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NADH(NADPH):(ACCEPTOR) OXIDOREDUCTASE ACTIVITIES OF THE BOVINE ADRENAL CHROMAFFIN GRANULES

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SUMMARY

1. An analysis of the NADH(NADPH):(acceptor) oxidoreductase activities of adrenal chromaffin granules depleted of low molecular weight substances (notably catecholamines and ATP), has been made in terms of the flavoprotein(s) and cytochrome b_{561} previously reported (Flatmark, T., Terland, O. and Helle, K. B. (1971) *Biochim. Biophys. Acta* 226, 9–19).

2. The overall NADH oxidation of freshly prepared granule preparations was rather low (0.06–0.32 nmole NADH oxidized per min per mg of protein; $n=21$), but approximately 10 times higher than the NADPH oxidation (0–0.04 nmole NADPH oxidized per min per mg of protein; $n=6$).

3. Of the total NADH oxidase activity of the granule preparation approximately 45% was attributed to an oxidative sequence involving a *p*-hydroxymercuribenzoate (PHMB)-sensitive flavoprotein, cytochrome b_{561} and a cyanide-sensitive oxidase. This NADH oxidase activity was greatly stimulated by mammalian cytochrome *c* ($K'_m = 1.6 \cdot 10^{-6}$ M) and resembled the microsomal activity, *e.g.* of liver, in its insensitivity to antimycin A. Approximately 45% of the NADH oxidase activity was attributed to an oxidative sequence probably involving a flavoprotein and an endogenous electron acceptor. This activity was inhibited only at high concentrations of PHMB and was stimulated by fumarate. An additional 10% of the NADH oxidation was completely insensitive to PHMB as well as to cyanide and revealed the same specific activity with NADPH.

4. The NADH(NADPH): (acceptor) oxidoreductase activities vary according to the structural state of the membrane phase. Thus, the oxidative reactions suffer profound changes upon disintegration of the membrane structure by various means, *e.g.* by dilution (particularly at higher temperatures) and by detergent (Triton X-100). One of the most remarkable features of this transition was the appearance of a fumarate-stimulated oxidation of NADH which did not require molecular oxygen, but could be accounted for on the basis of the presence of an endogenous electron acceptor of molecular weight $<25\,000$. The nature of this electron acceptor is discussed.

Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

INTRODUCTION

Recent studies on chromaffin granule membranes from bovine adrenal medulla[1,2] as well as from bovine splenic nerves[3] have revealed the presence of at least two electron carriers in addition to dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine:oxygen oxidoreductase (hydroxylating), EC 1.14.2.1), *i.e.* flavoprotein(s)[1-3] and a unique high-potential *b*-type cytochrome (termed b_{561})[1-3]. We have found that the flavoprotein(s) and cytochrome b_{561} are largely confined to the membrane phase and appear to be integral parts of the granule membrane structure[1]. To clarify the biological significance of these electron carriers, we have primarily focused upon the possibility that they may be linked to the main biosynthetic function of these organelles, *i.e.* the hydroxylation of dopamine to form noradrenaline. Preliminary observations[4] supporting this possibility have made it necessary to achieve a more detailed characterization of the oxidative reactions as well as of the individual electron carriers of the membrane and the matrix phase.

The present study was undertaken to establish the acceptor and donor specificities of the individual electron carriers in some more details, particularly those of the flavoprotein(s). In addition, the effect of aging as well as of various physical and chemical treatments on the oxidative reactions have been studied to get a more clear picture of the stability of the granule membrane enzymes and the possibility of introducing artifacts during isolation, storage and solubilization of the chromaffin granules.

MATERIALS AND METHODS

Chromaffin granules. Isolation and subfractionation

The chromaffin granules were isolated from fresh bovine adrenal glands, which were kept on ice immediately after extirpation. The isolation procedure was exactly as previously described[5]; the pellet P_c was used. The chromaffin granules thus isolated were given hypoosmotic shock and gently homogenized by an all-glass Potter–Elvehjem homogenizer to induce a release of their soluble content essentially as previously described[2]. A 50-mM potassium phosphate buffer of pH 6.5 was used as the medium for this procedure as well as for the subsequent dialysis. The dialysis buffer (1 l) was changed from 6 to 8 times over a period of 36 h. By this method more than 99% of the initial catecholamines were removed. Even prolonged dialysis with more frequent buffer changes did not remove the catecholamines completely. During dialysis the protein concentration decreased from the initial values of approx. 150 to 30–40 mg of protein per ml as a result of an increase in volume (Donnan effect). The final retentate was resuspended by careful homogenization and stored at 4 °C until use[2].

