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# HYDROGEN PEROXIDE GENERATING SYSTEM IN HOG THYROID MICROSOMES

SACHIYA OHTAKI, KEIMEI MASHIMO and ISAO YAMAZAKI

*2nd Department of Medicine, School of Medicine and Biophysics Division, Research Institute of Applied Electricity, Hokkaido University, Sapporo (Japan)*

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SUMMARY

1. The NADH oxidation caused by hog thyroid microsomes was studied. Time courses of the oxidation of NADH and the reduction and oxidation of microsomal cytochrome $b_5$ were followed simultaneously by the use of a dual-wavelength spectrophotometer.

2. The turnover number of the bound cytochrome $b_5$ in the NADH oxidation was calculated to be 0.13 $s^{-1}$ according to Chance. From the kinetic trace of the cytochrome $b_5$ oxidation the rate of the oxidation was calculated to be 0.037 $s^{-1}$. The discrepancy between these two values was discussed.

3. The $H_2O_2$ generation was confirmed by use of hydroperoxidase inhibitors and cytochrome peroxidase (ferrocytochrome *c*:hydrogen-peroxide oxidoreductase, EC 1.11.1.5). By the latter method, the amount of $H_2O_2$ generation was determined to be 40% of the amount of NADH oxidized.

While there appears to be substantial evidence for the presence of a peroxidase (iodide:hydrogen-peroxide oxidoreductase, EC 1.11.1.8) in the thyroid, which can, under favorable conditons, oxidize iodide in the presence of an $H_2O_2$-generating system, certain questions remain concerning the operation of such a system *in vivo*[1,2]. Reduced forms of pyridine nucleotides and flavin nucleotides are considered to be possible sources of $H_2O_2$ on the basis of observations that iodination by thyroid preparations can be enhanced by added NADH or NADPH[3,4] and by added flavin nucleotides[3–6]. Klebanoff and co-workers[7,8] suggested another possibility, based on the NADH oxidase activity of thyroid and other preparations. They showed that purified peroxidase may serve as an $H_2O_2$-generating system in an $Mn^{2+}$-dependent aerobic oxidation of NADH or NADPH.

On the other hand, Bhagvat *et al.*[9] found monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4) activity in thyroid extracts and Fischer *et al.*[10] claimed that bovine microsomal monoamine oxidase with tyramine in the thyroid gives rise to sufficient $H_2O_2$ to account for the iodination which occurs. We have recently confirmed this assumption in both *in vitro* and *in vivo* experiments for iodination and KSCN oxidation in the thyroid tissues[11]. Further studies have shown that thyroid monoamine oxidase has an additive effect on thyroid hormono-

genesis in a thyroid-stimulating hormone-stimulated preparation (Ohtaki, S. and Rosenberg, I. N., unpublished results).

More recently, Bénard and Brault[12], using fluorimetry, observed that thyroid-stimulating hormone causes $H_2O_2$ production in dispersed cells from calf and hog thyroid. Suzuki[13] reported that cytochrome *c* reductase may serve as an $H_2O_2$ generator in the iodination reaction by a thyroid particulate fraction, vitamin $K_3$ (menadione) being an activator, and also that the oxidation of cytochrome $b_5$ is too slow to be involved in the iodination reaction. Recently Nagasaka *et al.*[14] also reported a similar observation on the effect of soluble cytochrome *c* reductase on the iodination reaction in a soluble system.

Thus, as suggested by Ogata *et al.*[15], the level of reduced pyridine nucleotides influenced by thyroid-stimulating hormone seems most important in thyroid hormone biosynthesis. In this paper, we will describe an oxidative pathway from NADH to oxygen in hog thyroid microsomes, which might be related to thyroid hormone biosynthesis.

