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INHIBITION BY TANNIC ACID OF SUCCINATE AND MALATE TRANSLOCATION ACROSS THE MITOCHONDRIAL MEMBRANE

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Abstract—The effect of tannic acid on succinate oxidation and on succinate and malate translocation has been studied in rat liver mitochondria. Tannic acid inhibits succinate oxidation in mitochondria but not in submitochondrial particles. The inhibition of succinate oxidation appears as competitive by using the double reciprocal plot and as partially competitive by using the Dixon plot. Tannic acid inhibits the swelling of mitochondria in the presence of ammonium succinate or ammonium malate and the valinomycin-induced swelling with succinate as accompanying anion. It is concluded that tannic acid inhibits succinate oxidation by preventing the operation of the carrier of dicarboxylic acids on the mitochondrial membrane. The reported evidence indicates that tannic acid induces a conformational change reducing the affinity of the carrier for succinate.

THE MECHANISM of penetration of anions across the mitochondrial membrane has recently received particular attention. Studies initiated by Chappell and co-workers[1–3] and developed by other groups[4–7] have led to the conclusion that mitochondria are impermeable even to small anions and that substrate anions can penetrate the mitochondrial membrane by means of specific exchange diffusion carriers.

Extensive research evidence suggests that the oxidation of tricarboxylic acid cycle intermediates is regulated by the rate of their penetration into mitochondria. The rate of penetration is in turn dependent upon the operation of the specific exchange–diffusion carriers located presumably in the inner membrane of mitochondria.

In order to gain more information on the nature and the properties of the exchange–diffusion carriers specific inhibitors have been used.

Drugs employed for this purpose can be divided into two main groups: (a) analogues of the substrate transported (e.g. butylmalonate preventing the penetration of malate[8] or methylsuccinate preventing the penetration of succinate[9]) acting at concentrations similar to those of the substrate transported (having an apparent K_i similar to the apparent K_m); (b) drugs interacting with functional groups of proteins (e.g. mersalyl[10] or *p*-hydroxymercuribenzoate[11] inhibitors of the penetration of inorganic phosphate into mitochondria). Inhibitors belonging to the second group are active at concentrations below those of the substrate transported.

The present paper gives a detailed account of the inhibition by tannic acid,[12] a known inhibitor of anion penetration in red blood cells,[13,14] on succinate and malate transport across the mitochondrial membrane. Part of the results presented in this paper appeared as a preliminary note.[15]

METHODS AND MATERIALS

Rat liver mitochondria were isolated by conventional techniques in a medium of 0·25 M sucrose, 4 mM Tris–HCl. The twice washed mitochondria were suspended in the same medium at a protein concentration of 80–100 mg protein/ml, stored at 0° and used within 3 hr.

Submitochondrial particles were prepared by ultrasonic disruption of rat liver mitochondria according to Kielley and Bronk[15] in a medium containing 10 mM succinate and 30 mM phosphate buffer pH 7. Respiration rates were measured with a Clark oxygen electrode.[16] Mitochondrial swelling was followed by measuring absorbancy changes at 546 nm in an Eppendorf photometer equipped with a recorder. ATPase activity was measured as described previously.[17]

Mitochondrial protein was estimated by the biuret method.[18] Inorganic phosphate was measured by the Fiske and Subbarow method.[19]

Pure tannic acid was obtained by extraction with ethyl acetate from a neutralized solution of commercial tannic acid.[20] The purity was assessed by paper chromatography.[21]

Valinomycin was a gift from Prof. V. V. Zakusov (Moscow), all other reagents were analytical grade.

RESULTS

Effect of tannic acid on mitochondrial respiration. The effect of tannic acid on mitochondrial respiration stimulated either by ADP or by uncoupling agents in the presence of various substrates is shown in Table 1. Forty nmoles/mg protein of tannic acid almost completely prevented the oxidation of succinate both in the presence of DNP and in the presence of Pi acceptor. The same concentration of tannic acid produced only a slight (10–15 per cent) inhibition with NAD-linked substrates like glutamate and was devoid of any effect on the oxidation of ascorbate plus TMPD, indicating

TABLE 1. EFFECT OF TANNIC ACID ON OXYGEN UPTAKE BY RAT LIVER MITOCHONDRIA

Substrate	Tannic acid 40 nmoles/mg protein	Oxygen uptake μgAO/mg protein/min	
		ADP stimulated	DNP stimulated
Succinate (5 mM)	−	110	150
	+	13	13
Glutamate (5 mM)	−	80	87
	+	70	74
Ascorbate (2 mM) + TMPD (0·2 mM)	−	160	—
	+	160	—

Oxygen uptake was measured using the following medium: 100 mM KCl, 20 mM Tris-HCl pH 7·4, 5 mM phosphate and rotenone (2 μg) when succinate was the substrate. ADP was 0·25 mM, DNP was 0·1 mM. Mitochondrial protein was 3–4 mg. Final volume 2 ml. Temperature 30°.