A membrane preparation as well as the soluble matrix phase of chromaffin granules were isolated as described[1]; the membrane preparation was washed twice. Electron micrographic examination of the final sediment of disrupted granule membranes were carried out as described[5] and revealed the presence of linear membrane fragments as well as electron-lucent vesicles.

The different granule preparations were denoted G*xy*T and G*xy*M; G for granule, *xy* is the specific preparation number, and T and M refer to the total dialyzed granule preparation and the membrane preparation, respectively.

Cytochrome c

Native, monomeric cytochrome *c* was prepared from bovine heart muscle as described[6]. Native, monomeric cytochrome c_2 was prepared from light-grown cells of *Rhodospirillum molischianum* as described[7].

Chemicals and buffers

Fumaric acid, *p*-hydroxymercuribenzoate (PHMB), antimycin A, menadione, Triton X-100, bovine serum albumin (fatty acid free) and the disodium salts of NADH and NADPH were the products of Sigma Chemical Co., U.S.A. Fusaric acid was a gift from Professor T. Nagatsu, Department of Biochemistry, Aichi-Gakuin University, Nagoya, Japan. All other chemicals were of reagent grade. Deionized, double-distilled (quarts) water were used exclusively. Oxygen was removed from commercial nitrogen by passage through an alkaline solution of pyrogallol[8]. Dialysis tubings were extensively washed before use.

Ultrafiltration of the matrix phase

The matrix phase was subjected to membrane ultrafiltration with a collodion bag (SM 13 200 from Sartorius-Membranefilter GmbH, Göttingen, Germany) with a cut-off at a molecular weight around 25 000.

Analytical methods

Protein concentrations were determined by the method of Eggstein and Kreutz[9], using bovine serum albumin as a standard[1].

NADH and NADPH concentrations were determined from their molar absorption coefficient $6.22 \cdot 10^3$ at 340 nm[10]. The concentrations of cytochrome *c* were determined by using the molar absorption coefficient $29.04 \cdot 10^3$ at 550 nm (reduced)[11] and of cytochrome c_2 from *R. molischianum* by using the molar absorption coefficient $29.76 \cdot 10^3$ at 550 nm (reduced)[7].

NADH:ferricyanide oxidoreductase activities were measured as described[1]. Other NADH(NADPH):(acceptor) oxidoreductase activities were also measured spectrophotometrically at 340 nm. All measurements have been carried out in a Shimadzu MPS-50L recording spectrophotometer. Anaerobic conditions were achieved in Thunberg cuvettes by alternately (5 times) evacuating and flushing with oxygen-free nitrogen; care was taken to avoid bubbling the reaction mixture. The chromaffin granule preparations were incubated at 25 °C in a medium containing in a volume of 1.25 ml: 50 mM potassium phosphate buffer, pH 6.5; 25 μM NADH(NADPH). The granule preparation was preincubated for 10 min at 25 °C at the desired protein concentration before the coenzymes were added to initiate the reaction. Alterations from this procedure and other additions are indicated in the legends to figures and tables; fumarate and inhibitors (PHMB, cyanide and fusaric acid) were present during the preincubation period unless otherwise stated. The quoted reaction rates are all corrected for non-enzymic oxidation of the coenzymes.

The effect of Triton X-100 on the light scattering of the granule preparation was measured in the Shimadzu MPS-50L recording spectrophotometer. Using 340 nm light, the intensity of the direct beam was measured, *i.e.* $A' = \log (I_o/I_p + I_d)$ where I_o, I_p and I_d represent the incident light, the parallel transmitted and the diffuse transmitted light, respectively.

The measurement of the oxidation–reduction level of cytochrome b_{561} was performed in cuvettes of 10 mm light path using an Aminco-Chance dual-wavelength spectrophotometer with both monochromators calibrated with reduced cytochrome *c* at an accuracy of $\pm$ 0.2 nm[1].

A Clark oxygen electrode (Yellow Spring Instrument Co., U.S.A.) was employed for measurements of oxygen consumption[12].

Electron microscopy was performed essentially as described[5].

The Michaelis constants were calculated graphically[13].

