**HISTOCHEMICAL STUDY ON THE INTRAVITAL
REDUCTION POTENCY OF COENZYME Q
IN THE RESPIRATORY CHAIN
OF THE HAIR CELLS OF THE SPIRAL ORGAN
IN NORMAL GUINEA PIGS
AND ITS ALTERATION
IN ACUTE STARVATION AND VITAMIN B₁ DEFICIENCY**

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The present study revealed that the intravital reduction of Nitro-BT can occur in the absence of any exogenous substrate in the living hair cells of the cochlea in nembutal anaesthetized guinea pigs. Electron transfer inhibitors, such as amytal and malonate considerably impeded the intravital reduction of Nitro-BT. The reduction of Nitro-BT was completely blocked after heating at 100°C for 15 minutes and cooling at -20°C for 30 minutes. The reduction of Nitro-BT continued to some extent in the hair cells left at room temperature at least for 60 minutes after sacrifice. Acute complete starvation caused considerable decrease in the intravital reduction of Nitro-BT and vitamin B₁ deficiency did rather slightly. The intravital reduction of Nitro-BT seemed to be due to the intravital multi-enzymatic tetrazolium reducing reaction. The present study suggested that the intravital enzymatic tetrazolium reducing reaction may depend on the amount of electrons which are transferred by CoQ in the respiratory chain to Nitro-BT. The amount of electrons transferred by CoQ to Nitro-BT was considered the intravital reduction potency of CoQ. The intravital reduction potency of CoQ seemed to depend on cellular metabolism and its rate in the hair cells.

Crane *et al.* (1959) (1), and Lester and Fleischer (1961) (2) developed a simple efficient method for removing Coenzyme Q (CoQ or ubiquinone, UQ) from mitochondrial preparation and for restoration with CoQ and clarified the role of CoQ as well as lipid in the electron transport system. Thereafter the effects of CoQ on the histochemical reaction for the activity of succinate dehydrogenase (SDH) in the tissue sections were intensively studied by Wattenberg and Leong (1960) (3), Straud and Wattenberg (1962) (4), Wolman and Bubis (1967) (5) and Horwitz *et al.* (1967) (6). Horwitz *et al.* (1967) (6) studied the role of CoQ in the succinate tetrazolium reductase reaction in the fresh cryostat sections of normal rat tissues after acetone extraction of CoQ and restoration with CoQ-lecithin complex. They concluded that the succinate tetrazolium reductase reaction requires intermediate electron carrier, mainly CoQ and nitro blue tetra-

zolium (Nitro-BT) cannot accept electrons (or hydrogen atoms) directly from the flavins but from the intermediate electron carriers including CoQ.

On the other hand, Klingenberg and Kröger (1967) (7) biochemically investigated the functional behaviour of UQ over a wide variety of redox reactions in the rat heart mitochondria and revealed that UQ is a full member on the main path of the electron transfer in the respiratory chain and forms a common pool collecting electrons from the various flavoprotein dehydrogenases.

Akiyoshi and Sato (1967) (8) successfully demonstrated histochemically the intravital activity of succinate dehydrogenase of the hair cells of the cochlea by intracochlear perfusion of the incubation medium in the anaesthetized guinea pigs with nembutal. Furthermore, they revealed that the intravital reduction of Nitro-BT within the living hair cells can occur in the absence of any exogenous substrate in guinea pigs under nembutal narcosis (9).

On the basis of these data it was assumed that during active metabolism of the living hair cells electrons may be successively transferred from the various NAD-linked and non-NAD-linked substrates, such as succinate, to CoQ and then these electrons were transferred by the CoQ to Nitro-BT, resulting in reduction.

The present study was made to investigate histochemically the intravital reduction potency of CoQ in the respiratory chain by means of the intravital enzymatic tetrazolium reducing reaction in the living hair cells of the cochlea in normal guinea pigs and its alteration in acute starvation and vitamin B₁ deficiency.