The abbreviations used are: DNP, 2,4-dinitrophenol; TMPD, tetramethylparaphenylenediamine; EGTA, ethylene glycol-bis-(2-aminoethyl)tetracetate.

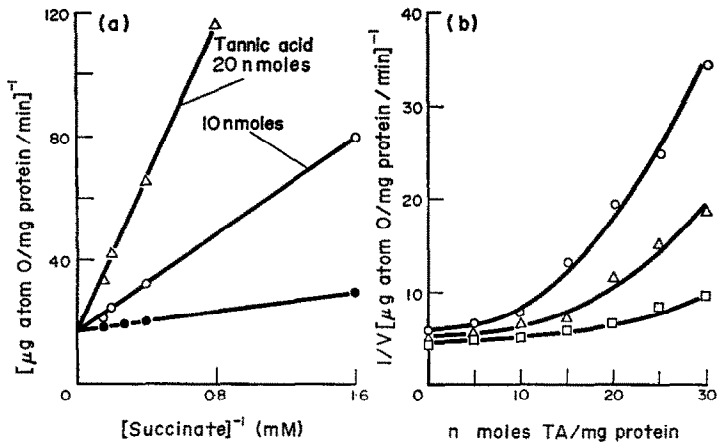

FIG. 1. Lineweaver and Burk plot (a) and Dixon plot (b) of succinate oxidation in the presence of tannic acid. Experimental conditions as described in Table 1. The respiration was stimulated by 0·1 mM DNP. (□) 10 mM succinate, (△) 5 mM succinate; (○) 2·5 mM succinate.

that neither the NADH-cytochrome *c* segment of respiratory chain or cytochrome oxidase are affected by the drug.

In Fig. 1 a Lineweaver and Burk[22] plot is presented showing apparent competition between succinate and tannic acid (Fig. 1a). The respiration is stimulated in this case by DNP but the same result is obtained both in coupled mitochondria or uncoupled by so-called ionophorous antibiotics. However, in agreement with a recent report by Tyler and Newton,[23] by plotting the results according to Dixon ($1/v$ against i) a non-linear relationship is obtained. This behaviour indicates[24] a partially competitive inhibition.

The inhibition of succinate oxidation together with the relative insensitivity of the oxidation of NAD-linked substrates and ascorbate–TMPD could indicate a direct interference of this drug with the operations of the succinate–cytochrome b segment of the respiratory chain. However, tannic acid, at the concentration producing maximum effect on the oxidation of succinate in intact mitochondria, was without effect on the oxidation of succinate in a phosphorylating preparation of sonicated rat liver mitochondria. Only after increasing the concentration of tannic acid above 50 nmoles/mg of protein was succinate oxidation as well as NADH oxidation inhibited in sub-mitochondrial particles.[25] Inhibition of succinate oxidase activity by high concentrations of tannic acid has already been reported.[26]

Effect of tannic acid on swelling of mitochondria in ammonium salts. The inhibition of succinate oxidation in intact mitochondria and the insensitivity of succinate oxidase activity in submitochondrial particles by comparable amounts of tannic acid indicated an interference by this drug with the penetration of succinate across the mitochondrial membrane.

In order to test directly the hypothesis of an inhibition by tannic acid of the transfer of succinate across the mitochondrial membrane, its effect on mitochondrial swelling has been studied.

In agreement with the findings previously reported[12] it has been shown that tannic acid prevented the swelling due to penetration of ammonium succinate and ammonium