RESULTS

Enzymic oxidation of NADH(NADPH) by chromaffin granules depleted of low molecular weight substances

It has previously been demonstrated[1] that the oxidation of NADH is catalyzed by washed membrane preparations of chromaffin granules in the absence of exogenous electron acceptors. The same catalytic activity has been found in the total preparation of chromaffin granules (Fig. 1A, Curve a), *i.e.* in a preparation containing the macromolecules of the matrix phase as well as of the membrane phase. The overall NADH oxidation was rather low and varied largely from one preparation to the other, *i.e.* from 0.06 to 0.32 nmole NADH oxidized per min per mg of protein (21 different preparations). In contrast to the washed membrane preparations[1], the total preparation of chromaffin granules revealed a small NADPH oxidation (Table II), *i.e.* from 0 to 0.04 nmole NADPH oxidized per min per mg of protein (six different preparations). This activity can, however, be induced/increased by different physical and chemical treatments of the granule preparation (see below) suggesting the possibility that this activity in freshly prepared chromaffin granules at least partly may be a preparative artifact.

Effect of fumarate and fusaric acid on the overall NADH oxidation

Fumarate and other dicarboxylates are known to stimulate partly purified dopamine β-hydroxylase about 6-fold at a concentration of 10 mM[14], and in the present study we have used fumarate to see if some of the overall NADH oxidation could be attributed to this enzyme. From Fig. 1A, Curve b it is seen that in freshly prepared chromaffin granules fumarate has a stimulatory effect on the rate of NADH oxidation, which was not inhibited by fusaric acid at a concentration sufficient to inhibit soluble dopamine β-hydroxylase[15]. It is, however, also known that flavoproteins, *e.g.* D-amino acid oxidase, are stimulated by fumarate and other dicarboxylates[16] suggesting the possibility that the fumarate-stimulated NADH oxidation observed may be an effect on a flavoprotein, which is known to be present in chromaffin granule membranes[1]. The K'_m value for fumarate in our assay system was found to be $5.5 \cdot 10^{-5}$ M, but in all experiments described in the following text saturating amounts (10 mM) of fumarate have been used. It is also seen from Fig. 1A, Curve b, that this activity is progressively inhibited by high concentrations of PHMB. Furthermore, there is no activity with NADPH in this reaction.

Effect of PHMB on NADH:(acceptor) oxidoreductase activities

Evidence has accumulated in recent years for the importance of sulphydryl groups in the function of chromaffin granules, *e.g.* for the ATP-dependent and -independent catecholamine fluxes over the granule membrane[17,18]. Furthermore,

