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THE EFFECT OF TANNIC ACID ON THE PHOSPHORYLATION AND ATPase ACTIVITY OF MITOCHONDRIA FROM BLOWFLY FLIGHT MUSCLE

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Abstract—Low concentrations (below 10^{-6}M) of tannic acid reduced the respiratory control index of blowfly flight muscle mitochondria, apparently reducing the rate of phosphorylation, although ADP:O ratios were normal. At higher concentrations, no stimulation in respiration was found when ADP was added. Respiration uncoupled with DNP was only slightly affected by tannic acid, even at high concentration. However, when DNP is added after tannic acid, its effect is dependent both on the concentration of tannic acid and on the time that elapses before the addition of DNP. Tannic acid also inhibited the mitochondrial ATPase activity, the DNP-stimulated activity being particularly sensitive to low concentrations. Comparisons are made with the action of oligomycin and the results are discussed in relation to the effect of tannic acid on erythrocytes.

TANNIC acid has a variety of effects on mammalian erythrocytes, but probably the most striking is the marked and instantaneous reduction in permeability to anions produced at low concentrations.[1–3] Herz and Kaplan[4, 5] have shown that tannic acid also inhibits irreversibly the acetylcholinesterase located on the outer surface on intact red cells; the activity of this enzyme may therefore be implicated in the control of anion permeability. However, we have also shown (Bowler and Duncan, in preparation) that low concentrations of tannic acid have a dramatic effect on ATPase activity of erythrocyte ghosts. Thus, tannic acid at 5×10^{-5}M down to 5×10^{-7}M activates both the Mg^{2+}-ATPase and the Na^+ -K^+ -Mg^{2+}-ATPase, whereas higher concentrations inhibit ATP hydrolysis. DNP is also able to stimulate the Mg^{2+}-ATPase of erythrocyte ghosts 3-fold,[6] and there is an interesting parallel with the effect of this agent on mitochondria, where it both uncouples oxidative phosphorylation and stimulates ATPase activity. In this paper we report on the effect of tannic acid on oxidative phosphorylation and on the ATPase activity of mitochondria from the flight muscle of the blowfly (*Calliphora erythrocephala*).

EXPERIMENTAL

Materials

Calliphora erythrocephala were reared from stock originally obtained from Pest Infestation Laboratory, Slough.[7] They were maintained throughout at 24° and 50%

Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); BSA, Bovine serum albumin; DNP, 2,4-dinitrophenol; RCI, respiratory control index.

r.h. and the imagoes were given a liver/sugar/water diet. Only 10-day-old males were used.

Preparation of mitochondria

Thirty flies were immobilized by cooling with cardice and the heads and abdomina were rapidly removed. Thoraces were collected on ice and then gently crushed for 2 min by a flat-footed glass rod in a glass mortar containing 2 ml of the isolation medium (0·32 M sucrose, 2% BSA, 10 mM Tris-HCl buffer pH 7·3; see ref. 8). After dilution with 1 ml medium the resulting pulp was forcibly squeezed through boiled muslin and the filtrate was centrifuged at 100 *g* at 0° for 4 min. The pellet was rejected and the supernatant centrifuged at 2200 *g* for 10 min at 0°. The pellet was resuspended in isolation medium lacking BSA and recentrifuged at 2200 *g* (10 min at 0°). The resulting pellet was suspended in 0·75 ml ice-cold 150 mM KCl containing 1 mM EDTA (pH 7·3).

Oxygen electrode

Oxygen consumption was measured in a Clark Oxygen Electrode (Rank Bros., Cambridge) containing 3 ml reaction medium (50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl at pH 7·3, 30 mM phosphate buffer at pH 7·3). The reaction was followed at 24° (acclimatization temperature of flies) and was started by the addition of 50 μl of mitochondrial suspension. Substrate rate oxygen consumption was followed after the addition of 50 μl 2Mα-glycerophosphate. Tannic acid was made up in distilled water, neutralized with NaOH and added in 20 μl aliquots. The oxygen content of the medium and the ADP assay followed the method of Chappell.[9]

ATPase activity

The reaction medium contained 75 mM KCl, 0·5 mM EDTA, 50 mM sucrose, 1·5 mM $MgCl_2$, 2 mM ATP, 50 mM Tris-HCl (pH 7·3). 0·1 mM 2,4 dinitrophenol was included where indicated. Three ml of reaction medium was thermoequilibrated at 24° and ATPase activity was initiated by the addition of 50 μl mitochondrial suspension followed immediately by 0·1 ml of neutralized tannic acid or distilled water. The reaction was stopped after 10 min by the addition of 1 ml ice-cold 12% trichloroacetic acid. The precipitated protein was eliminated by centrifugation at 0° and inorganic phosphate was determined by the method of Fiske and Subbarow.[19]

Protein determination

These were made by the Folin method,[11] using BSA, fraction V, as a standard.