## MATERIALS AND METHODS

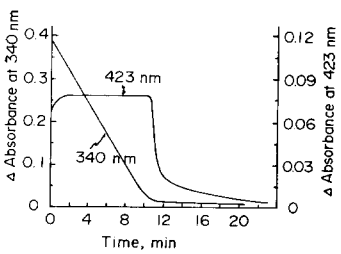
The subcellular particulate fractionation was carried out by a modification of the method of Hosoya and Morrison[16]. Connective tissue and fat were removed from hog thyroid glands which were freshly supplied from a slaughter house. The thin slices were washed several times with isotonic saline solutions. About 60–80 g of this material was homogenized for 30 s in a Waring blender with 3 vol. of 0.25 M sucrose solution containing 1 mM EDTA and 0.1 M phosphate buffer, pH 7.4. This homogenization was repeated three times at brief intervals and the pooled homogenate was then passed through a Potter–Elvehjem homogenizer and centrifuged at $8500 \times g$ for 15 min. Centrifugation of the supernatant was performed at $33700 \times g$ for 100 min with a Hitachi Model 65P ultracentrifuge with a rotor, RPB 23 BTA. The pellets were re-homogenized with the above sucrose solution and again subjected to ultracentrifugation. This procedure was repeated three times. Finally, in order to minimize contamination with hemoglobin and catalase, the pellets were further washed with 0.025 M sucrose solution containing 0.1 M phosphate buffer (pH 7.4) and 0.01 mM EDTA. The ultracentrifugation procedure was the same as above. The pellets were suspended in 0.1 M phosphate buffer (pH 7.4) and kept frozen overnight. All these procedures were performed at 0–2 °C. The pellets were thawed and gently re-homogenized with a Potter–Elvehjem homogenizer before use.

Absorbance changes were followed with a Hitachi Model 356 dual-wavelength spectrophotometer at 25 °C. The following absorbance coefficients were used to determine the concentration of cytochrome $b_5$ and cytochrome peroxidase (Ferrocytochrome *c*:hydrogen-peroxide oxidoreductase, EC 1.11.1.5): for cytochrome $b_5$, $\Delta\varepsilon$ at 423 nm (reduced *minus* oxidized) = 100 $mM^{-1} \cdot cm^{-1}$ (ref. 17); and for cytochrome peroxidase, at 408 nm = 93 $mM^{-1} \cdot cm^{-1}$ and $\Delta\varepsilon$ (peroxide compound *minus* free enzyme) at 424 nm = 49 $mM^{-1} \cdot cm^{-1}$ (ref. 18) and at 438 nm = 19.1 $mM^{-1} \cdot cm^{1}$. The latter wavelength was an isosbestic point of reduced and oxidized cytochrome $b_5$ of hog liver. The protein concentration was determined by the method of Lowry *et al.*[19] using bovine serum albumin as standard. $O_2$ consumption was measured with a Clark electrode. All reactions were carried out at 25 °C.

Cytochrome peroxidase from bakers' yeast was generously supplied by Dr T. Yonetani. Hog liver cytochrome $b_5$ and green pea superoxide dismutase were kindly supplied by Dr Iyanagi[20] and Mr Sawada[21], respectively. NADH was obtained from Boehringer Mannheim. All other chemicals were obtained from commercial sources in the highest available state of purity.

## RESULTS

The addition of NADH to hog thyroid microsomes caused a rapid increase of absorbance at 423 nm, which appeared to be related to the oxidation of NADH as can be seen in Fig. 1 as kinetic traces measured simultaneously at two wavelengths with a two-wavelength spectrophotometer. The NADH oxidase activity of our preparation was somewhat lower than that of microsomes prepared from calf thyroids[22]. Fig. 2 shows that the increase of absorbance at 423 nm was due mostly to the reduction of cytochrome $b_5$. A similar difference spectrum has been reported by Hosoya and Morrison[16,23]. The kinetics of reduction and oxidation of cytochrome $b_5$ caused by adding NADH has been studied with midgut homogenates of *Cecropia*[24] and with liver microsomes under aerobic conditions[25,26]. The reduction of cytochrome $b_5$ by NADH appeared to be as rapid as in the case of rat liver microsomes[25,26], but a slow phase followed the initial rapid change in the reaction of hog thyroid microsomes. The slow phase was not affected by $MnCl_2$ (10–100 $\mu$M). Ascorbate (50–100 $\mu$M) partially reduced the cytochrome but did not affect the slow phase of the cytochrome reduction when NADH was added. $H_2O_2$ (100 $\mu$M) prolonged the slow phase of the cytochrome reduction and the time required to reach a steady-state level was delayed. Fig. 2 also shows that a further reduction of hemoproteins present in the microsomes was caused by the addition of sodium dithionite, showing a difference absorption peak around 428 nm. This difference spectrum,