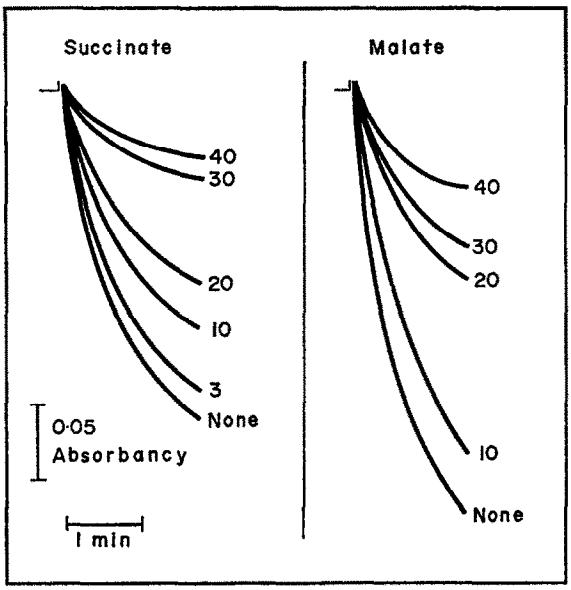

FIG. 2. Effect of tannic acid on mitochondrial swelling in ammonium succinate and in ammonium malate. The swelling was measured as described under Materials and Methods. The incubation medium had the following composition: ammonium succinate (or malate) 100 mM, EGTA 0·1 mM, rotenone (2 μg) and antimycin (2 μg) in a final volume of 3 ml. The swelling was initiated by the addition of 3 mM ammonium phosphate. Mitochondrial protein was 3 mg. Temperature 30°. The numbers on the right indicate nanomoles of tannic acid/mg protein.

malate when initiated by the addition of a small amount of ammonium phosphate (Fig. 2).

Tannic acid did not prevent the penetration of ammonium phosphate in a medium of ammonium chloride; on the contrary it was observed that tannic acid enhanced the swelling due to penetration of phosphate into mitochondria. Inhibition of penetration of succinate and malate was also observed in the presence of the sodium salts of these anions which, as shown by Mitchell and Moyle[27] are able to induce mitochondrial swelling though less extensively than the correspondent ammonium salts.

Concentrations of tannic acid which completely prevents the penetration of succinate and malate are without effect on the swelling induced by other anions like glutamate, acetate or adenosine triphosphate.

Effect of tannic acid on swelling induced by valinomycin. Penetration of anions can be studied also by rendering the mitochondrial membrane highly permeable to cations by means of the so-called ionophorous antibiotics like valinomycin[28] or gramicidin.[29] The swelling due to addition of valinomycin is linked to penetration of K^+ together with an accompanying anion. It has been shown that the anions taken up by mitochondria in this system are either phosphate, acetate or substrate anions[30] like succinate or malate. In order to study solely the specific role of succinate as penetrating anion, valinomycin was added to anaerobic mitochondria (KCN) utilizing ATP as energy source for K^+ accumulation.

As shown in Fig. 3 concentrations of tannic acid in the same range as those active

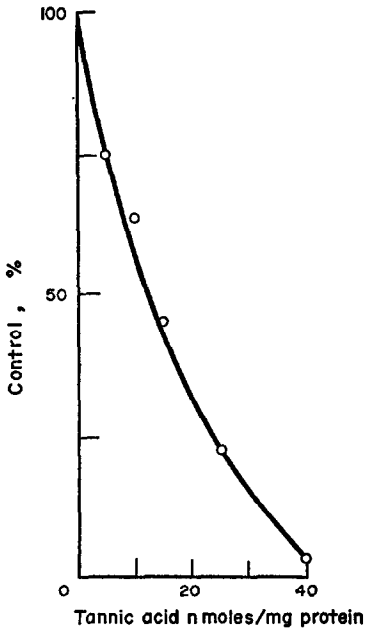

FIG. 3. Effect of tannic acid on mitochondrial swelling induced by valinomycin. The swelling was measured as described under Materials and Methods. The incubation medium had the following composition: 200 mM sucrose, 10 mM Tris pH 7·4, 0·1 mM EGTA, 2 mM KCN, 1 mM ATP, 5 mM succinate (tris–salt) and valinomycin 0·2 μg in a final volume of 3 ml. Mitochondrial protein 3 mg. Temperature 30°. Extent of swelling was measured 2 min. after the addition of valinomycin.

on respiration prevented the accumulation of K^+ accompanied by penetration of succinate induced by valinomycin.

A possible inhibition by tannic acid of ATPase activity had to be excluded in order to make significant the response of the experiment described above. The effect of tannic acid has been therefore tested on ATPase activity induced by DNP or by

TABLE 2. INFLUENCE OF CONCENTRATION OF MITOCHONDRIA ON THE EFFECT OF TANNIC ACID

Mitochondrial protein (mg)	Oxygen uptake (μgAO/min)			
	Control	Tannic acid 0·015 mM	Difference	Inhibition (%)
		Succinate 2 mM		
3·4	300	125	−175	58
5·1	470	285	−185	39
6·8	560	385	−165	30
		Succinate 4 mM		
3·4	370	230	−140	38
5·1	578	445	−130	22
6·8	730	650	−130	16

Oxygen uptake was measured as described in Table 1. Respiration was stimulated by 0·1 mM DNP.

valinomycin. In none of these conditions did tannic acid show significant inhibition of ATP hydrolysis.