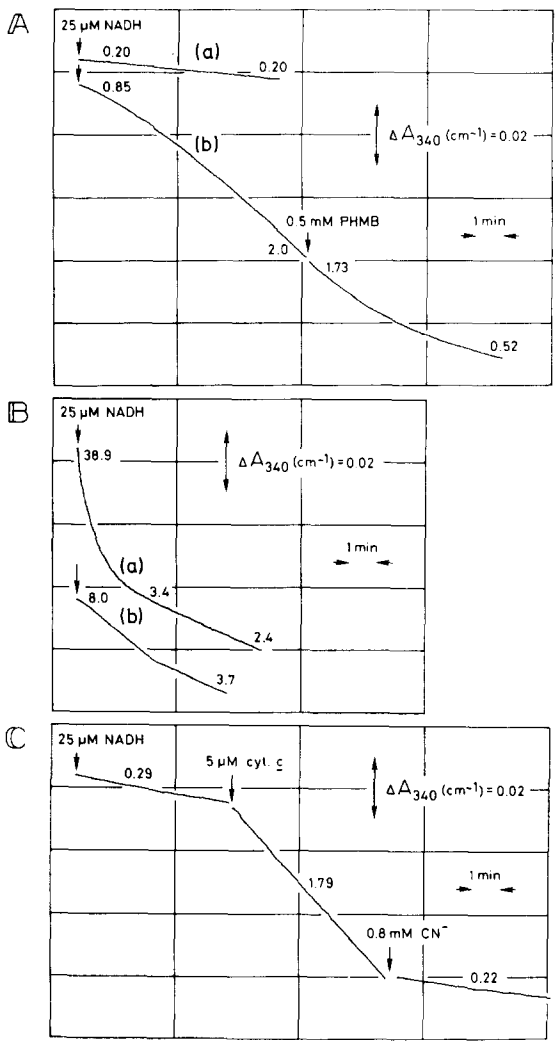

Fig. 1. (A) The time course of the aerobic oxidation of NADH catalyzed by freshly prepared chromaffin granules in the absence (Curve a) and presence (Curve b) of 10 mM fumarate. The chromaffin granule preparation (G27T) was diluted to 1.7 mg of protein per ml in a 50 mM potassium phosphate buffer, pH 6.5, at 25 °C and preincubated for approx. 5 min before addition of the coenzyme. (B) Effect of prolonged storage of the chromaffin granules on the time course of the fumarate-stimulated NADH oxidation in the absence (Curve a) and presence (Curve b) of 0.38 mM PHMB. The chromaffin granule preparation (G44T) was diluted to 0.66 mg of protein per ml in the standard incubation buffer containing 10 mM fumarate and incubated for 5 h at 4 °C. For experimental details see (A). PHMB was added 10 min before the coenzyme. (C) Stimulation of the NADH oxidation by horse heart cytochrome *c* and its inhibition by cyanide. For experimental details, see (A), Curve a, except that the protein concentration was 1.0 mg per ml (preparation G33T). The numbers immediately above and below each trace represent the specific oxidation rates (nmoles NADH oxidized per min per mg of protein).

we have recently observed[1] that PHMB inhibits the NADH:ferricyanide oxidoreductase activity of isolated membrane preparation, suggesting the presence of functional thiol groups in this dehydrogenase reaction. In the present work the effect of PHMB on different NADH:(acceptor) oxidoreductase activities has been studied in more detail.

The inhibition of the overall NADH oxidation by PHMB always revealed a biphasic dose–response curve (Fig. 2A) when the mercurial was tested over a wide concentration range. Furthermore, preincubation of the chromaffin granules with PHMB at 25 °C for at least 8 min was necessary to achieve inhibition in the high concentration range (*i.e.* 10–200 μM), whereas the inhibition in the low concentration range (<8 μM) was exactly the same whether or not the chromaffin granules were preincubated with the mercurial. It is also seen from Fig. 2A that cyanide (2.7 mM) inhibits only that part of the overall NADH oxidation which is inhibited by low concentrations of PHMB. From Fig. 2B it is also seen that the NADH:ferricyanide oxidoreductase activity previously described[1] is inhibited by

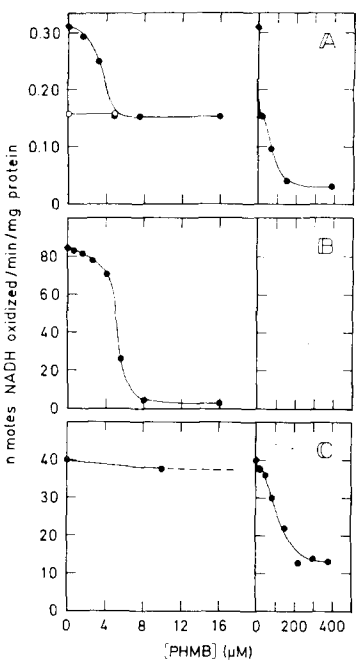

Fig. 2. The effect of increasing concentrations of PHMB on the aerobic oxidation of NADH (A), the NADH:ferricyanide oxidoreductase activity (B) and the fumarate-stimulated NADH oxidation (C). (A) The chromaffin granule preparation (G51T) was diluted to 0.8 mg of protein per ml in the standard incubation buffer at 25 °C containing PHMB at the concentrations indicated (●) as well as 0.27 mM cyanide (○). For experimental details, see Fig. 1A, Curve a. (B) The chromaffin granule preparation (G26T) was preincubated for 5 min at 25 °C with 25 μM NADH and PHMB at the concentrations indicated; the protein concentration was 0.15 mg per ml. The reaction was started by adding 77 μM ferricyanide. (C) The experimental conditions were as described in Fig. 1B, Curve b, except for the concentration of PHMB. The specific oxidation rates (nmoles NADH oxidized per min per mg of protein) were calculated from the initial part of the progress curves.

