidoreductase (EC 1.6.99.3) with ferricyanide, 2,6-dichlorophenolindophenol (DCIP), bovine heart ferricytochrome *c* and the endogenous ferricytochrome b_{561} as acceptors. Furthermore, the membrane fraction catalyzes the rapid oxidation of bovine heart ferrocytochrome *c*. This enzymic activity, which is completely inhibited by CN^- ($3 \cdot 10^{-4}$ M) and CO, is not attributed to cytochrome $a + a_3$ (ferrocytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1).

INTRODUCTION

The catecholamines of the adrenal medulla are mainly stored within specific cytoplasmatic granules other than mitochondria[1,2]. A characteristic feature of these particles, termed chromaffin granules, is their high content of adenine nucleotides, mainly ATP[3-8], which is present in a stoichiometric amount of the granular-bound pool of catecholamines[9]. It was suggested by BLASCHKO[10] that the rate-limiting step in the biosynthesis of the pressor amines is the hydroxylation of the side chain of dopamine (3,4-dihydroxyphenylalanine) to form noradrenaline. KAUFMAN[11] and KAUFMAN *et al.*[12] have shown that this hydroxylation is catalyzed by a copper-protein enzyme (dopamine β-hydroxylase) of the chromaffin granule and that the reaction fits into the general equation for mixed-function oxidases. Thus, the reaction is characterized by a requirement for an external electron donor, and the oxygen in the hydroxylated product comes from molecular O_2 and not from water[12]. Ascorbate has been shown[13] to stimulate the β-hydroxylation of dopamine even in adrenal particles, and the requirement for ascorbate becomes more complete as the enzyme is purified[13].

The presence of a *b*-type cytochrome in the chromaffin granules of bovine adrenal medulla has been reported[14-17], and it has been termed cytochrome *b*-559 (ref. 17). Except for spectral properties of a partially purified cytochrome, very little is known about the physico-chemical properties and physiological significance of this heme

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

protein, and the existence of other electron transfer pigments in these organelles has not previously been reported. The purpose of the present study is to describe the identification of known and new electron transfer pigments and functions of the chromaffin granules. The possibility of their participation in hydroxylation of dopamine to noradrenaline is discussed.

MATERIALS AND METHODS

Chromaffin granules

The chromaffin granules were isolated from fresh bovine adrenals using a slight modification[18] of the procedure of SMITH AND WINKLER[19]. Unless otherwise stated pellet P_c was used .The chromaffin granules, obtained as a pink sediment in 1.6 M sucrose, were lysed by careful resuspension in ice-cold 50 mM potassium phosphate buffer (pH 6.5) (approx. 1 ml/mg of protein), by homogenization in a Potter–Elvehjem-type homogenizer. The homogenate was centrifuged ($1 \cdot 10^6 \times g_{av} \cdot$min), and the sediment of disrupted granule membranes and a few intact granules were resuspended twice with ice-cold 50 mM potassium phosphate buffer (pH 6.5), homogenized and centrifuged as described above. The final sediment of disrupted granule membranes was resuspended by homogenization in 50 mM potassium phosphate buffer (pH 7.4) to give a concentration of approx. 15 mg of protein per ml.

For the assay of glucose-6-phosphatase (EC 3.1.3.9) activity, the water-insoluble membrane fraction was prepared by lysis and washing in histidine–EDTA buffer (pH 6.5) (see below).

Preparation of microsomes

The $3 \cdot 10^5 \times g_{av} \cdot$min supernatant of the standard procedure[18] was recentrifuged at $3 \cdot 10^5 \times g_{av} \cdot$min (Sorvall No. SS-34 rotor), and the resulting supernatant was centrifuged at $9 \cdot 10^6 \times g_{av} \cdot$min (Spinco No. 40 rotor) to yield a supernatant and a microsomal pellet. This pellet was resuspended in 10 times its volume of 0.3 M sucrose by a manually operated Teflon–glass homogenizer and centrifuged at $9 \cdot 10^6 \times g_{av} \cdot$min. The washing procedure was repeated once with sucrose and twice with a suitable buffer, *e.g.* histidine–EDTA buffer for assay of glucose-6-phosphatase activity (see below).