Reagents

Tannic acid was obtained from BDH Chemicals Ltd., Poole, England. (Specification: sulphated ash $< 0{\cdot}2\%$; gums, dextrin, sugar and salts not detected.) All inorganic salts were AnalaR grade and all other reagents were obtained from Sigma Chemical Co., St. Louis. All solutions were made up in glass distilled water. Since tannic acid has a high affinity for clean glass,[2] all glassware was siliconed and all reaction tubes were equilibrated with tannic acid solutions of appropriate concentration before use.

RESULTS

Tannic acid and respiration

The effect of a given concentration of tannic acid was governed by a number of

factors; both the number of micromoles tannic acid per unit weight of mitochondrial protein and the time between the addition of the tannic acid and subsequent reagents produced small differences in the effects observed at any one concentration of tannic acid. However, only small differences in protein concentrations between different mitochondrial preparations were found and only a small variability in the critical concentration of tannic acid was observed.

The rate of oxygen consumption by mitochondria is dependent on the presence of substrates and acceptor molecules. In polarographic experiments these rates are defined as follows: "Substrate rate" is the rate of oxygen consumption in the presence of exogenous substrate alone (see Fig. 1, trace 1). In our experiments the substrate

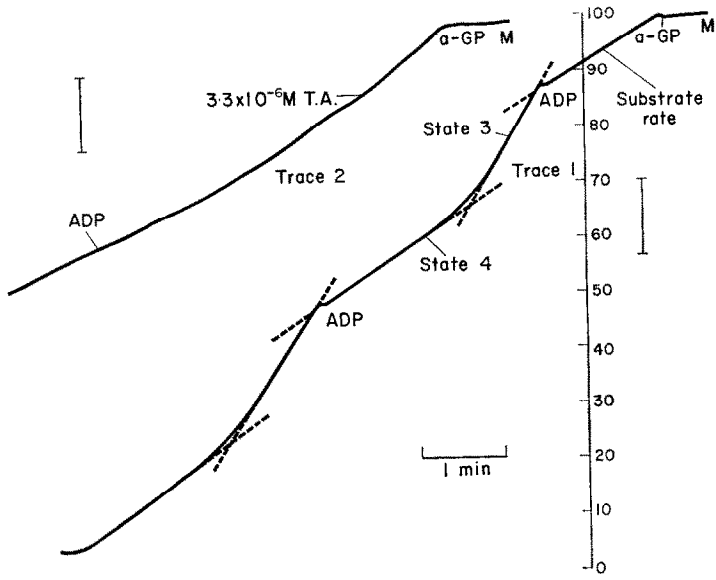

FIG. 1. Polarographic records: sarcosomes from blowfly flight muscle: 3 ml reaction medium: 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl, 30 mM Phosphate buffer, pH 7·3. Temperature = 24°. Additions: M = 50 μl mitochondrial suspension, (0·4–0·5 mg protein); 50 μl of 2 M α-glycerophosphate (α-GP); tannic acid (T.A.) 20 μl to give final concentration of $3{\cdot}3 \times 10^{-6}$M; ADP 10μl, (0·5 μmoles). Vertical lines indicate 0·2 μg atoms oxygen. Trace 1: control. Trace 2: effect of tannic acid.

used was α-glycerophosphate. The substrate rate is stimulated by the addition of ADP, the new rate being termed state 3 respiration. The rate following the expenditure of ADP is termed state 4 respiration. The respiratory control index (RCI) is a sensitive index of mitochondrial integrity and is a measure of the enhancement of respiratory rate that is caused by the addition of the phosphoryl acceptor. Since the additions of tannic acid frequently prevented any stimulation in the respiration rate when ADP was added, RCI has been expressed throughout as $\frac{\text{State 3 respiration}}{\text{Substrate rate}}$ rather than as the more conventional $\frac{\text{State 3 respiration}}{\text{State 4 respiration}}$. State 4 respiration in control experiments

always proved to be very slightly greater than the substrate rate, so that the values given for RCI are marginally increased (see Fig. 1).

Additions of tannic acid at concentrations of 10^{-5}M (69 μmoles tannic acid/g protein) down to $3{\cdot}3 \times 10^{-6}$M produced a gradual decline in the rate of respiration; the rate falls by over 40 per cent over 2 min at the latter concentration (see Fig. 1). Addition of ADP (State 3) now produced no acceleration in oxygen consumption and, at 10^{-5} and $5{\cdot}0 \times 10^{-6}$M tannic acid, was followed by a further small fall in the rate, RCI becoming less than 1.