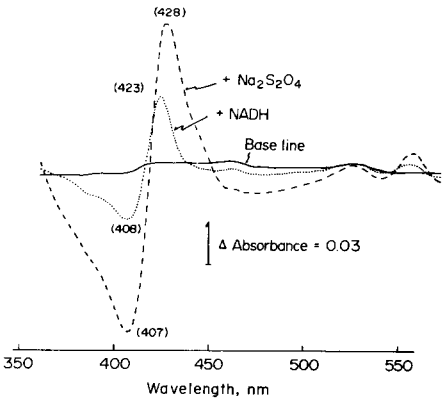




Fig. 1. Time courses of the NADH oxidation (340 nm) and the oxidation–reduction state of microsomal cytochrome $b_5$ (423 nm). The reaction was started by the addition of NADH (53 $\mu$M) to a thyroid microsomal suspension (4.1 mg protein/ml) in 0.1 M phosphate buffer (pH 7.4).

Fig. 2. Difference spectra of thyroid microsomes. Both sample and reference cuvettes contained thyroid microsomes (4.1 mg protein/ml) in 0.1 M phosphate buffer (pH 7.4). NADH difference spectrum; 53 $\mu$M NADH was added to the sample cuvette. Dithionite spectrum; crystals of sodium dithionite were added to the sample cuvette after NADH had been oxidized.

however, was evidently different from that of cytochrome $b_5$ and indicated a considerable contribution from other hemoproteins such as thyroid peroxidase and contaminating hemoglobin as pointed out by Hosoya and Morrison[16].

The oxidation of the cytochrome $b_5$ started almost at the time when NADH had disappeared, as is illustrated by the absorbance trace at 423 nm in Fig. 1. The oxidation proceeded at a considerably higher speed as compared with that reported by Suzuki[13]. The oxidation reaction, too was found to be composed of rapid and slow phases. It was difficult to measure the exact rate of cytochrome $b_5$ oxidation in the rapid phase because of its concurrent reduction by the remaining NADH. The approximate value was estimated as 0.037 $s^{-1}$ in the middle where half of the cytochrome $b_5$ was oxidized.

Turnover number of the bound cytochrome $b_5$ in the NADH oxidation by washed homogenates of the *Cecropia* midgut was calculated by Chance and Pappenheimer[24] according to the following equation,

$$k_3 = \frac{[\text{NADH}]_0}{[b_5{}^{2+}]_m \cdot t_{\frac{1}{2}\ \text{off}}} \tag{1}$$

and a value of 0.13 $s^{-1}$ was reported. In this equation, $[\text{NADH}]_0$ is the initial NADH concentration, $[b_5{}^{2+}]_m$ is the maximal concentration of the reduced cytochrome $b_5$, and $t_{1/2\ \text{off}}$ is the time for cytochrome $b_5$ to be oxidized to 1/2 $[b_5{}^{2+}]_m$. Similar kinetics might be applicable to the present results as shown in Fig. 3. The turnover number was then calculated to be 0.13 $s^{-1}$. Fig. 3 shows that the reduction of the cytochrome by NADH was very fast and the reduction of cytochrome $b_5$ reached a constant level at low NADH concentrations. The present value was similar to that of Chance and Pappenheimer[24], but different from that obtained for rat liver microsomes. The value of $k_3$ for rat liver cytochrome $b_5$ was estimated to be 0.03 $s^{-1}$ by Modirzadeh and Kamin[25] and by Oshino and Sato[26]. In the present experiments,