Preparation of mitochondria

The interphase band obtained by sucrose density centrifugation of the large granule fraction[18] was resuspended in 10 times its volume of 0.3 M sucrose and centrifuged at $16 \cdot 10^3 \times g_{av} \cdot$min to remove rests of cell debris *etc.*, and the resulting supernatant was centrifuged at $12 \cdot 10^4 \times g_{av} \cdot$min (Sorvall No. SS-34 rotor). The mitochondrial pellet was washed twice by repeating the above procedure.

Cytochrome c

Cytochrome *c*, extracted from bovine heart muscle by dilute H_2SO_4 at pH 4 and 4°, was obtained in the monomeric form by gel filtration on Sephadex G-75 (ref. 20). From the preparation thus obtained, the four different forms of the protein (Cy I–Cy IV) were isolated by disc electrophoresis on polyacrylamide gel[21]; the percentage distribution of these forms was essentially as reported previously for a similar prepara-

tion[21]. Ferro- and ferricytochrome *c* were prepared essentially by the method of YONETANI AND RAY[22]; Sephadex G-10 was used for the molecular sieve chromatography in order to remove the reducing and oxidizing agents.

Chemicals and buffers

O_2 was removed from commercial N_2 by passage through an alkaline solution of pyrogallol[23]. CO was purchased from Baker Chemical Co., U.S.A. The sodium salts of NADH and NADPH were obtained from C. F. Boehringer and Söhne, Germany, and Triton X-100 as well as 2,6-dichlorophenolindophenol (DCIP) were obtained from Sigma Chemical Co., U.S.A.

Extraction and purification of heme

The heme was extracted from a pellet of chromaffin granule membranes with the acid-acetone method (acetone containing 5 % by volume of concentrated HCl) and purified by the method of FALK[24].

Thin-layer chromatography of heme

Thin-layer chromatography of the extracted heme was carried out on silica gel (MN-Polygram, Type Sil N-HR from Macherey-Nagel & Co., Germany) by using the 2,6-lutidine–water system of CHU AND CHU[25].

Measurement of light absorption spectra

Absolute and difference spectra were measured in a Shimadzu multipurpose recording spectrophotometer (Model MPS-50L) calibrated against several emission bands of a mercury arc and equipped with external base-line compensator. Cuvettes of 10-mm light path were used and the cell compartment was thermostatically controlled at 25°.

Analytical methods

The concentrations of NADH and NADPH were determined on the basis of the molar absorption coefficient of $6.22 \cdot 10^3$ at 340 nm (ref. 26). The concentrations of bovine heart cytochrome *c* were determined by using the molar absorption coefficient $29.04 \cdot 10^3$ at 550 nm (reduced)[27].

Protein concentration was determined colorimetrically according to the method of EGGSTEIN AND KREUTZ[28], using bovine serum albumin from Sigma as a standard. The concentration of albumin in the standard stock solution was determined by amino acid analyses of acid hydrolysates (6 M HCl) on a Biochrom amino acid analyzer (BioCal, Germany) using a modification (T. FLATMARK, unpublished results) of the stepwise four-buffer elution program of DUS *et al.*[29]. The amino acid composition of bovine serum albumin given by MOORE AND STEIN[30] was used as reference.

NADH(NADPH): (acceptor) oxidoreductase activities were measured spectrophotometrically. Standard components of the reaction mixtures were as described in Figs. 3 and 4 and Table II. The reaction was started with the addition of enzyme (membranes), and the change in absorbance at 340, 550 and 600 nm was followed using ferricyanide, ferricytochrome *c* and DCIP as acceptors, respectively. The non-enzymic reduction of the acceptor by NADH (NADPH) was determined each time and subtracted from the value obtained with the complete system. The specific activities were

expressed as μmoles of donor oxidized or acceptor reduced per min per mg of protein.

Succinate oxidase activity was assayed polarographically as previously described[31]. Native bovine heart cytochrome *c* (Cy I)[31] was added at a concentration which gave maximum stimulation of mitochondrial O_2 consumption.