PHMB in the same concentration range as the most sensitive part of the overall NADH oxidation; neither in this case preincubation with the mercurial was necessary to achieve inhibition. On the other hand, the fumarate-stimulated NADH oxidation was inhibited only by high concentrations of PHMB (Fig. 2C), *i.e.* similar to the most resistent part of the overall NADH oxidation in the absence of added carboxylic acid. Preincubation with the mercurial was, however, necessary to achieve full inhibition of this activity. If the mercurial was added during NADH oxidation, a progressive inhibition by time was observed (Fig. 1A, Curve b).

Effect of cytochrome c on the NADH oxidation

From Fig. 1C it is seen that native horse heart cytochrome *c* revealed a remarkably high stimulatory effect on the rate of NADH oxidation and that this effect was completely inhibited by cyanide (Fig. 1C) and low concentrations of PHMB (figure not shown). The stimulatory effect of cytochrome *c* was, however, completely insensitive to antimycin A. On the other hand, cytochrome c_2 from *R. molischianum*, which is chemically and structurally very similar to mammalian cytochrome *c* and has a similar oxidation–reduction potential[7], did not show any stimulation of NADH oxidation which indicates a specificity of the stimulatory effect of horse heart cytochrome *c*. The apparent K_m of cytochrome *c* was found to be $1.6 \cdot 10^{-6}$ M as an average value of three different preparations.

Stability of the NADH:(acceptor) oxidoreductase activities

Storage of the total preparation of chromaffin granules at 4 °C and pH 6.5 (50 mM potassium phosphate buffer) resulted in a gradual decrease in the NADH: ferricyanide oxidoreductase activity (Fig. 3A). The rate of decay was higher the lower the protein concentration, and the washed membrane preparation (Fig. 3B) lost the activity more rapidly than the total preparation (Fig. 3A). Thus, the rate

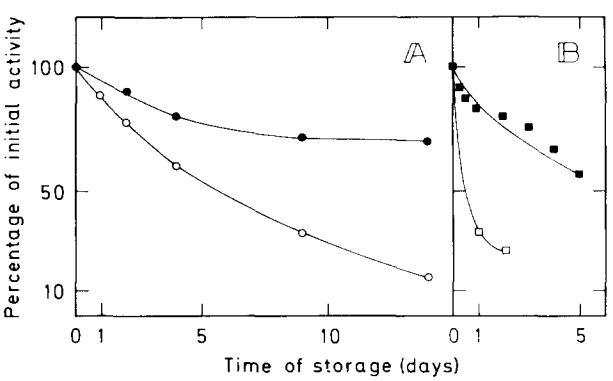

Fig. 3. The effect of protein concentration on the decay of the NADH: ferricyanide oxidoreductase activity in a total preparation (A) and washed membrane preparations (B) of chromaffin granules. The granule preparations were stored at 4 °C in 50 mM potassium phosphate buffer, pH 6.5, and samples were withdrawn at the times indicated and assayed for NADH: ferricyanide oxidoreductase activity at 25 °C by the standard procedure; the concentrations of the reactants were 25 μM (NADH) and 77 μM (ferricyanide). (A) The granule preparation (G38T) was stored at a protein concentration of 8.0 mg per ml (●) and 4.0 mg per ml (○). 0.03 mg of protein was used in the assay. (B) The washed membrane preparations (G5M and G3M) were stored at a protein concentration of 18.0 mg per ml (■) and 3.0 mg per ml (□), respectively. 0.06 mg of protein was used in the assay.

of decay is almost the same in the total preparation at a concentration of 4 mg of protein per ml as in the washed membrane preparation at 18 mg of protein per ml.

The most PHMB-sensitive part of the overall NADH oxidation was stable for at least 1 week in the total preparation at high protein concentrations (*e.g.* at 30 mg of protein per ml) when stored at 4 °C in the standard buffer. At 25 °C and a protein concentration of about 1 mg of protein per ml (the standard assay conditions) the activity was stable for at least 30 min, but it was found to be lost within 3 min if the temperature was increased to 38 °C. On the other hand, the overall NADH oxidation was enhanced under the same experimental conditions, particularly the fumarate-stimulated activity (Fig. 1B). This activity revealed a biphasic progress curve (Fig. 1B, Curve a) and only the initial rapid phase was inhibited by PHMB (Fig. 1B, Curve b) which indicated the presence of two principally different reaction mechanisms for the rapid and the slow reaction phases.

