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TRIPHENYLTETRAZOLIUM AND ITS DERIVATIVES ARE ANISOTROPIC INHIBITORS OF ENERGY TRANSDUCTION IN OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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Triphenyltetrazolium and its derivatives inhibited energy transduction in mitochondria but not in submitochondrial particles, which are inside-out relative to the membranes of mitochondria. Triphenyltetrazolium incorporated into the inside of submitochondrial particles inhibited ATP synthesis in the particles. Triphenyltetrazolium also inhibited the reduction of NAD by succinate coupled with oxidation of succinate by O_2 and hydrolysis of ATP. Energization of mitochondrial inner membranes with succinate and with ATP induced sites on the membranes for triphenyltetrazolium and its derivatives. The maximum amounts of energy-dependent binding sites for triphenyltetrazolium on membranes energized with succinate and ATP, respectively, were 14 and 4 nmol/mg protein. Triphenyltetrazolium also induced H^+ ejection from the energized membranes. The maximum amounts of H^+ ejection from membranes energized with succinate and ATP, respectively, were 4 and 2.4 nmol/mg protein. Triphenyltetrazolium also decreased the membrane potential up to about half the control value and caused shrinkage of mitochondria in an energy-dependent fashion. Comparison of the Hammett's sigma constants of triphenyltetrazolium derivatives with various substituents on the 3-benzene ring showed that lower concentrations of triphenyltetrazolium derivatives with a stronger positive charge were required for inhibition of energy transduction. The present findings show that triphenyltetrazolium and its derivatives act as anisotropic inhibitors of energy transduction by binding to negative charges created on the outer side (C-side) of energized mitochondria, and that the positive charge of these inhibitors is one of important factors for their inhibitory activity. These negative charges may be an essential part of the H^+ pump.

Introduction

Tetrazolium salts, which are readily reduced by various dehydrogenase systems to insoluble highly colored formazans, have been widely used for histochemical localization of enzymes in tissue slices and suspensions [1–13]. The interactions of tetrazolium salts with the respiratory chain in mitochondria have also been studied [5–8,11,12].

It has been shown in this laboratory [14–21] that positively charged anisotropic inhibitors (e.g., ethidium and tetraphenylarsonium) inhibit energy transduction in oxidative phosphorylation in mitochondria by binding to negative charges created on the outer side (C-side) of the membranes, and that these compounds have no inhibitory activity on the inner side (M-side) of the membranes. Recently, we found in experiments on photoaffinity labeling of mitochondria with ethidium monoazide that ethidium specifically

binds to two kinds of new hydrophobic proteins (8 and 13 kDa, named chargerin) of mitochondria energized with succinate [22,23,45].

These findings suggest that the negative charges appearing on the C-side of energized mitochondria are part of an H^+-pumping device [19]. This idea suggests that some amphipathic cations inhibit energy transduction in oxidative phosphorylation by binding to negative charges generated on the C-side. The present paper shows that triphenyltetrazolium and its derivative cations act as anisotropic inhibitors of energy transduction in mitochondria and that the intensity of the charge of these cations is one of important factors in determining their inhibition of energy transduction.

Materials and Methods

Various tetrazolium salts were synthesized by Tokyokasei, Tokyo (Japan). Valinomycin was obtained from Sigma Chemical Co., St. Louis, MO. ^{86}Rb, [^{14}C]sucrose and [^{3}H]H_2O were products of Amersham Japan Co., Tokyo. Tetrazolim salts were used as solutions in water. Other reagents were as described previously [20].

Rat liver mitochondria were isolated by the method of Hogeboom [24], as described by Myers and Slater [25], except that 0.25 M sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and two washings [26]. Sonicated submitochondrial particles were prepared by a modification of the method of Hansen and Smith [27] as described previously [20].