Acid phosphatase (EC 3.1.3.2) activity was assayed using a slight modification of the *p*-nitrophenol method of LOWRY *et al.*[32].

Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed as described by DE DUVE *et al.*[33]. The P_i liberated was determined by the method of RATHBUN AND BETLACH[34] with slight modification. Thus, the concentrations of the stock solutions of ammonium molybdate and $SnCl_2$ were 5 % (w/v) and 15 mM, respectively, as compared to 2 % (w/v) and 6.75 mM in the original procedure[34].

The measurement of reduction and oxidation of membrane-bound cytochrome b_{561} was performed in cuvettes of 10 mm light path using an Aminco–Chance dual-wavelength spectrophotometer with the reference wavelength set at 550 nm (isosbestic point) and the measuring wavelength set at 561 nm. The temperature was kept constant by a thermostatically controlled cell housing.

RESULTS

Light absorption spectra of the membrane fraction

In agreement with the results of other workers[14–17], the light absorption spectrum of a suspension of chromaffin granule membranes revealed the presence of a heme protein. Difference spectra were recorded in order to reduce spectral errors due to

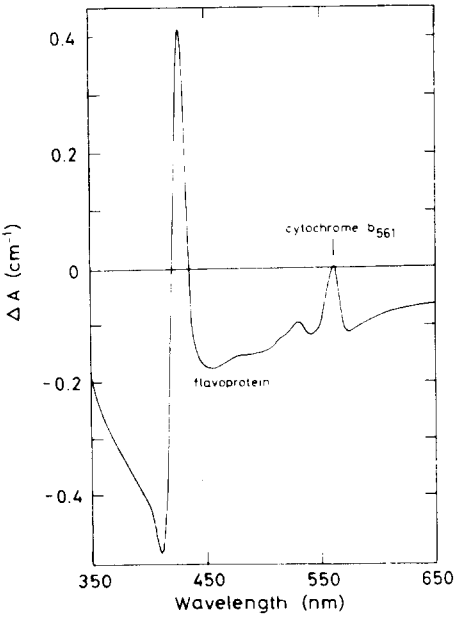


Fig. 1. The spectrophotometrically detectable electron transfer pigments of chromaffin granule membranes. These changes of absorbance occur when the oxidized pigments (ferricyanide) become reduced upon addition of dithionite. The cuvettes contained: 50 mM potassium phosphate buffer at pH 6.5 and 25°; membranes, 0.74 mg of protein per ml.

TABLE I

COMPARISON OF SOME ENZYME ACTIVITIES IN THE MEMBRANE FRACTION OF CHROMAFFIN GRANULES AND IN ADRENAL MEDULLA MICROSOMES

Experimental conditions were as described in the text.

	Succinate oxidase * (*nmoles* O_2*/min per mg of protein*)	*Oxidation of cytochrome c* ** (*μmoles/min per mg of protein*)	*Glucose-6-phosphatase* *** (*μmoles* P_i*/60 min per mg of protein*)	*NADH : ferricyanide oxidoreductase* *** (*μmoles/min per mg of protein*)
Membrane fraction of chromaffin granules	0	0.037	0.044	0.027
Microsomal fraction	0	0.014 §	0.272 §§	0.043

* The protein concentration was always in the range 0.5–0.8 mg protein/ml.

** The results represent the mean value of four assays, *i.e.* at four different protein concentrations. The initial concentration of ferrocytochrome *c* was 10.7 μM.

*** The results represent the mean value of five assays.

§ The corresponding value for bovine liver microsomes was 0.019 μmole cytochrome *c* oxidized per min per mg of protein.

§§ The corresponding value for bovine liver microsomes was 4.54 μmoles P_i/60 min per mg of protein.

turbidity of the membrane suspension. The reduced *minus* oxidized difference spectrum (Fig. 1) of the membrane fraction has absorption maxima at 430, 532 and 561 nm at pH 6.5–7.0 (50 mM phosphate buffer). In addition, a broad negative peak around 455 nm was found, indicating the presence of flavoprotein(s). The same absorption maxima and minima were observed when the membranes were solubilized either by Triton X-100 (0.4 %, v/v) or by sonication.