Demonstration of an endogenous electron acceptor for the enzymic oxidation of NADH

From Fig. 2A it is seen that the overall oxidation of NADH catalyzed by the total granule preparation is only partially inhibited by cyanide, indicating the presence of either (1) an autooxidizable flavoprotein, (2) a cyanide-insensitive oxidase, or (3) an endogenous electron acceptor of an NADH-linked dehydrogenase. The concept of an endogenous electron acceptor is supported by the observation that upon prolonged storage of the granule preparation the fumarate-stimulated

TABLE I

DEMONSTRATION OF AN ENDOGENOUS ELECTRON ACCEPTOR APPEARING UPON STORAGE OF THE CHROMAFFIN GRANULE PREPARATION

The chromaffin granule preparation (G51T) was stored at a concentration of 0.88 mg of protein per ml at 4 °C for 60 h (Experiment A) and for approx. 5 min (Experiment B) prior to high-speed centrifugation (for details, see Methods). The two supernatants (termed Preparations A and B, respectively) were assayed for fumarate-stimulated NADH oxidation (control value) as described in Fig. 1B, Curve b, except that the fumarate concentration was 1 mM. 7 ml of the supernatant were then subjected to membrane ultrafiltration (see Methods) to give 6.9 ml filtrate and 0.1 ml retentate. 1.1 ml of the filtrate (from A or B), 0.04 ml of the retentate (from A or B) and 0.06 ml of a fumarate solution (final concentration 1 mM) were preincubated for 5 min before the addition of 25 μM NADH.

	*nmoles NADH oxidized per min per mg of protein**
Preparation A (control)	59.4
Preparation B (control)	4.5
Filtrate A + retentate A	65.3
Filtrate B + retentate B	8.3
Filtrate A + retentate B	65.9
Filtrate B + retentate A	4.5

* Each number represents the mean value of two experiments. Neither the filtrates (with components of molecular weight < 25 000) nor the high molecular weight retentates (containing the dehydrogenase) revealed any activity alone.

oxidation of NADH clearly revealed a biphasic progress curve (Fig. 1B, Curve a) where the initial 'rapid' phase, but not the slow phase, was inhibited by PHMB (Fig. 1B, Curve b and Fig. 2C). Furthermore, simultaneous measurements of NADH oxidation and oxygen consumption showed that molecular oxygen was not consumed during this reaction and it proceeded at the same rate under aerobic and anaerobic conditions (not shown). Finally, the initial 'rapid' phase activity was confined to the granule matrix phase and by ultrafiltration neither the filtrate (with components of molecular weight $<25\,000$) nor the high molecular weight retentate (containing the dehydrogenase) revealed any activity alone. The activity requires the retentate as well as the filtrate fraction, and was almost quantitatively recovered by recombination of the two separated fractions (Table I). It is clearly seen that it is the ultrafiltrable electron acceptor which progressively increases upon prolonged storage of the diluted granule preparations and not the enzyme catalyzing the reaction. Fumarate-stimulated NADH oxidase activity is also found in the matrix phase upon storage of the granule preparation at high protein concentrations, but the activity then increased very slowly.

Effect of solubilization of the granule membranes by Triton X-100

Solubilization of the membrane phase of the total granule preparation by the non-ionic detergent Triton X-100 resulted in marked changes of the oxidoreductase activities. Thus, the portion of the overall NADH oxidation which was inhibited by low concentrations of PHMB was rapidly lost as the membranes were solubilized at low concentrations of the detergent, and the total rate of NADH oxidation was reduced correspondingly (Fig. 4). On the other hand, at higher detergent concentrations this activity increased in parallel with an increase in the oxidation of NADPH as well (Fig. 4). The NAD(P)H oxidase activities, however, did not rise in parallel with the drastic reduction in size of the granule membrane particles as followed by the decrease in the amount of light scattering (Fig. 4).

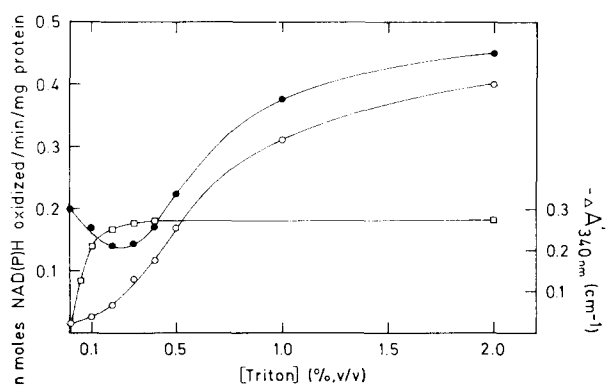

Fig. 4. Effect of solubilization by Triton X-100 on the rate of NADH (●) and NADPH (○) oxidation as well as on light scattering (□) of a chromaffin granule preparation (G49T). The dialyzed granules, at a concentration of 1.4 mg of protein per ml, were solubilized by the detergent at the concentrations indicated. The preincubation period was 10 min. For experimental details, see Fig. 1A, Curve a, and Methods section.