Concentrations of tannic acid in the range 10^{-6}M (6·9 μmoles tannic acid/g protein) down to 10^{-7}M produced detectable falls in respiration (approximately 20 per cent over 2 min at 10^{-6}M), but subsequent addition of ADP (0·5 μmoles) to the preparation now stimulated oxygen consumption, giving evidence of oxidative phosphorylation. However, RCI values at these concentrations of tannic acid were depressed below control values, falling from about 2·5 to 1·7 at 10^{-6}M tannic acid (Table 1). RCI

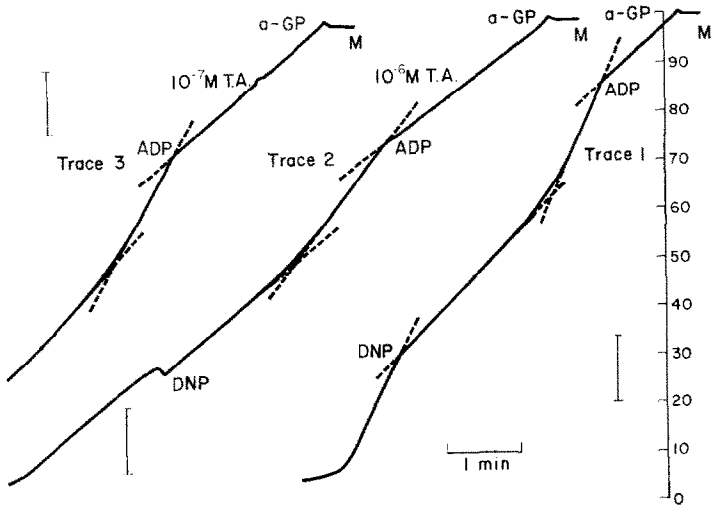

FIG. 2. Polarographic records. Details and abbreviations as Fig. 1. Other additions: 100 μl of 1 mM DNP. Trace 1: control. Trace 2: 10^{-6}M tannic acid. Trace 3: 10^{-7}M tannic acid.

increases as the concentration of tannic acid is reduced. These slower rates of oxidative phosphorylation are shown in Fig. 2.

The efficiency of mitochondria is also measured by the quantity of oxygen consumed for each molecule of ADP phosphorylated to ATP. In this study the ADP:O ratio is determined experimentally as micromoles ADP added per microgram atoms oxygen utilized in phosphorylating the added ADP. The theoretical maximum ADP:O ratio is 2, using α-glycerophosphate as substrate. The obligatory link between substrate oxidation and phosphorylation in coupled mitochondria can be uncoupled with such agents as DNP, whereupon respiratory control is lost, oxygen consumption increases (Fig. 2, trace 1) and phosphorylation ceases.

Although RCI values at 10^{-7} to 10^{-6}M tannic acid are clearly below control values,

ADP:O ratios are little affected (Table 1). The probable effect of tannic acid at these concentrations, therefore, is to reduce the rate of phosphorylation, without impairing the efficiency of the mitochondria. Clearly at concentrations of $3{\cdot}3 \times 10^{-6}$M tannic acid and above (where additions of ADP do not stimulate substrate oxygen consumption), RCI = 1, and ADP:O ratio was not measurable (see Fig. 1, trace 2).

To determine whether the tannic acid was primarily affecting oxidative enzymes, the mitochondria were uncoupled with 0·04 mM DNP. Addition of either $3{\cdot}3 \times 10^{-6}$ or $6{\cdot}7 \times 10^{-6}$M tannic acid now produced only a very small reduction in the accelerated respiration (< 10 per cent). The uncoupling by DNP was therefore essentially

TABLE 1. EFFECT OF TANNIC ACID ON MITOCHONDRIA FROM BLOWFLY FLIGHT MUSCLE

Conc. of tannic acid (M)	*n*	RCI	ADP:O
0	21	2·6 (2·2–3·2)	1·8 (1·5–2·1)
10^{-7}	7	2·5 (2·0–3·0)	1·7 (1·5–2·0)
5×10^{-7}	8	2·1 (1·7–2·6)	1·7 (1·5–1·8)
10^{-6}	10	1·6 (1·2–2·1)	1·7 (1·4–2·1)
$3{\cdot}3 \times 10^{-6}$	6	1	Not measurable

Conditions as described in Experimental. Temperature = 24°. Measurements made from polarographic records. Means of RCI and ADP:O ratios given, together with range in brackets.