No spectral peaks characteristic of the mitochondrial cytochromes were observed even when membrane suspensions of very high protein concentration were used for recording of difference spectra.

Characterization of heme

The heme extracted from the membrane pellet of chromaffin granules revealed a single spot upon thin-layer chromatography on silica gel and the R_F value was identical with that of protoheme IX isolated from bovine hemoglobin by the same procedure. The identity of the two hemes was confirmed by the absorption spectra in ether; the main absorption peak was obtained at 381 nm for both hemes.

Some enzymic properties of the membrane fraction

Previous electron microscopic examinations as well as assays of various marker enzymes on chromaffin granules prepared by the present method have revealed[18] that the preparation is essentially free from contamination with mitochondria, lysosomes and fragments of endoplasmic reticulum. These observations have been confirmed in the present study by enzymic assays (Table I and Fig. 2) as well as by spectroscopic studies (Fig. 1). Thus, no succinate oxidase activity has been observed in any of our preparations, and the specific glucose-6-phosphatase activity is low, *i.e.* only approximately 15 % of that determined for the unfractionated microsomal fraction. The significance of this activity in the granule membrane fraction is, however, not yet clear, though it seems certain that it cannot be attributed to microsomal contamination[18].

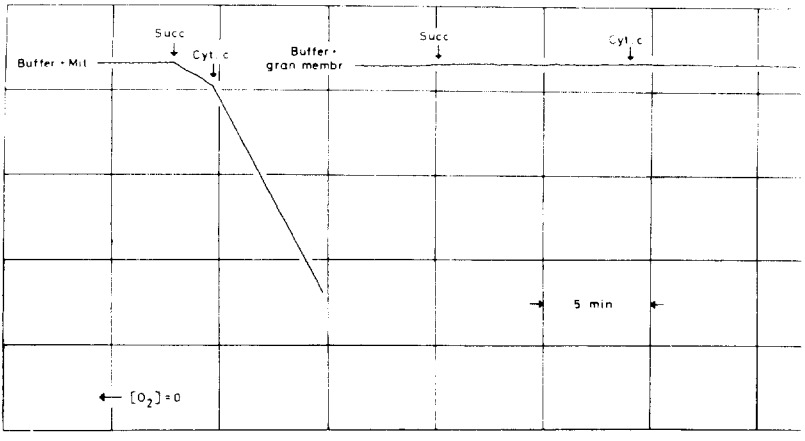


Fig. 2. Comparison of succinate oxidation by mitochondria of adrenal medulla (Mit.) and chromaffin granule membranes (gran. membr.) in 26 mM potassium phosphate buffer (pH 7.4); protein concentrations 0.83 mg/ml (Mit.) and 0.54 mg/ml (gran. membr.); potassium succinate, 35 mM; cytochrome *c* (Cy I), 1 μM; O_2, 278 μM; 25°.

From Fig. 3 and Tables I and II it is seen that the membrane fraction catalyzed the rapid oxidation of bovine heart ferrocytochrome *c* and that the specific activity is approx. 2.5 times higher for the granule membrane fraction than for unfractionated adrenal medulla microsomes. Thus, the activity ratio oxidation of cytochrome *c*/glucose-6-phosphatase is 16 times higher in the granule membrane fraction than in the microsomal fraction. The oxidation of cytochrome *c* was completely inhibited by CO and CN^- ($3 \cdot 10^{-4}$ M), and solubilization of the membranes with 2.5 % (v/v) Triton X-100 (1 min of preincubation) resulted in a 50 % reduction of this catalytic activity.

From Fig. 3 and Table II it is also seen that a rapid reduction of ferricytochrome *c* is obtained with NADH (but not with NADPH) as the electron donor.