MATERIALS AND METHODS

Forty-five albino guinea pigs (about 350 g body weight) of the Hartley strain were divided into 3 groups: normal group; vitamin B₁ deficiency one and starvation one. Of them, 20 guinea pigs were fed on normal synthetic diet (Oriental Yeast Co., Ltd.) and the other 15 were fed on vitamin B₁ deficient synthetic diet (Oriental Yeast Co., Ltd.) for 21 days. The remaining 15 animals were exposed to complete starvation for 72 hours.

Incubation medium for the intravital enzymatic tetrazolium reducing reaction was composed of 5.0 ml Nitro-BT (1 mg/ml) in distilled water and 5.0 ml phosphate buffer (0.2 M, pH 7.4). Exogenous substrate was not added to the medium.

General procedures for the intravital enzymatic tetrazolium reducing reaction in the living hair cells of the spiral organ: In all groups, animals were almost always anaesthetized with nembutal and their cochleas were surgically exposed. The incubation medium aforementioned was slowly injected into both the perilymphatic and endolymphatic spaces of the cochlea through the round window membrane, and allowed to discharge from the small opening in the apex of the cochlea (Fig. 1). By this procedure the incubation medium could diffuse to the hair cells of the spiral organ (Fig. 2) and then easily penetrate into mitochondria through the cell membrane of the living hair cells, resulting in reduction of Nitro-BT. The intracochlear reaction was ceased by perfusion of 10% formalin after the incubation for 15 minutes. After removal of the bony capsule of the cochlea the spiral organ was dissected under stereomicroscope and the specimens containing the hair cells were mounted on the slide glass and observed microscopically.

Procedures for control: For the block of nicotinamide adenine dinucleotide

FIG. 1. Midmodiolar section of the cochlea in the guinea pig. A thick long arrow indicates the site of insertion of the needle at the round window membrane, whereas a thin short arrow indicates the site of opening in the apex of the cochlea. Triangles show the spiral organ in the endolymphatic space. The incubation medium is injected from the thick arrow, then flows upwards and discharged from the short arrow.

FIG. 2. Ductus cochlearis containing endolymph. The spiral organ is composed of the inner hair cells (IHC), outer hair cells (OHC), supporting cells and pilars. These hair cells are directly or indirectly surrounded by Cortilymph and subtektorial lymph. The incubation medium diffuses to these lymphs and penetrates through the cell membrane into mitochondria of the hair cells.

(NAD)-linked oxidase system, amytal as an inhibitor of electron transfer was administered intraperitoneally. For competitive inhibition of SDH, malonate (0.2 M) was added to the incubation medium for the intravital enzymatic tetrazolium reducing reaction. In addition, heating at 100°C for 15 minutes and cooling at 6°C or -20°C for 30 minutes of the specimens were also conducted before the enzymatic tetrazolium reducing reaction. For evaluation of the effect of numbutal on the intravital enzymatic tetrazolium reducing reaction in the living hair cells, nonanaesthetized animals were decapitated and the cochlea were perfused with the same incubation medium in the same way immediately and 60 minutes after sacrifice. The enzymatic tetrazolium reducing reaction was carried out at 37°C for 15 minutes after various procedures for control. On the other hand, the effects of the extraction of CoQ and lipid, and restoration with CoQ on the succinate tetrazolium reductase reaction were investigated in the specimens of the spiral organ: for the extraction of CoQ aqueous acetone dilution (96%) was used as prefixative bath according to Horwitz *et al.* (1967) (6) and for restoration of CoQ CoQ₇ (suspension in distilled water, Takeda Chemical Industries, Ltd.) was added to the acetone-extracted specimens. The incubation medium for SDH consisted of: 5 ml sodium succinate (0.2 M) in distilled water; 5.0 ml phosphate buffer (0.2 M, pH 7.6); 10.0 ml Nitro-BT (1 mg/ml) in distilled water.