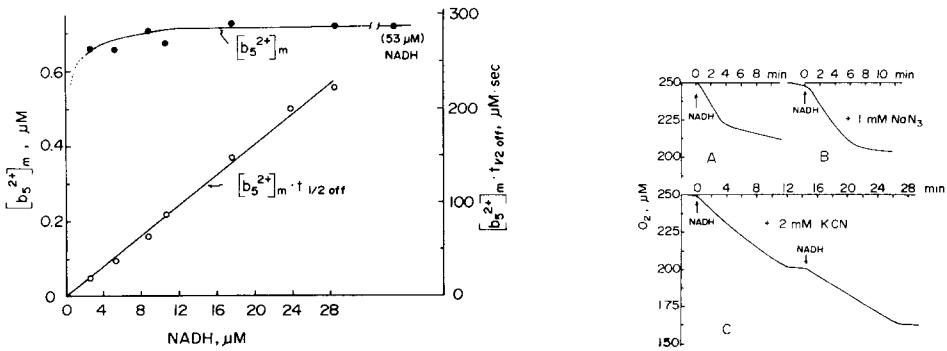



Fig. 3. The effect of initial NADH concentration on the maximum amount of reduced cytochrome $b_5$ and upon the half-time of cytochrome $b_5$ oxidation. The experimental conditions were as described in Fig. 1.

Fig. 4. The effect of $NaN_3$ and KCN upon the $O_2$ consumption. The experimental conditions were as described in Fig. 1, except that the microsome concentration was 12.3 mg protein per ml. Inhibitors were added 5 min before NADH addition. (A) Without inhibitor (B) 1 mM $NaN_3$ (C) 2 mM KCN. In C, the second NADH (53 μM) was added after the first NADH had disappeared.

however, there was a marked discrepancy between the values measured from the rate of the cytochrome oxidation and the overall kinetics of NADH oxidation. At any rate, these values are quite large compared with a value of 0.268 $min^{-1}$ reported by Suzuki[13] for cytochrome $b_5$ bound in hog thyroid microsomes.

The rate of NADH oxidation was augmented by tyramine (0.5–1.0 mM), while no effect was produced by the addition of catalase or superoxide dismutase.

The stoichiometry of the reaction between NADH and $O_2$ in the presence of the thyroid microsomes was studied using a 3-times more concentrated suspension of the microsomes than that used for spectrophotometric experiments. Under these conditions it was possible to observe the consumption of $O_2$. Table I shows that the ratio of the initial velocity of $O_2$ consumption to that of NADH oxidation and the ratio of the amount of $O_2$ consumed to that of the initial NADH concentration were both increased by addition of KCN and $NaN_3$. Fig. 4 shows a typical experiment on the effect of KCN and $NaN_3$ upon the $O_2$ consumption caused by adding NADH to an aerobic suspension of the microsomes. The data were not strictly reproducible but it could be concluded that $H_2O_2$ is a product in the NADH oxidation and is decomposed by peroxidase or catalase in the absence of KCN and $NaN_3$. In the ideal case, where $H_2O_2$ is an obligatory reduction product of $O_2$ and the reactions of peroxidase and catalase are completely inhibited by KCN and $NaN_3$, the ratios defined in Table I should be 0.5 and 1.0 in the absence and presence of such inhibitors, respectively.