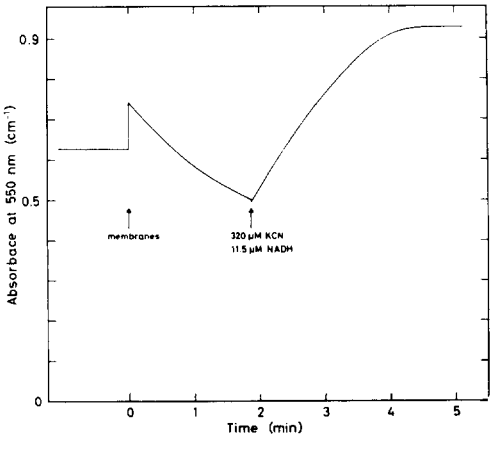


Fig. 3. Oxidation and reduction of bovine heart cytochrome *c* catalyzed by the membrane fraction of chromaffin granules. The cuvette contained: 50 mM potassium phosphate buffer (pH 7.4); cytochrome *c*, 33.0 μM; membranes, 0.10 mg of protein per ml; 25°.

TABLE II

SOME ELECTRON TRANSPORT ACTIVITIES OF CHROMAFFIN GRANULE MEMBRANES

Experimental conditions were as described in the text; 50 mM phosphate buffer (pH 7.4); 25°. Activities are expressed as μmoles of NADH(NADPH) oxidized and cytochrome *c* reduced/oxidized per min per mg of protein. Mean values of at least three experiments.

Electron donors	*Electron acceptors*			
	Ferricyanide (79 μM)	*DCIP* (38 μM)	*Ferricytochrome c* (32 μM)	O_2 (260 μM)
NADH (27 μM)	0.0270	0.0264	0.0628 *	0.0015 **
NADPH (27 μM)	0	0	0	0
Ferrocytochrome *c* (20 μM)	—	—	—	0.0435 **

* In the presence of $3 \cdot 10^{-4}$ M KCN.
** Complete inhibition by $3 \cdot 10^{-4}$ M KCN and CO. No inhibition by 6 mM EDTA.

NADH: (acceptor) oxidoreductase activities

The membrane fraction catalyzes the oxidation of NADH using various electron acceptors, whereas no activity was observed with NADPH as electron donor (Fig. 4 and Table II). Solubilization of the membranes by preincubation in Triton X-100 resulted in a slight initial increase in the specific NADH-linked ferricyanide oxidoreductase activity. However, even in the presence of this detergent no NADPH-linked activities could be detected (Fig. 5). The initial decrease in absorbance at 340 nm is totally explained as solubilization of the membranes. Thus, the steady state of NADPH oxidation after solubilization was the same as prior to the addition of membranes.

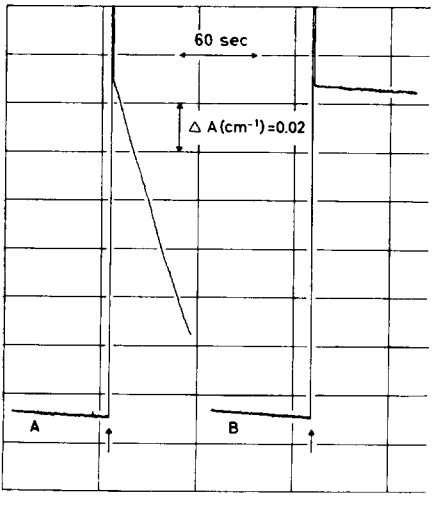
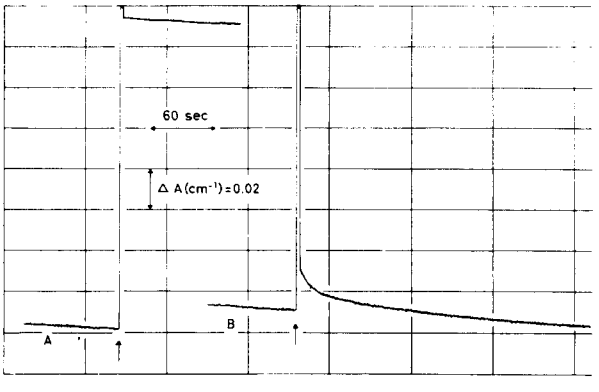


Fig. 4. Typical progress curves of the oxidation of NADH (Curve A) and NADPH (Curve B) in the standard procedure of NADH(NADPH): ferricyanide oxidoreductase assay. The cuvettes contained: 50 mM potassium phosphate buffer (pH 7.4); potassium ferricyanide, 79 μM; NADH (NADPH), 25.2 μM; 25°. Membranes (0.072 mg of protein per ml) were added as indicated by arrows. The rate of NAD^+ ($NADP^+$) formation was measured at 340 nm.