Procedures for demonstration of CoQ in the living hair cells: Incubation medium was prepared according to the following prescription of Tranzer and Pearse (1963) (10): 0.25 ml 3-(4,5-dimethylthiazolyl-2)-5-diphenyl tetrazolium bromide (MTT, 1 mg/ml) in distilled water; 0.05 ml cobalt chloride (0.5 M); 0.40 ml Tris buffer (0.2 M, pH 7.4); 0.10 ml hydroquinone (40 mg/ml) in Tris buffer (0.2 M, pH 7.4); 0.20 ml distilled water; 2 mg catalase (Boehringer & Söhne, Mannheim). Intravital perfusion of the cochlea with the incubation medium for CoQ was performed in the same way as in the intravital enzymatic tetrazolium reducing reaction of the hair cells.

RESULTS

1. *Intravital perfusion of the cochlea with the incubation medium for SDH, and effect of acetone extraction of CoQ, and restoration with CoQ₇ on the succinate tetrazolium reductase reaction*

Intravital perfusion (for 15 minutes) of the cochlea with the incubation medium for histochemical reaction of SDH resulted in deposition of a large amount of formazan granules in the cytoplasm of the inner and outer hair cells of the spiral organ (Fig. 3). The nuclei of these hair cells were always negative for the histochemical reaction. On the other hand, extraction of CoQ and lipid from the specimens of the spiral organ with 96% aqueous acetone almost completely abolished the production of formazan granules in the cytoplasm of the both hair cells (Fig. 4). However, reincubation of the specimens which were extracted in 96% aqueous acetone and then treated with CoQ₇, restored considerably the production of formazan granules in the hair cells (Fig. 5). These findings suggested that in the succinate tetrazolium reductase reaction of the hair cells, CoQ may play rather important role of intermediate electron carrier.

2. *Intravital enzymatic tetrazolium reducing reaction of the living hair cells of the cochlea*

Intravital perfusion of the cochlea with the incubation medium consisting of

Nitro-BT and phosphate buffer resulted in the production of a considerable amount of formazan granules in the cytoplasm of the outer hair cells (Fig. 6) and inner hair cells (Fig. 7). The nuclei of these cells, however, did not show any deposition of formazan granules and the supporting cells in the spiral organ also almost always were free from the formazan granules. The result suggested that CoQ in the respiratory chain of the living hair cells might have a sufficient amount of electrons to reduce Nitro-BT even in the absence of exogenous substrate.

3. *Effect of electron transfer inhibitors (amytal and malonate) on the intravital enzymatic tetrazolium reducing reaction*

Intravital perfusion of the cochlea with the incubation medium containing Nitro-BT, phosphate buffer and malonate in the guinea pigs anaesthetized with amytal resulted in remarkable inhibition of the production of formazan granules in the living hair cells (Fig. 8). It indicated that in the intravital enzymatic tetrazolium reducing reaction in the living hair cells, CoQ uninterruptedly accepts rather large amount of electrons from the NAD-linked substrates on the one hand and succinate on the other hand. When the electron transfer from these endogenous substrates is blocked by amytal and malonate, so CoQ in the respiratory chain probably cannot accept sufficient amount of electrons to reduce Nitro-BT (Diagram I).

4. *Effect of heating and cooling on the enzymatic tetrazolium reducing reaction in vitro*

Heating of the specimens at 100°C for 15 minutes almost completely abolished the reduction of Nitro-BT, so that formazan granules were not produced in the hair cells (Fig. 9). Cooling of the specimens at 6°C or -20°C for 30 minutes also resulted in considerable or complete inhibition of the production of formazan granules in the hair cells (Fig. 10). However, when the specimens kept at room temperature (about 23°C) for 60 minutes after sacrifice were incubated in the incubation medium containing Nitro-BT and phosphate buffer, so formazan

