The in Vitro Effects of Tannic Acid on Rat Liver Mitochondrial
Respiration and Oxidative Phosphorylation

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The in vitro effects of tannic acid on the membrane structure and function of rat liver
mitochondria were investigated. The respiratory control ratio (RCR) decreased by about 50% on
addition of 50 μg/ml tannic acid to highly coupled mitochondria, but the adenosine-5'-diphos-
sphate/oxygen (ADP/O) ratio was constant. The uncoupler-induced respiration was also inhibited
in the same manner as the RCR. Moreover, the respiratory control disappeared and the ADP/O
ratio could not be measured at concentrations of tannic acid above 100 μg/ml. On the other hand,
the oxygen consumption rate of succinate-dependent respiration decreased on addition of more
than 100 μg/ml tannic acid (50% inhibitory concentration (IC50) = 150 μg/ml tannic acid) to
mitochondria. These findings suggest that tannic acid at lower concentrations inhibits the electron
transport system to decrease the RCR, but does not impair the membrane, retaining the coupled
reaction, while at higher concentrations it impairs the structural integrity of mitochondrial
membranes, and directly inhibits the electron transport system.

Tannic acid inhibited the succinate oxidase, reduced nicotinamide adenine dinucleotide
(NADH) oxidase, succinate dehydrogenase, and NADH dehydrogenase activities of submito-
ochondrial particles (SMP). The IC50 values of tannic acid toward these enzyme systems were
estimated to be 35, 45, 30, and 15 μg/ml, respectively. Tannic acid competitively inhibited succinate
dehydrogenase and NADH dehydrogenase. However, it did not show significant inhibition of the
cytochrome oxidase activity of SMP. It is thus concluded that tannic acid exerts its effect on
mitochondrial respiration and oxidative phosphorylation through action on the membrane and on
both succinate dehydrogenase and NADH dehydrogenase of mitochondria.

Keywords—tannic acid; mitochondria; dehydrogenase; respiratory control; submitochondri-
drial particle

Introduction

Tannin, a general term for water-soluble polyphenols included in plants, is a major
component in various oriental medicinal plants. Tannic acid, which is a kind of tannin (also
called Chinese gallotannin), is readily available as a commercial reagent prepared from
Chinese nutgall. This compound possesses protein-aggregating, astringent, and antibacterial
actions.1) As regards toxicity, it was reported that tannic acid might be absorbed from the
gastrointestinal tract, denuded surfaces, and mucous membranes and then cause severe
centrilobular necrosis of the liver.2–4) We have recently observed that tannic acid exerted its
antibacterial effects on Photobacterium phosphoreum through inhibition of nicotinamide
adenine dinucleotide (NADH) dehydrogenase and the terminal oxidase of the respiratory
chain.5) From these results, we supposed that tannic acid might influence the respiratory chain
of mitochondria in animal cells. In this work, the in vitro effects of tannic acid on rat liver
mitochondria were investigated.
Materials and Methods

Preparation of Mitochondria—Rat liver mitochondria were prepared according to the method of Hogeboom with some modification. Wistar rats (male, 250–300 g weight), purchased from Sankyo Labo. Co., were used in this study. In order to obtain intact mitochondria with high respiratory control and normal adenosine-5'-diphosphate/oxygen (ADP/O) ratio, we chose the following procedure for the preparation of the rat liver mitochondria. The liver was quickly excised and thoroughly washed with ice-cold 0.25 M sucrose following decapitation of the rat. The liver was minced into small pieces with a pair of sharp, chilled scissors. The finely minced tissue was gently homogenized with a loose-fitted Potter–Elvehjem homogenizer in 0.25 M sucrose, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 5 mM Tris–HCl, pH 7.4 (solution A), at the rate of 10 ml/g of liver. The homogenate was centrifuged at 7000 × g for 10 min. The supernatant obtained was centrifuged at 7000 × g for 10 min. The precipitate was washed once with solution A by centrifugation, and then washed twice with 0.25 M sucrose, adjusted with KOH to pH 7.4 (solution B), by repeating the above centrifugations. The final mitochondrial pellet was suspended in a minimal volume of solution B. The above procedures were carried out at 4°C.

Preparation of Submitochondrial Particles (SMP)—The rat liver SMP was prepared according to the method of Gregg with some modification. The isolated mitochondria were sonicated with a Tomy Seiko UR-200P ultrasonic disrupter in 50 mM phosphate buffer, pH 7.4, with cooling in ice water. The treated suspension was centrifuged at 7000 × g for 10 min to remove undisrupted mitochondria, and the supernatant thus obtained was then centrifuged at 100000 × g for 1 h. The packed pellet was suspended in 50 mM phosphate buffer, pH 7.4, 50% glycerol by gentle homogenization. The SMP were stored at −20°C before use.

Tannic Acid—Tannic acid mainly used in this study was purchased from Wako Pure Chemical Industries (Lot No. KWP7592). In order to check the difference of activities of tannic acids from various makers, several other tannic acids were used (Nakarai Chemicals, Lot No. M4R1112; Kanto Chemical, Lot No. 001H5101; Sigma Chemical, Lot. No. 6F-0049; Wako Pure Chemical, Lot No. EPR7215). Tannic acid was adjusted with NaOH to pH 7.0 before use.