Oxidation and reduction of membrane-bound cytochrome b^{561}

Pellets of intact chromaffin granules in 1.6 M sucrose are pink coloured, and storage for several days at 4° does not change this appearance. On the other hand, when the granules are lysed, *e.g.* by hypo-osmotic shock, the colour of the pellets changes from pink to brown, and in freshly prepared membranes the cytochrome b_{561} is found to be present mainly in the oxidized form (see Figs. 6A and 6B).



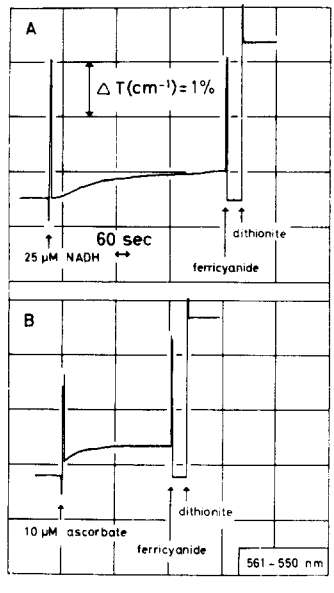


Fig. 5. The effect of the detergent Triton X-100 on the NADPH: ferricyanide oxidoreductase activity. A. Standard assay procedure (for details see Fig. 4). B. Standard assay procedure + Triton X-100, 0.4% (v/v). Membranes (0.066 mg of protein per ml) were added as indicated by arrows. The rate of $NADP^+$ formation was measured at 340 nm.

Fig. 6. Reduction of membrane-bound cytochrome b_{561} by NADH (A) and by ascorbate (B) at 25°. The cuvettes contained: 50 mM potassium phosphate buffer (pH 6.5); 6 mM EDTA; membranes, 0.61 mg of protein per ml; 25°. The rate of formation of reduced (oxidized) cytochrome b_{561} was measured with an Aminco–Chance dual wavelength spectrophotometer.

Membrane-bound cytochrome b_{561} is completely oxidized by ferricyanide and completely reduced by dithionite in 'instant' oxidation–reduction reactions (Figs. 6A and 6B). The cytochrome is, however, only partially reduced by ascorbate and the progress curve for this reaction is more complex. Thus, at all concentrations of ascorbate tested so far (from 10 μM to 4 mM) the time curve is biphasic, *i.e.* it is composed of an initial rapid phase and a final slower phase (Fig. 6B). As seen from Fig. 6A the membrane -bound cytochrome b_{561} is even more slowly reduced by 25 μM NADH, but the percentage degree of reduction is almost the same as with 10 μM ascorbate. The rate slightly increased when EDTA was present in the reaction mixture and this chelating agent was therefore included in the standard procedure. Exclusion of O_2 also slightly increased the rate as well as the extent to which cytochrome b_{561} was reduced by NADH, whereas *p*-chloromercuribenzoate inhibited the reaction. Thus, it may be concluded that the membranes contain a cytochrome b_{561} oxidoreductase activity linked to NADH.