It is seen from Table II that the overall NADH oxidation is only partially inhibited by high concentrations of PHMB even after preincubation with the mercurial, and the PHMB-insensitive part of this oxidation is numerically equal to the oxidation of NADPH which was completely insensitive to PHMB.

TABLE II

EFFECT OF PHMB AND TRITON X-100 ON THE NADH(NADPH) OXIDATION CATALYZED BY CHROMAFFIN GRANULES

For experimental details, see Fig. 1A.

Electron donor	*Additions*	*nmoles of NADH(NADPH) oxidized per min per mg of protein**			
		Preparation number:			
		G29T	*G30T*	*G49T*	*G49T***
NADH	None	0.150	0.090	0.116	0.350
	PHMB***	0	0.024	0.004	0.245
NADPH	None	0	0.023	0.008	0.240
	PHMB	—	0.022	0.006	0.220

* Each number represents the mean value of two experiments.
** Solubilized with Triton X-100 (2%, v/v); see Fig. 4.
*** The chromaffin granules were preincubated with 0.5 mM PHMB for 6 min before adding coenzyme.

DISCUSSION

Several points should be considered in the determination of oxidoreductase activities in chromaffin granule preparations. Perhaps the most serious problem lies in the fact that these organelles contain very high concentrations of redox active compounds, *i.e.* the catecholamines. Thus, the study of NADH(NADPH):(acceptor) oxidoreductase activities in intact granules is almost impossible, *e.g.* due to the disturbing non-enzymic reaction between the coenzymes and the oxidized forms of the catecholamines[19]. This difficulty can, however, largely be eliminated either by hypoosmotic lysis followed by repeated sedimentations and resuspensions in a hypoosmotic buffer[1] or by extensive dialysis against such a buffer[2]. These procedures have been found to be effective[1,2] although we have not so far been able to remove the catecholamines quantitatively. That the rest of the catecholamines may be of significance also in the present study will be discussed below. The other important point is the problem of stability of the various oxidoreductase activities during isolation, storage and solubilization of the chromaffin granules as well as their partition between the membrane and the matrix phase.

Using the NADH:ferricyanide oxidoreductase activity as a marker on the stability of the membrane-bound flavoprotein(s)[1], it is evident that functional properties of the membranes are rather sensitive to storage even at 4 °C when the protein concentration is low (Fig. 3B). A marked stabilizing effect on the membranes was, however, induced by the matrix proteins. Thus, the total preparation

at a protein concentration of 4 mg per ml was just as stable as purified membranes at a concentration of 18 mg of protein per ml (Fig. 4). Furthermore, it is generally accepted that at least one of the granule enzymes, *i.e.* the dopamine β-hydroxylase is partitioned between the membrane and the matrix phase[20–26], and that approximately one half of the total enzymic activity can be recovered in the matrix phase. At sufficiently high protein concentrations, however, as much as 90% of the total dopamine β-hydroxylase activity appears to be associated with the particulate fraction (Terland, O., unpublished results). This observation, together with the marked stabilizing effect shown in Figs 3A and 3B, has led us to use the total preparation, *i.e.* the unfractionated, dialyzed membrane phase *plus* matrix phase, in the present study.