TABLE I

THE EFFECT OF KCN AND $NaN_3$ ON THE MOLAR RATIO BETWEEN NADH OXIDIZED AND $O_2$ CONSUMED

Reactions were carried out in 0.1 M phosphate buffer, pH 7.4. The microsomal concentration was 12.3 mg protein per ml (A) and 16.2 mg protein per ml (B). Spectrophotometric experiments were carried out with one third of these concentrations. The microsomal preparation used in Expt B had been stored for 5 days in a freezer at −30 °C.

| *Expt* | *Addition* | *μM NADH/min** | *μM $O_2$/min* | *Ratio ($O_2$/NADH)* | *NADH(μM)* | *$O_2$(μM)* | *Ratio ($O_2$/NADH)* |
|---|---|---|---|---|---|---|---|
| A | — | 21.6 | 8.7 | 0.40 | 63.8 | 29.9 | 0.47 |
| | 2 mM KCN | 7.2 | 4.8 | 0.67 | 63.8 | 42.1 | 0.66 |
| | 1 mM $NaN_3$ | 15.9 | 7.9 | 0.50 | 63.8 | 45.2 | 0.71 |
| B | — | 18.3 | 6.7 | 0.37 | 138 | 85.8 | 0.63 |
| | 4 mM KCN | 9.3 | 8.4 | 0.90 | 138 | 117 | 0.85 |
| | 4 mM $NaN_3$ | 14.6 | 8.6 | 0.59 | 138 | 96.2 | 0.70 |

* 3-fold of the value measured spectrophotometrically.

In order to demonstrate the peroxide generation during the NADH oxidation by the thyroid microsomes, further studies were carried out using cytochrome peroxidase as a scavenger of $H_2O_2$ which has high specificity for both electron donor and acceptor. The peroxide compound of cytochrome peroxidase is relatively stable in the absence of suitable electron donors. The formation of the peroxide compound was confirmed as a difference spectrum between microsome + NADH and microsome

+NADH+cytochrome peroxidase as shown in Fig. 5. Under the experimental conditions cytochrome peroxidase was only partially converted into its peroxide compound in the presence of 5.7 $\mu$M cytochrome peroxidase (Fig. 5A), while most of the enzyme was observed in the form of its peroxide compound when half of the concentration was used (Fig. 5B).

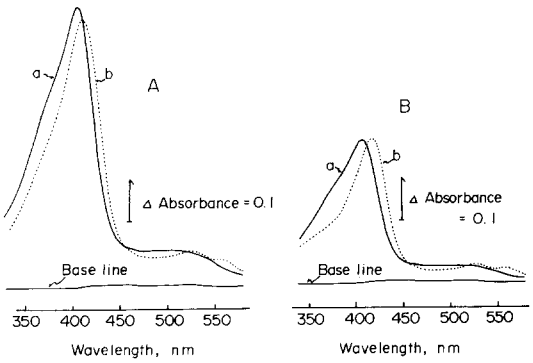



Fig. 5. Absorption spectra of the peroxide compound of cytochrome peroxidase formed in the presence of NADH and thyroid microsomes. Both reference and sample cuvettes contained thyroid microsomes (4.1 mg protein per ml) in 0.1 M phosphate buffer (pH 7.4). a, spectra of cytochrome peroxidase. Cytochrome peroxidase was added to the sample cuvette. b, spectra of the peroxide compound of cytochrome peroxidase. The wavelength was scanned from red 2 min after 53 $\mu$M NADH was added to both reference and sample cuvettes of a. Concentration of cytochrome peroxidase was 5.7 and 2.8 $\mu$M in A and B, respectively.