DISCUSSION

A simple method for the large-scale preparation of chromaffin granules from adrenal medulla was introduced by SMITH AND WINKLER[19], but the isolated granules were reported to be slightly contaminated by mitochondria (or mitochondrial fragments) and lysosomes. In our modification[18] of their preparation procedure, ultrastructural studies of the pellets of chromaffin granules obtained have shown that the pellets were essentially free of contamination by mitochondria and lysosomes as well as by fragments of endoplasmic reticulum. This conclusion is confirmed in the present study. Thus, no succinate oxidase activity was observed even when the assays were carried out at a high protein concentration, and no spectral peaks characteristic of mitochondrial cytochromes were observed. The presence of the same enzymic activities in the granule membranes and in the microsomal fraction (Table I) might suggest a contamination of the granules with microsomes. However, the experimental evidence against this view is as follows: (1) The ultrastructural studies of the pellets of intact chromaffin granules have revealed that they were essentially free of contamination by smooth- and rough-surfaced microsomal structures[18]. (2) The pellets of intact chromaffin granules obtained by our procedure[18] are opaque and homogeneously pink coloured, and storage for several days at 4° did not change this appearance (see RESULTS). On the other hand, the microsomal fraction is obtained as a transparent dark red-brown pellet. (3) The specific NADH: ferricyanide oxidoreductase activities did not change significantly even after three washes of the chromaffin granules in keeping with the finding that the glucose-6-phosphatase activity remained essentially the same following this procedure[18]. (4) The ratio, oxidation of cytochrome *c*/glucose-6-phosphatase (Table I) is 16 times higher and the ratio, NADH: ferricyanide oxidoreductase/glucose-6-phosphatase, is 4 times higher in the granule membrane fraction than in the microsomal fraction. The presence of the same enzymic activities in the granule membrane as in the microsomal fraction is analogous to the similarities in enzyme composition between the outer mitochondrial membrane and the endoplasmic reticulum[35–37]. Thus, we consider that the most significant of our criteria of purity of the chromaffin granules are the ultrastructural studies[18]. It may also be noted that the electrophoretic patterns given by the proteins of the chromaffin granule membrane isolated by a procedure[19] similar to ours are distinct from those given by microsomal membrane proteins[38].

The rapid oxidation of cytochrome *c*, catalyzed by the membrane fraction, calls for a special comment. This activity, which is inhibited by CO and CN^-, is not due to cytochrome $a + a_3$ since the contamination of the chromaffin granules with mitochondria (or mitochondrial fragments) was found to be essentially zero as discussed above. This oxidation is possibly explained by the presence of a copper-protein enzyme in the membranes, *i.e.* the dopamine β-hydroxylase isolated and studied by KAUFMAN[11] and KAUFMAN *et al.*[12]. They showed that CO and CN^- bind to the copper of this enzyme, a reaction which inhibits its catalytic function. Thus, compounds that are inhibitors of this mixed-function oxidase also inhibited the oxidation of cytochrome *c* catalyzed by the membrane fraction used in the present work.

Spectroscopic studies of the subcellular fractions of bovine adrenal medulla have established that fragments of the endoplasmic reticulum (the microsomes) as well as the chromaffin granules contain a *b*-type cytochrome[14–17]. The position of the

α-peak, however, varies in the literature from 559 to 562 nm. In the present study on membrane fragments of the chromaffin granules we have found the α-peak at 561 nm (Fig. 1) which is in agreement with the wavelength reported by Tsou[39] who carried out spectroscopic studies of intact adrenal medulla tissue from horse. In addition, the reduced *minus* oxidized difference spectrum of the membrane fraction (Fig. 1) indicated the presence of flavoprotein(s), and this result was confirmed by the enzymic activities. Thus, in freshly prepared membranes a NADH: (acceptor) oxidoreductase of high donor but apparently low acceptor specificity was found. It should be emphasized that the demonstration of an absence of NADPH-dependent activities requires freshly prepared membranes. Upon prolonged storage at 0° NADPH-linked oxidoreductase activities appear slowly, and at the same time, the NADH-linked oxidoreductase activities decrease (O. TERLAND AND T. FLATMARK, unpublished results). Furthermore, dilution of the membrane fraction or addition of the detergent Triton X-100 increases the rate of this change in enzymic activities (O. TERLAND AND T. FLATMARK, unpublished results). This marked effect of dilution and of detergent on the donor specificity suggests that the structural state of the membrane may be of general importance in the characterization of enzyme activities in the chromaffin granule membrane.

The NADH-linked reduction of cytochrome b_{561} (Fig. 6) and its oxidation by dopamine (T. FLATMARK AND O. TERLAND, unpublished results) may suggest that the membrane-bound flavoprotein(s) and cytochrome b_{561} are linked to an electron transfer chain where the copper-protein enzyme dopamine β-hydroxylase serves as the terminal oxidase. Further studies on this reaction are now in progress.

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