Assay of Electron Transport Activity of Mitochondria—The succinate-dependent respiration rate of mitochondria was determined polarographically in an oxygen monitor equipped with a Clark type oxygen electrode as described by Estabrook. Freshly prepared mitochondria were incubated in the reaction chamber containing 1 ml of assay mixture (125 mM sucrose, 20 μM cytochrome c, 50 mM KCl, 6 mM MgCl₂, 20 μM rotenone, and 15 mM phosphate buffer, pH 7.0) at 25°C for 3 min before addition of 10 mM succinate. The substrate-induced oxygen consumption was plotted on a strip chart recorder, and then 400 μM ADP was added to the assay system to stimulate the respiration to state 3. After the consumption of ADP the respiration will change to state 4. The respiratory control ratio (RCR) was expressed as the ratio of the respiration rate of state 3 to that of state 4.

Succinate Oxidase and NADH Oxidase Activity of SMP—Substrate (10 mM succinate or 4 mM NADH) was added to the assay mixture (0.7 mg of SMP, tannic acid, 20 μM cytochrome c, 1 mM EDTA, and 0.1 M phosphate buffer, pH 7.4) at 25°C, and oxidase activity was determined polarographically.

Cytochrome Oxidase Activity of SMP—Ascorbate (16 mM, adjusted to pH 7.4) as the electron donor was added to the assay mixture (0.7 mg of SMP, tannic acid, 20 μM cytochrome c, and 0.1 M phosphate buffer, pH 7.4) at 25°C, and oxidase activity was determined polarographically.

Succinate Dehydrogenase and NADH Dehydrogenase Activities of SMP—Substrate (20 mM succinate or 1 mM NADH) was added to the assay mixture (0.7 mg of SMP, tannic acid, 0.06 mM 2,6-dichloroindophenol (DCIP), and 0.3 mM KCN) at 25°C, and the dehydrogenase activity was determined spectrophotometrically by measuring the absorbance change of DCIP at 600 nm.

Effect of Tannic Acid on Redox Behavior of Cytochromes b and c₁ + c—In order to investigate the effect of tannic acid on electron transport between cytochrome b and cytochrome c₁ + c in succinate oxidoreductase, we employed the following procedure. In the control experiment, an excess amount of sodium succinate was added to the suspension containing SMP (3.0 mg/ml), and the succinate reduced-minus-oxidized difference spectrum was recorded on an Aminco DW-2C spectrophotometer. In the inhibitory experiment, 30 μg/ml tannic acid was added to the same suspension before the addition of an identical amount of sodium succinate. The complete reduction of cytochromes was carried out by the addition of sodium dithionite.

Other Methods—Various polyphenols included in commercial tannic acid were fractionated by Sephadex LH-20 (Pharmacia Fine Chemical) column chromatography according to the method of Nishio et al. The protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard.

Results

The Effects on the Respiratory Control and Oxidative Phosphorylation of Mitochondria

Addition of ADP induced an approximately fivefold increase in the rate of succinate-dependent respiration (Fig. 1). However, in the presence of 50 μg/ml tannic acid this
respiratory control ratio decreased to about 50%. This effect was found to be dose-dependent. On addition of more than 100 µg/ml tannic acid, this respiratory control disappeared (Fig. 2). On the other hand, the ratio between the amount of ADP added and the oxygen consumed in state 3 (ADP/O ratio) was maintained at about 2.0 in the presence of tannic acid until its respiratory control disappeared (Fig. 2). The increase in oxygen consumption rate with an uncoupler, carbonylcyanide m-chlorophenylhydrazone (CCCP), was inhibited by up to 100 µg/ml tannic acid and its 50% inhibitory concentration (IC50) was about 150 µg/ml (Fig. 2). We also found that the substrate-induced respiration (state 4) was slightly increased by low concentrations of tannic acid.

The Effect of Tannic Acid on the Electron Transport Activities of the Respiratory Chain of SMP

In view of the findings that tannic acid inhibited the succinate-dependent respiratory...
activity of mitochondria, we investigated the effects of tannic acid on SMP to determine the inhibitory site of tannic acid on the respiratory chain. As shown in Fig. 3, tannic acid inhibited succinate oxidase and NADH oxidase activities (IC_{50}: 35 and 45 µg/ml, respectively), but did not significantly inhibit cytochrome oxidase activity. The results obtained suggested that the crossover point was prior to the cytochrome oxidase. We found that the spectral features of both cytochromes b and c_{1} + c in the succinate reduced-minus oxidized difference spectrum of SMP were not changed after incubation with tannic acid (Fig. 4). It appears that tannic acid does not inhibit the electron transport between cytochromes b and c_{1} + c, and thus the crossover point is prior to cytochrome b. Furthermore, it was shown that tannic acid inhibited succinate dehydrogenase and NADH dehydrogenase activities (Fig. 5) (IC_{50}: 30 and 15 µg/ml, respectively). The above results suggest that the inhibitory sites of tannic acid on the respiratory chain are both succinate dehydrogenase and NADH dehydrogenase.
Enzyme Kinetics

Since the inhibitory effects of tannic acid on the respiratory chain were found to be located at succinate dehydrogenase and NADH dehydrogenase, we performed some kinetic studies to establish the mechanism of inhibition by tannic acid. The results are illustrated by double-reciprocal plots in Figs. 6 and 7, indicating that tannic acid is a competitive inhibitor of both succinate dehydrogenase and NADH dehydrogenase.