irreversible with tannic acid. However, when the DNP was added after the tannic acid, the effect was dependent on the concentration of tannic acid used. No stimulation of oxygen consumption was found after 5×10^{-6}M tannic acid, and after 10^{-5}M tannic acid the rate of respiration continued to fall in the presence of DNP. DNP produced a normal acceleration in respiration (200 per cent) when added after 10^{-7}M tannic acid. However, the effect of DNP following the addition of tannic acid in the intermediate concentration range 5×10^{-7} to $3{\cdot}3 \times 10^{-6}$M was markedly dependent on the time elapsing between the exposure of the mitochondria to the tannic acid and the subsequent addition of DNP. Thus at $3{\cdot}3 \times 10^{-6}$M tannic acid, DNP produces a very small increase in oxygen consumption (115 per cent) when added within 90 sec. At 10^{-6}M tannic acid, however, the acceleration in respiration is almost normal when DNP is added within 60 sec, but the stimulation is reduced to 112 per cent if 200 sec elapse before DNP addition, and no stimulation was found at all if 270 sec are allowed to elapse before addition. Similarly at 5×10^{-7}M tannic acid, the effect of DNP is normal when added within 120 sec of the tannic acid, but if 200 sec elapse stimulation falls to 112 per cent only.

ATPase activity

Another measure of the intactness of mitochondria is the ATPase activity of the preparation;[12] mitochondria in which oxidative phosphorylation is tightly coupled exhibit low activity, whereas ATPase activity increases markedly when the mitochondria are uncoupled.

The ATPase activity of mitochondria in the presence and absence of 0·1 mM DNP

was studied under conditions designed to simulate those of the oxygen electrode and the results are shown in Fig. 3. The DNP factor is the ratio of ATPase activity in mitochondria uncoupled with DNP to the activity of coupled mitochondria. The DNP factor in these experiments was 3·5. In the intact mitochondrion tannic acid at concentrations of 10^{-7} to $3{\cdot}3 \times 10^{-6}$M produced a consistent, small and progressive inactivation of the ATPase (25 per cent at $3{\cdot}3 \times 10^{-6}$M). Between $3{\cdot}3 \times 10^{-6}$ and 10^{-5}M inactivation increased much more rapidly with increase in concentration. The DNP-stimulated ATPase, however, was much more sensitive to tannic acid at the lower concentrations. Inactivation at $3{\cdot}3 \times 10^{-6}$M was 73 per cent and the DNP factor fell to 1·3. Above $3{\cdot}3 \times 10^{-6}$M tannic acid, the inactivation resembled that shown by intact mitochondria.

DISCUSSION

Tannic acid has a number of effects on mammalian erythrocytes. In addition to the familiar reduction in anion permeability it modifies the facilitated entry of glycerol,

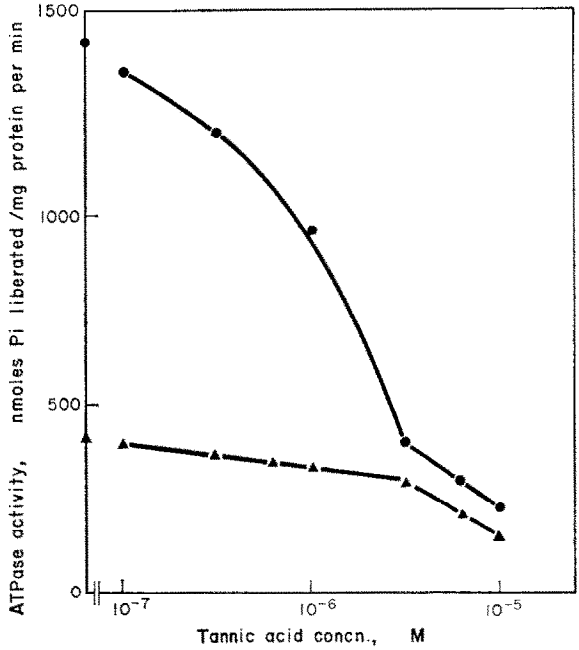

FIG. 3. Effect of tannic acid on ATPase activity of sarcosomes from blowfly flight muscle. Details: see Experimental; —●— DNP-stimulated ATPase; —▲— latent ATPase.

although at higher concentrations,[3,13] and produces changes in osmotic resistance,[2,3,14] and in disc to sphere transformations.[2] At the concentrations used in this study (5×10^{-7} to 10^{-5}M) tannic acid markedly activated the ATPase activity of the erythrocyte ghosts, whereas higher concentrations inhibit (Bowler and Duncan, unpublished). The action of tannic acid on the erythrocyte at low concentrations is believed to be restricted to the surface membrane[2] and does not penetrate the cell,

interiorly-located enzymes being unaffected.[5] Tannic acid readily cross-links and "solidifies" proteins[15] and it seems most likely that this large polyphenolic derivative of glucose becomes bound to the protein layer of mitochondrial membranes.