Triphenyltetrazolium was incorporated into submitochondrial particles as follows. Rat liver mitochondria were suspended at a concentration of 40 mg protein per ml in medium (pH 7.4) consisting of 0.25 M sucrose, 10 mM Tris, 1 mM ATP, 1 mM $MgCl_2$ and kept at $-15°C$ overnight. The mitochondrial suspension was thawed just before preparation of the particles. The mitochondria (1 mg protein/ml) were incubated for 5 min at 25°C in medium (pH 7.4) consisting of 150 nmol triphenyltetrazolium per mg protein, 2 mM ATP, 0.4 μg rotenone per mg protein, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 150 mM sucrose and 25 mM Tris. The resulting suspension was rapidly cooled to about 0°C and then centrifuged at $18\,000 \times g$ for 10 min. The precipitate was used as starting material for preparing submitochondrial particles as described before [20], except that the mitochondria were sonicated in the medium used for preparation of submitochondrial particles containing 100 nmol triphenyltetrazolium per mg protein.

For measurement of binding of triphenyltetrazolium to mitochondria, the mitochondria (1 mg protein per ml) were incubated for 5 min with a known concentration of triphenyltetrazolium in the presence of 10 mM succinate (+0.75 μg rotenone) or 2 mM ATP (+0.25 μg antimycin A), 5 mM $MgCl_2$, 2 mM EDTA, 25 mM Tris and 180 mM sucrose at pH 7.4 in a final volume of 1.5 ml at 25°C. The incubation mixture was shaken during the reaction. Then the mixture was rapidly at $8000 \times g$ for 2 min in an Eppendorf, model 3200 microcentrifuge, and the remaining cation was determined spectrophotometrically by measuring the red shift in the absorbance maximum of ethidium on its reaction with tetraphenylboron using a Hitachi, model 556, two-wavelength double-beam spectrophotometer, by a similar method to that for determination of tetraphenylarsonium [16].

The transmembrane potential $\Delta\psi$ was determined by the method of Rottenberg [28] as follows. Two parallel samples, a and b, were prepared, each containing [^{3}H]H_2O (50 μCi/ml). Sample a, for determination of the sucrose-impermeable space (matrix space), containing [^{14}C]sucrose (1.7 μCi/ml) in medium A (0.15 M sucrose, 10 mM Tris, 100 mM NaCl and 5 mM $MgCl_2$ at pH 7.4). Sample b, for determination of $\Delta\psi$, contained $^{86}Rb^+$ (0.13 μCi/ml) in medium A. After adding 0.1 μM valinomycin, the samples were stood for 1 min, and then the reaction was started by adding 10 mM succinate or 2 mM ATP and stopped 3 min later by centrifugation at $12\,000 \times g$ for 3 min in an Eppendorf, model 5412, microcentrifuge. A sample of the supernatant (50 μl) and the pellet were dissolved in 0.5 ml of 14% perchloric acid. The mixtures were stood overnight, and then stirred with a vortex mixer and centrifuged at $12\,000 \times g$ for 3 min in a microcentrifuge. A sample of 0.35 ml of the resultant supernatant was mixed with 5 ml of scintillation liquid and ^{3}H, ^{14}C and ^{86}Rb were counted as described in Ref. 20.

The sucrose space (external water space) was calculated using Eqn. 6 in Ref. 28. The transmembrane potential was calculated from Eqns. 4 and 10 in Ref. 28.

Protein was determined from the contents of cytochromes $a + a_3$ in mitochondria and submitochondrial particles, as described previously [29]. The amount of $^{32}P_i$-labeled substances were determined by the method of Nielsen and Lehninger [30] as modified by Avron [31].

Results and Discussion

Anisotropy of mitochondrial inner membranes with respect to inhibition of energy transduction by triphenyltetrazolium

Tetrazolium salts are known to act as acceptors of electrons from the respiratory chain in mitochondria [1–13]. However, this activity of triphenyltetrazolium is very weak compared with the respiratory activity [6], possibly because the standard redox potential of triphenyltetrazolium is extremely low (E_0' at pH 7.0 = −460 mV [32]. Under the present experimental conditions, no formazan was produced from triphenyltetrazolium (data not shown).