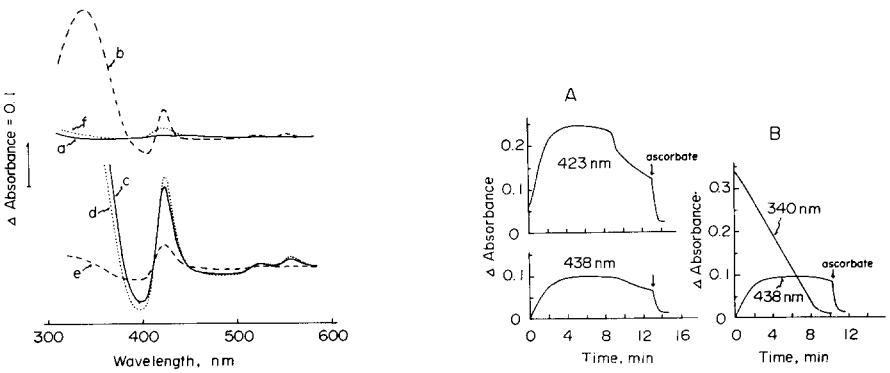



Fig. 6. Difference spectra of thyroid microsomes. a, base line. Both reference and sample cuvettes contained thyroid microsomes (4.1 mg protein per ml) in 0.1 M phosphate buffer (pH 7.4). b, spectrum after 53 $\mu$M NADH was added to the sample cuvette alone (the same experiment as that of Fig. 2). c, spectrum right after 53 $\mu$M NADH was added to the sample cuvette. 5.7 $\mu$M cytochrome peroxidase had been added to the both cuvettes. d, e and f, spectra were measured 5, 15 and 30 min after the NADH addition (continuation of c), respectively.

Fig. 7. Time courses of the reduction of cytochrome $b_5$, formation of the peroxide compound of cytochrome peroxidase and oxidation of NADH. The reaction solution contained thyroid microsomes (4.1 mg protein per ml) and 5.7 $\mu$M cytochrome peroxidase in 0.1 M phosphate buffer (pH 7.4). The reaction was started by the addition of 53 $\mu$M NADH. Absorbance changes at two wavelengths, 423 and 438 nm (left) and 340 and 438 nm (right) were followed simultaneously. 438 nm was an isosbestic point of reduced and oxidized cytochrome $b_5$. 6 $\mu$M ascorbate was added at arrows.

Fig. 6 shows difference spectra of cytochrome peroxidase and its peroxide compound which overlapped the spectral changes of cytochrome $b_5$. Both reference and sample cuvettes contained the microsome preparation. When NADH was added to the sample cuvette, a difference spectrum of the reduced cytochrome $b_5$ to the oxidized one was observed as in the case of Fig. 2. After cytochrome peroxidase was added to both cuvettes, NADH was added to the sample cuvette. Then, a larger increment in absorbance at 423 nm appeared and reached its maximum level within several minutes (Fig. 7A). This increment in absorbance beyond that in the absence of cytochrome peroxidase could be ascribed to the formation of its peroxide compound. The formation of the peroxide compound and the reduction of cytochrome $b_5$ are known to give rise to peaks of difference spectra at 424 and 423 nm, respectively. Their different contribution to the absorbance increase at 423 nm was, however, clearly demonstrated in Fig. 7A. Rapid reduction and oxidation of cytochrome $b_5$ were observed at the beginning and the end of NADH oxidation, as in the case without cytochrome peroxidase. Slow phases which followed the rapid ones were obviously ascribed to the formation and decomposition of the peroxide compound of cytochrome peroxidase. Ascorbate caused a rapid recovery of the free enzyme from its peroxide compound.

438 nm was an isosbestic point of the reduced and oxidized cytochrome $b_5$. The formation and decay of the peroxide compound of cytochrome peroxidase could thus be preferentially followed at this wavelength. Fig. 7B shows the time courses for the formation of the peroxide compound of cytochrome peroxidase and NADH oxidation. From these results, the initial velocities of NADH oxidation and $H_2O_2$ generation were calculated to be 6.6 and 2.6 $\mu M \cdot min^{-1}$, respectively.

## DISCUSSION

Since Serif and Kirkwood[27] and Alexander and co-worker[28,29] found that thyroid peroxidase plays a pivotal role in the biosynthesis of thyroid hormone, there have been many discussions regarding the source of $H_2O_2$. It may not be difficult to confirm the $H_2O_2$ generation in oxidative reactions provided that $H_2O_2$ is accumulated in the reaction system. This is, however, not always the case with reactions of tissue homogenates and subcellular fractions which usually contain high peroxidase and catalase activities. In this case, KCN or $NaN_3$ can be used as a specific inhibitor for these hydroperoxidases as was reported by Rossi *et al.*[30]. Recently, Chance and co-workers[31,32] have reported a successful approach for detecting the $H_2O_2$ generation in a subcellular fraction consisting of both peroxisomes and mitochondria. They found that horse radish peroxidase and scopoletin can be used as fluorescent $H_2O_2$ indicators and also that cytochrome peroxidase can be used as a direct spectrophotometric $H_2O_2$ indicator.