DIAGRAM I. Diagram of respiratory chain and possible main route for electron transportation to Nitro-BT in intravital enzymatic tetrazolium reducing reaction in the living hair cells. M: Site of action of malonate, A: Site of action of amytal, FP: Flavoprotein, AA: Site of action of antimycin A.

granules appeared in the hair cells (Fig. 11). These findings suggested that cellular metabolism in the hair cells might continue to some extent at room temperature at least for 60 minutes after sacrifice and during the metabolism electrons from the various endogenous substrates in the metabolic pathways might be continuously transferred to CoQ. The cooling at -20°C might stop the cellular metabolism and then results in discontinuation of the electron transfer to CoQ. Consequently, CoQ might be unable to reduce Nitro-BT in the hair cells.

5. *Effect of nembutal narcosis on the intravital enzymatic tetrazolium reducing reaction*

In the present study, the intravital enzymatic tetrazolium reducing reaction in the living hair cells was almost always carried out in the guinea pigs under nembutal narcosis, because surgical procedure for the reaction and intracochlear perfusion could not be performed in the non-anaesthetized animals. In the enzymatic tetrazolium reducing reaction performed immediately after sacrifice of guinea pigs under nembutal narcosis, a considerable amount of formazan granules were produced in the cytoplasm of the inner and outer hair cells (Fig. 12). On the other hand, in the enzymatic tetrazolium reducing reaction performed immediately after sacrifice of the nonanaesthetized guinea pigs, the deposition of formazan granules in the cytoplasm was rather prominent (Fig. 13). There was no distinct difference in the production of the formazan granules between two kinds of the reactions. It suggested that nembutal may not inhibit so remarkably the electron transfer as amytal, consequently no remarkable decrease in the reduction of Nitro-BT occurred in nembutal anaesthetized guinea pigs.

FIG. 3. Intravital succinate tetrazolium reductase reaction in the living hair cells of the cochlea in guinea pig. A large amount of formazan granules are produced in the cytoplasm of the inner hair cells (IHC) and outer ones (OHC). Strongly positive reaction in some of supporting cells.

FIG. 4. Succinate tetrazolium reductase reaction in the dissected specimen of the spiral organ after extraction of CoQ and lipids with 96% acetone. Neither inner hair cells (IHC) nor outer hair cells (OHC) contains any formazan granule in their cytoplasm.

FIG. 5. Succinate tetrazolium reductase reaction in the dissected specimen of the spiral organ which previously underwent acetone extraction and then was restored with CoQ. The production of formazan granules is more prominent in the outer hair cells (OHC) than in the inner hair cells (IHC).

FIG. 6. Intravital enzymatic tetrazolium reducing reaction in the living hair cells of the spiral organ. The reaction relatively strongly can occur only in the cytoplasm of the hair cells including the outer hair cells (OHC), but not in the other cells.

FIG. 7. In the intravital enzymatic tetrazolium reducing reaction in the living hair cells, formazan granules are produced in the cytoplasm of the inner hair cells (IHC) and outer hair cells (OHC). OHC are out of focus.

FIG. 8. Intravital enzymatic tetrazolium reducing reaction is strongly blocked by electron transfer inhibitors, such as amytal and malonate. Considerable suppression of the production of formazan granule in the cytoplasm of the inner hair cells (IHC) and outer ones (OHC).

FIG. 9. Enzymatic tetrazolium reducing reaction in the spiral organ after heating at 100°C for 15 minutes. No formazan granule in the cytoplasm of the outer hair cells (circles).

FIG. 10. Enzymatic tetrazolium reducing reaction in the spiral organ after cooling at 6°C for 30 minutes. Almost no formazan granule in the cytoplasm of the inner hair cells (triangle) and outer hair cells (circles).

FIG. 11. Enzymatic tetrazolium reducing reaction in the spiral organ left at room temperature for 60 minutes after sacrifice. Formazan granules are produced moderately in the cytoplasm of the inner hair cells (asteroid) and slightly in the outer hair cells (circles).