Effect of increasing the concentration of mitochondria on the effect of tannic acid. As shown in Table 2 the inhibition of oxygen uptake induced by tannic acid is decreased on increasing the concentration of mitochondrial protein whereas the difference between the respiration in the absence and that in the presence of tannic acid is unchanged. Plotting these results by the procedure of Ackermann and Potter[31] the inhibition by tannic acid appears as irreversible (Fig. 4). However, by comparing the plots of two different concentrations of succinate it can be seen that on increasing the concentration of substrate the affinity between the enzyme and the inhibitor decreases. The slope of the inhibited respiration tends to overlap the control at infinite substrate concentration.

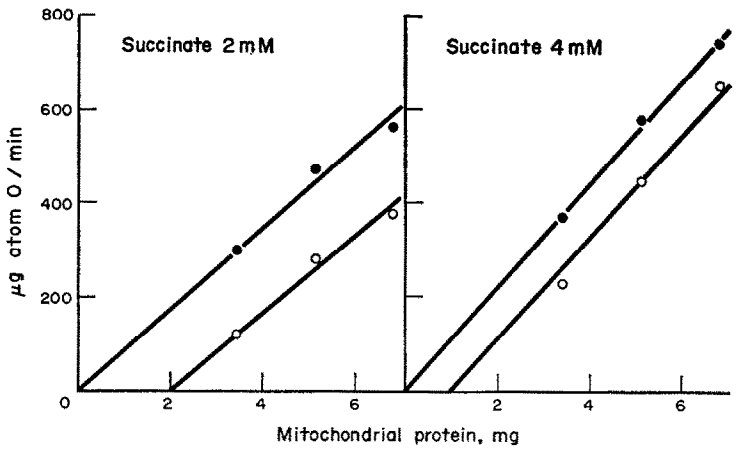

FIG. 4. Ackermann and Potter plot of the data reported in Table 2.

DISCUSSION

The results presented in this paper clearly indicate that succinate oxidation is inhibited by tannic acid only when the integrity of mitochondrial membrane is preserved, whereas no inhibition is present when access to the succinate oxidase system is independent from the penetration across the mitochondrial membrane.

The observation that the entry of both succinate and malate is inhibited by tannic acid indicates an interaction of this drug with the carrier operating the translocation of dicarboxylic acids. Both the nature of the inhibition and the binding of tannic acid to the mitochondrial membrane seem relevant to the mechanism of tannic acid effect on the carrier of succinate and malate. The partial competition between succinate and tannic acid can be explained since the two substances are structurally unrelated. It is therefore unlikely that they both occupy the same binding site as happens with substrate analogues like butylmalonate. On the other hand the results of the experiment with various concentrations of protein plotted according to Ackermann and Potter[31] indicate that tannic acid induces a reduction of the affinity between the carrier and the substrate behaving as "irreversible" inhibitor at fixed substrate concentration.

However, the inhibition is reversed by increasing the concentration of succinate, that is by inducing a modification of the catalytical properties of the carrier. This indicates that substrate and inhibitor are bound at different though not independent sites.[24] The well-known property of tannic acid of interacting with functional groups of proteins, e.g. ϵ-amino groups of lysine,[32,33] supports (on chemical grounds) this possibility.

It should be mentioned that the conformational modification of translocators has been invoked also to explain the inhibition of adenine nucleotide transport in mitochondria by bonkrekic acid,[34] atractyloside[35] and carboxyatractyloside.[36]

The observation that concentrations of tannic acid over 50 nmoles/mg protein inhibits also the oxidation of succinate in submitochondrial particles indicates an interference of this drug with succinate oxidase activity in addition to the inhibition of penetration. Whether these two effects are completely unrelated or the consequence of an interaction between the drug and the same enzymatic site in both preparations is difficult to state at present. The higher concentration of tannic acid required to inhibit succinate oxidase activity as compared to translocation favors the first alternative unless a higher sensitivity of the intact mitochondria than submitochondrial particles toward tannic acid is postulated. This appears to be the case for thenoyltrifluoroacetone[25] a known inhibitor of succinate oxidase activity.[37] If this postulation is proved valid the effect of tannic acid might be interpreted in terms of negative regulation of succinate oxidase activity requiring the structural integrity of the mitochondrial membrane. Regulators of succinate dehydrogenase, such as ATP, acting only in intact mitochondria have been demonstrated by Gutman *et al.*[38]

The effect of tannic acid on oxidative phosphorylation in blowfly mitochondria has been recently investigated by Duncan *et al.*[39] Part, but not all, of their results can be interpreted in terms of inhibition of substrate anion translocation taking into account the different permeability properties of blowfly mitochondria with respect to rat liver.

Inhibition by tannic acid of succinate translocation in rat liver mitochondria has been recently confirmed by Diwan,[40] using [^{14}C]succinate.

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