In the present experiment the $H_2O_2$ generation was demonstrated by use of hydroperoxidase inhibitors and cytochrome peroxidase in the NADH–thyroid microsome–$O_2$ system. It can be concluded that there is an oxidative path from NADH to $O_2$ *via* cytochrome $b_5$, as formulated in the sequence of reactions,

$$\text{NADH} \rightarrow \text{flavoprotein} \rightarrow \text{cytochrome } b_5 \rightarrow O_2$$

If all of the electrons travel through this path, the turnover number of cytochrome $b_5$ may be calculated from the following equation instead of Eqn 1.

$$k'_3 = \frac{2[\mathrm{NADH}]_0}{[b_5^{2+}]_m \cdot t_{\frac{1}{2}\,\mathrm{off}}} \tag{2}$$

Evidently, the turnover number of cytochrome $b_5$ thus calculated is twice that of $k_3$ and becomes 7 times larger than the value measured directly in the middle of cytochrome $b_5$ oxidation, where the reduction of the cytochrome by NADH is assumed to be negligibly small. This assumption, however, is a very important criterion for evaluating the turnover number measured directly from the oxidation of cytochrome $b_5$, since this apparent value would be markedly decreased by the presence of electron pools which supply electrons for the cytochrome at that moment. If the turnover number of cytochrome $b_5$ is really 0.037 $s^{-1}$, the discrepancy in the value obtained from the overall kinetics may be explained as follows. (1) The reaction sequence postulated above is not correct and the electrons leak to $O_2$ through electron carriers besides cytochrome $b_5$. (2) There are different binding forms of cytochrome $b_5$, one of which is extremely autooxidizable. The time course of cytochrome $b_5$ oxidation (Fig. 1) clearly shows that there are at least two groups of cytochrome $b_5$, their turnover numbers being calculated to be 0.037 $s^{-1}$ and 0.0025 $s^{-1}$. If the NADH oxidation occurs only through the autooxidation of cytochrome $b_5$, the third group of the cytochrome would occupy one third of the total cytochrome $b_5$, the cytochrome of this groups having a turnover number of about 1 $s^{-1}$.

A chain reaction containing the superoxide anion radical ($O_2^-$) as an intermediate, which was suggested by Yokota and Yamazaki[33] in the aerobic oxidation of NADH by peroxidase, does not appear to play a role in this reaction, since $Mn^{2+}$ and superoxide dismutase had no effect.

In the present experiment with cytochrome peroxidase, only 40% of reduced oxygen was detected as $H_2O_2$. The primary product of $O_2$ reduction is known to be $O_2^-$ and $H_2O_2$ but not water in the reactions of flavoprotein catalysis and autooxidation of biological molecules. Superoxide anion radicals disproportionate into $O_2$ and $H_2O_2$. So, when NADH is oxidized aerobically in the absence of cytochrome oxidase system, the generation of $H_2O_2$ equimolar to NADH will be expected. Cytochrome peroxidase reacts with $H_2O_2$ much faster than other peroxidases[34]. However, there is a possibility that $H_2O_2$ formed on the microsome surface occupies a suitable position so as to react preferentially with thyroid peroxidase. This assumption is of special interest for the physiological function of thyroid peroxidase. But, no spectral indication was obtained to support the formation of such a peroxide compound of thyroid peroxidase.

The amount of $H_2O_2$ detected in the present experiment appears to be sufficient to account for the iodination reaction in the thyroid hormone formation. It would be permissible to assume that this rate of $H_2O_2$ generation denotes the maximum latent capacity of iodination and that there are regulatory processes in the intact thyroid tissue which suppress the useless electron leakage.

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