Discussion

When succinate was used as a substrate, the respiratory control ratio and the ADP/O ratio of prepared mitochondria were about 4.5 and 1.9, respectively. These data indicated that the mitochondrial preparation was highly coupled and active in oxidative phosphorylation. When tannic acid was present in the assay medium, the RCR was decreased and this effect was dose-dependent, but the ADP/O ratio was constant. The decrease of RCR was caused by the alteration of state 3 respiration, since the state 4 respiration did not change greatly with tannic acid at concentrations below 75 μg/ml, and the inhibition of state 3 respiration was not released by the addition of uncoupler CCCP. These findings suggest that tannic acid inhibits the electron transport system of mitochondria, but does not much impair the membrane barrier, and does not inhibit coupled oxidative phosphorylation.

We found that the succinate-dependent respiration (state 4) was slightly increased by tannic acid, suggesting that the respiration was somewhat uncoupled due to leakiness of the mitochondrial membrane to ions induced by the inhibitor. Although this effect might be similar to that of an uncoupler of oxidative phosphorylation, it was very small and probably did not significantly affect the ADP/O ratio.

At high concentrations of tannic acid (above 100 μg/ml), the respiratory control was not observed, the ADP/O ratio could not be measured, and the succinate-dependent electron transport system was inhibited. These data indicate that tannic acid impairs the mitochondrial membrane and then directly inhibits the exposed electron transfer system. The inhibitory sites of tannic acid on the electron transport system are considered to be both succinate dehydrogenase and NADH dehydrogenase. This conclusion is supported by the findings that the succinate- and NADH-supported respiratory systems were inhibited by tannic acid but...
cytochrome oxidase was not influenced, and the crossover point was prior to cytochrome b from the difference spectral data. In fact, both the dehydrogenases of SMP were inhibited competitively by tannic acid (Figs. 5, 6), even though this inhibitor is not obviously structurally related to succinate and NADH. As a similar example, pyrophosphate, which is not structurally related of succinate, is a strong competitive inhibitor of succinate dehydrogenase.\textsuperscript{12} It is not yet clear how tannic acid reacts with the dehydrogenases.

The orientation of the mitochondrial inner membrane is opposite to that of SMP (inside-out), and in the case of mitochondria, there is an outer membrane outside of the inner membrane which carries the respiratory chain components. Regardless of these differences, the inhibitory effect of tannic acid on uncoupler-induced activity or state 3 respiration activity of mitochondria (IC\textsubscript{50} = 65 and 40 µg/ml, respectively; determined from the data in Fig. 2) was similar to that on succinate oxidase of SMP (IC\textsubscript{50} = 35 µg/ml).

As described above, the decrease of RCR was caused by the decrease of state 3 respiration while state 4 respiration remained almost constant in the presence of the inhibitor. One possible explanation is that tannic acid may hardly be taken up by mitochondria in the presence of membrane potential (state 4 respiration).

From the above results, we may draw the following conclusions. At lower concentrations of tannic acid, it inhibits the electron transport of mitochondria to decrease the respiratory control ratio but does not inhibit the coupled reaction in oxidative phosphorylation. At higher concentrations, tannic acid breaks the membrane barrier of mitochondria to dissipate the coupled reaction, and then competitively inhibits succinate dehydrogenase and NADH dehydrogenase.

Tannic acid prepared from Chinese nutgall is readily available from commercial sources, but this contains many structural analogues composed of glucose and gallic acid, and the composition of polyphenols may differ from lot to lot. Therefore, we investigated the effects of five different lots of tannic acid purchased from four companies on the NADH dehydrogenase activity of SMP. Their IC\textsubscript{50} values were almost the same (15—30 µg/ml) (data not shown). Furthermore, we tried to fractionate tannic acid by Sephadex LH-20 column chromatography and obtained 15 fractions, although it is difficult to isolate each polyphenol from tannic acid. Three of the 15 fractions separated by the chromatography had no inhibitory effect. On the other hand, the others (12 fractions) had strong inhibitory activities against succinate oxidase of SMP and their IC\textsubscript{50} values were 21—44 µg/ml, similar to that of the whole tannic acid. One of the former 3 fractions was the first fraction eluted from the column and contained mainly gallic acid and digallic acid. The other two were the last-but-one fraction and the final fraction, and contained mostly high-molecular-weight polyphenol. No inhibitory effect was observed with pure gallic acid, which is consistent with the above result. The isolation and characterization of each polyphenol are in progress in our laboratory.

References