A detailed examination in the present and previous[1] studies of the various redox reactions as well as the effect of inhibitors (PHMB and cyanide) makes it possible to reduce the different activities observed to a few electron transport systems. First, the overall oxidation of NADH is inhibited to the same degree by low concentrations of PHMB and cyanide (Fig. 2A) which suggests that this part of the reducing equivalents is transferred to a cyanide-sensitive electron-transfer component by way of a PHMB-sensitive dehydrogenase. In addition to a unique *b*-type cytochrome (b_{561}), an NADH:cytochrome b_{561} oxidoreductase has previously been detected in the chromaffin granule membrane[1]. This activity is inhibited by PHMB in the same concentration range as the most sensitive part of the overall NADH oxidation and is slightly increased by cyanide (Terland, O. and Flatmark, T., unpublished results). These findings suggest that all these electron transport components may be connected in a multicomponent electron transport system of the granule membrane. Furthermore, cyanide (Fig. 1C) and PHMB (figure not shown) in the same concentration range completely inhibit the cytochrome *c*-stimulated oxidation of NADH, which indicates that this cytochrome may function as an electron mediator (shunt) between the reductase and the cyanide-sensitive component. In addition, in the presence of cyanide, cytochrome *c* (which is then almost completely reduced[1]) increases the steady-state redox level of cytochrome b_{561} to approx. 23% reduction (figure not shown), which is an equilibrium value which compares well with a difference in the redox potentials of approx. 110 mV between the two cytochromes . Thus, cytochrome b_{561} appears to be localized between a PHMB-sensitive NADH dehydrogenase and a cyanide-sensitive electron-transfer component as schematically illustrated in Fig. 5. In addition, PHMB at low concentrations inhibits the NADH:ferricyanide oxidoreductase activity (Fig. 2B), and this activity may, therefore, be a measure of the common NADH dehydrogenase (termed cytochrome b_{561} reductase in Fig. 5). The common cyanide-sensitive com-

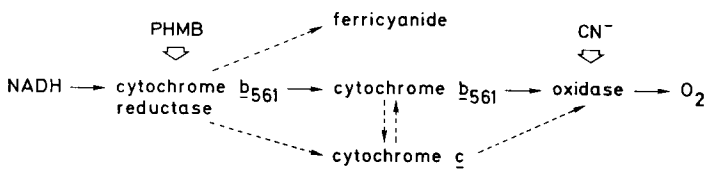

Fig. 5. Model for electron transport in adrenal chromaffin granule membrane. The solid arrows indicate the PHMB- and cyanide-sensitive NADH oxidase pathway which is greatly stimulated by exogenous cytochrome *c* (broken arrow).

ponent (Fig. 5) which seems to function as an oxygen-activating enzyme[1], is probably not dopamine β-hydroxylase, since this copper enzyme is inhibited to approx. 98% by fusaric acid[15], which has no effect on the oxidation of cytochrome b_{561} or the cytochrome *c*-stimulated NADH oxidation (Terland, O., unpublished). Thus, it is necessary to postulate the presence of an additional oxygen-activating enzyme in chromaffin granules.

It has been shown in the present study that strong sulphydryl-blocking reagents such as PHMB are able to block approx. 45% of the overall NADH oxidase activity (Fig. 2A) and almost completely the NADH:cytochrome b_{561} oxidoreductase activity at low concentrations (<8 μM) using different artificial electron acceptors, *e.g.* ferricyanide (Fig. 2B) and ferricytochrome *c*. At higher concentrations of the mercurial an additional part of the overall NADH oxidation is inhibited (Fig. 2A). The fact that the concentration of PHMB required for half maximal inhibition of this activity was the same as that required for half maximal inhibition of the fumarate-stimulated NADH oxidation (Fig. 2C) suggests that these two reactions are catalyzed by the same enzyme. As shown in Table I the fumarate-stimulated activity of the matrix phase depends on a high molecular weight component ($>25\,000$), *i.e.* the dehydrogenase, and an ultrafiltrable low molecular weight component which functions as an electron acceptor and is present at variable concentrations depending on the experimental conditions. Preliminary results (fluorescence and absorption spectra) indicate that the endogenous electron acceptor may have catecholamine nature (Terland, O., unpublished results).

A PHMB-insensitive NAD(P)H oxidase activity of approximately the same specific activity with the two coenzymes (Fig. 4 and Table II), rise in response to membrane solubilization. The increase in the two oxidoreductase activities did not, however, parallel the removal of the permeability barrier by Triton X-100, as measured by the decrease in light scattering. Thus, at 50% and 100% membrane solubilization the NADPH oxidation was only approx. 5% and 30% of the maximal activity, respectively (Fig. 4). A further disintegration of the 'subunit' structure of the membrane seems to be necessary to obtain maximal NADH(NADPH) oxidase activities and this transition requires high concentrations of the detergent. Not only solubilization by Triton X-100, but also dilution and other physical treatments of the granules provoke these activities.

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