FIG. 12. Enzymatic tetrazolium reducing reaction in the spiral organ performed immediately after sacrifice of guinea pig under nembutal narcosis. Considerable amount of formazan granules are produced in the cytoplasm of the inner hair cells (asteroid) and outer ones (circles).

FIG. 13. Enzymatic tetrazolium reducing reaction in the spiral organ performed immediately after sacrifice of non-anaesthetized guinea pig. Rather considerable amount of formazan granules are deposited in the cytoplasm of the inner hair cells (asteroid) and outer ones (circles).

6. *Histochemical demonstration of the intravital activity of CoQ in the hair cells*

The intravital activity of CoQ in the living hair cells was demonstrated histochemically by the intracochlear perfusion of the incubation medium for CoQ in guinea pigs under nembutal narcosis. The formazan granules in this reaction almost showed the same intracellular distribution as those in the succinate tetrazolium reductase reaction (Fig. 14). These findings suggested that the deposition of formazan granules most probably corresponds to the mitochondria of the hair cells, because SDH is a marker enzyme of mitochondria and formazan granules

produced in the succinate tetrazolium reductase reaction are considered to indicate mitochondria.

7. *Alteration in the intravital enzymatic tetrazolium reducing reaction in acute complete starvation*

Acute complete starvation for 72 hours brought about rather considerable decrease of formazan granules in the cytoplasm of the living hair cells in the intravital enzymatic tetrazolium reducing reaction (Fig. 15). It indicated that the

FIG. 14. Intravital reduction of MTT by the activity of CoQ (Hq-MTT reaction, Pearse 1972) in the living hair cells. Intracellular distribution of the formazan granules of the inner and outer hair cells seems to correspond to those in the intravital succinate tetrazolium reductase reaction.

FIG. 15. Intravital enzymatic reducing reaction in the living hair cells in guinea pig which was exposed to acute complete starvation. Considerable decrease in the production of formazan granules in the cytoplasm of the inner hair cells (asteroid) and outer ones (circles).

FIG. 16. Intravital enzymatic tetrazolium reducing reaction in the living hair cells in guinea pig which was fed on vitamin B₁ deficient diet. The production of formazan granules in the cytoplasm of the inner hair cells (asteroid) and outer ones (circles) is not so remarkably suppressed.

intravital reduction of Nitro-BT may be markedly suppressed in acute starvation, because the complete starvation brings about rapid decrease in concentration of the endogenous substrates, consequently the amount of electrons transferred from the endogenous substrates to CoQ also may become reduced in the living hair cells.

8. *Effect of vitamin B₁ deficiency on the intravital enzymatic tetrazolium reducing reaction of the living hair cells*

In the guinea pigs fed on vitamin B₁ deficient synthetic diet for 21 days, the intravital enzymatic tetrazolium reducing reaction resulted in slight decrease in deposition of formazan granules in the cytoplasm of the living inner and outer hair cells (Fig. 16). It suggested that impairment of oxidative decarboxylation of pyruvic acid, alphaketoglutaric acid and alpha-keto acid due to vitamin B₁ deficiency may not cause distinct suppression of the intravital reduction of Nitro-BT in the living hair cells.

DISCUSSION

1. *The role of CoQ in the electron transfer system or respiratory chain and in the succinate tetrazolium reductase reaction*

The studies on the electron transfer system by Lester and Smith (1961) (11) and Green and Fleischer (1963) (12) suggest that CoQ may be an essential intermediate electron or hydrogen carrier in the electron transfer system. On the other hand, Klingenberg and Kröger (1967) (7) reveal that the function of CoQ in the respiratory chain is to form a collecting pool for hydrogen from the various dehydrogenases. Horwitz *et al.* (1967) (6) suggest that CoQ acts as essential electron carrier in the succinate tetrazolium reductase reaction in fresh cryostat sections of the rat organs. The present authors also confirmed the role of CoQ

as intermediate electron carrier in the same reaction in the dissected specimens of the spiral organ. The function of CoQ as intermediate electron carrier is considered to depend on its reduction and reoxidation (Pearse, 1972) (13).