At low concentrations (below 10^{-6}M) tannic acid reduces the RCI of mitochondria from blowfly flight muscle and apparently slows down the rate of phosphorylation. No phosphorylation could be measured at higher concentrations and an RCI of 1 was obtained. Furthermore, at $6{\cdot}7 \times 10^{-6}$M, substrate rate oxygen consumption is also markedly depressed. However, since tannic acid has little effect when the mitochondria are previously uncoupled with DNP it seems improbable that its major site of inhibitory action is on the oxidative enzymes.

At the low concentrations used in this study, tannic acid clearly inhibits ATPase activity. In intact mitochondria this inactivation is clearly bi-phasic (Fig. 3); above $3{\cdot}3 \times 10^{-6}$M enzyme activity is markedly reduced and at these concentrations no stimulation of oxygen consumption was found when ADP was added. A small but consistent inhibition of ATPase activity was found at concentrations from 10^{-7} to $3{\cdot}3 \times 10^{-6}$M tannic acid, concentrations which also produced a depression of the RCI. The ATPase of the uncoupled mitochondrion was markedly more sensitive to tannic acid over the whole range 10^{-7} to 10^{-5}M than was that of the coupled mitochondrion. Since tannic acid has only a small effect on the oxygen consumption of the uncoupled mitochondria, even at high concentration, this observation further supports the view that the major site of action of tannic acid is not the oxidative enzymes.

The action of tannic acid may be compared with that of oligomycin. This reagent also inhibits coupled respiration but has no effect on uncoupled respiration. It inhibits the DNP-stimulated ATPase and the ATPase activity of mitochondrial fragments.[16] In the housefly (*Musca domestica*) the ability of DNP to abolish oligomycin inhibition was also time-dependent; no stimulation of respiration was observed when DNP was added 14 min after oligomycin.[12] Considerable variation was found between different mitochondrial preparations in the amount of oligomycin required for maximal inhibition of coupled respiration. For example, rat liver mitochondria required 0·2–0·4 μmoles/g protein, but much larger concentrations were necessary for sub-mitochondrial particles from heart, where 1 μmole/g protein stimulated phosphorylation but higher concentrations inhibited.[16] Approximately 2·5 μmoles oligomycin/g protein reduced α-glycerophosphate oxidation by 55 per cent in *Musca* mitochondria and completely inhibited phosphorylation.[12] There is therefore at least a superficial similarity in the effects of tannic acid and oligomycin on mitochondria.

The action of tannic acid could therefore be explained in three ways, each of which is associated with the functional integrity of the mitochondrial membranes:

(1) It modifies the permeability of mitochondria to ions, ionic movements being an obligatory and normal event in oxidative phosphorylation.[17] Changes in ion transport also occur during uncoupling.[18, 19]

(2) It affects the translocation of adenine nucleotides.[20] The conversion of added ADP into extramitochondrial ATP by oxidative phosphorylation in the inner space of the mitochondria requires the transport of ADP and ATP through the mitochondrial membranes. Whereas the outer membrane is readily permeable to nucleotides, passage through the inner membrane requires a specific translocator.[20] This translocation limits the overall reaction of oxidative phosphorylation.[21]

(3) Its major site of action is a direct one on the phosphorylating system.

These three possibilities are not mutually exclusive. There is a considerable body of evidence[18] that the ATPases identified in the mitochondrion are the terminal enzymes of oxidative phosphorylation which, in the intact mitochondrion, synthesise ATP from ADP, either via a phosphorylated intermediate (if it exists), or directly from inorganic phosphate by a mechanism coupled to the utilization of a potential gradient or other energized states.

Tannic acid at low concentrations does not affect the oxidative enzymes but does inhibit coupled respiration. We conclude that it is the phosphorylating system that is the most sensitive. In this respect tannic acid resembles oligomycin and furthermore both agents inhibit mitochondrial ATPase activity. It is probable, therefore, that the effect of tannic acid on the mitochondrion is to modify, either directly or indirectly, its ATPase activity, thereby affecting oxidative phosphorylation.

Note added in proof—Luciani,[22] working with mammalian mitochondria and using higher concentrations of tannic acid, has recently suggested that this agent inhibits the oxidation of succinate by preventing the penetration of this substrate into mitochondria.

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