2. *The function of Nitro-BT in the succinate tetrazolium reductase reaction and in the enzymatic tetraaolium reducing reaction in the living hair cells*

Nitro-BT is used as exogenous electron acceptor in the succinate tetrazolium reductase reaction of fresh cryostat sections of animals, because Nitro-BT has a higher redox potential than the cytochrome system. The intact membrane of the mitochondria is considered to prevent the penetration of tetrazolium (Pearse, 1972) (13), for this reason the succinate tetrazolium reductase reaction usually is carried out in fresh cryostat sections and not *in vivo*. Spöndlin and Balogh (1963) (14), however, performed intravital perfusion of the cochlea in cat with the incubation medium and demonstrated histochemically the activity of SDH of the hair cells *in vivo*. Akiyoshi and Sato (1967) (8) also made independently the succinate tetrazolium reductase reaction using the almost same perfusion method of the cochlea in nembutal anaesthetized guinea pigs and suggested that Nitro-BT and substrate easily penetrate through the cell membrane into the mitochondria of the living hair cells and there undergo histochemical reaction. The intravital enzymatic tetrazolium reducing reaction revealed that in the living hair cells, Nitro-BT can accept the electrons from the NAD-linked and non-NAD-linked endogenous substrates which would normally pass by way of succession of carriers in the cytochrome system to molecular oxygen, if not intercepted by the exogenous electron acceptor (Nitro-BT).

3. *The intravital reduction potency of CoQ in the respiratory chain of the living hair cells*

On the basis of the studies by Horwitz *et al.* (1967) (6) and Klingenberg and Kröger (1967) (7) and the present study, the reduction of Nitro-BT in the living hair cells seems to correspond to the amount of electrons carried by CoQ. This amount of electrons which are carried by CoQ to Nitro-BT may be considered the reduction potency of CoQ. The control reaction with electron transfer inhibitors, such as amytal and malonate indicates that considerable amount of the electrons originates from the NAD-linked and non-NAD-linked endogenous substrates in the metabolic pathways and were transferred by the flavoproteins to CoQ. It seems probable that the intravital reduction potency of CoQ mostly depends on the amount of the electrons carried by the flavoproteins to CoQ on the one hand and on the amount of CoQ itself in the respiratory chain on the other.

4. *The reduction potency of CoQ and cellular metabolism*

The enzymatic tetrazolium reducing reaction indicates that the reduction potency of CoQ in the hair cells is rather high in normal nembutal anaesthetized guinea pigs and continue in a greater or less degree at room temperature at least for 60 minutes after sacrifice. However, the enzymatic tetrazolium reducing reaction became negative after cooling at -20°C for 30 minutes. These results suggest that the reduction potency of CoQ in the respiratory chain greatly depends on the electrons which are associated with the cellular metabolism of the hair cells. It may be assumed that the cellular metabolism of the hair cells lasts to some extent at room temperature at least for 60 minutes after sacrifice and is stopped almost completely after cooling at 6°C or -20°C .

5. *Alteration of the intravital reduction potency of CoQ in acute complete starvation and vitamin B₁ deficiency*

Considerable decrease in the intravital reduction of Nitro-BT in the acute complete starvation suggests that the intravital reduction potency of CoQ in the respiratory chain markedly becomes suppressed. The amount of the electrons carried to CoQ may become decreased, because the acute complete starvation probably brings about decrease in the concentration of the endogenous substrates in the living hair cells.

In the vitamin B₁ deficiency, the intravital reduction of Nitro-BT in the living hair cells was slightly decreased. It suggests that electron transfer to CoQ is not so remarkably impaired and the suppression of the intravital reduction potency of CoQ may be slight. In these instances, however, possible decrease in the amount of CoQ itself may be not completely excluded.

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