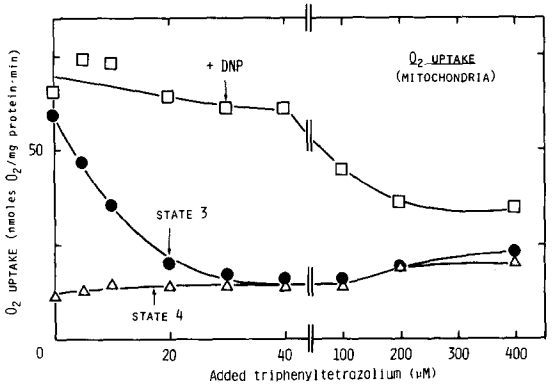


Fig. 1. Effect of triphenyltetrazolium on oxygen uptake by mitochondria. Mitochondria (1 mg protein/ml) were preincubated for 2 min at 25°C in the presence of 10 mM succinate/2 μg rotenone/20 mM potassium phosphate/5 mM $MgCl_2$/2 mM EDTA/15 mM KCl/50 mM sucrose/25 mM Tris and the indicated concentration of triphenyltetrazolium in a final volume of 4.5 ml at pH 7.4 (State 4). Then, ADP (State 3) and 2,4-dinitrophenol (DNP) were added. Oxygen uptake was measured polarographically with a Yellow Springs, model YSI-53, oxygen monitor.

Triphenyltetrazolium at 40 μM inhibited the increased rate of oxygen uptake with a phosphate acceptor (State 3) in mitochondria with succinate as substrate (Fig. 1). With up to 40 μM triphenyltetrazolium, this inhibition was released completely by addition of the uncoupler 2,4-dinitrophenol but with above 100 μM triphenyltetrazolium the inhibition was not completely released by the uncoupler (Fig. 1). Energy-dependent binding of triphenyltetrazolium to mitochondrial membranes energized with succinate occurred even with 350 μM triphenyltetrazolium. Therefore, these results show that triphenyltetrazolium does not inhibit electron transport, but inhibits energy transduction in oxidative phosphorylation. At concentrations of above 200 μM, triphenyltetrazolium slightly stimulated State 4 respiration, as reported previously by Clark and Greenbaum [11].

Fig. 2 shows that 60 μM triphenyltetrazolium completely inhibited ATP synthesis in mitochondria, but that concentrations of up to 300 μM did not inhibit ATP synthesis in submitochondrial particles, which are inside-out relative to the membranes of mitochondria [33–36].

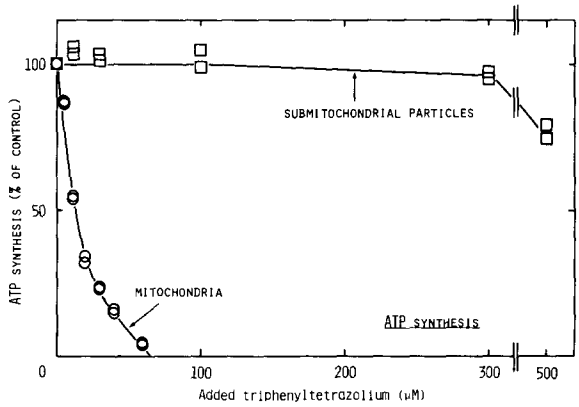


Fig. 2. Effect of triphenyltetrazolium concentration on ATP synthesis in mitochondria and submitochondrial particles. Mitochondria (1 mg protein/ml) or submitochondrial particles (1 mg protein/ml) were preincubated for 2 min at 25°C in the presence of 10 mM succinate/2 μg rotenone/20 mM potassium phosphate (+ about $5 \cdot 10^5$ cpm $^{32}P_i$)/10 mM glucose/0.1 mg hexokinase/5 mM $MgCl_2$/2 mM EDTA/15 mM KCl/50 mM sucrose/25 mM Tris and various amounts of triphenyltetrazolium in a final volume of 1.5 ml at pH 7.4. The reaction was started by adding 0.3 μmol ADP and was stopped 4 min later by adding 0.5 ml of 40% trichloroacetic acid. The ATP/O ratios (esterified phosphate/oxygen uptake) of mitochondria and submitochondrial particles were 1.9 and 0.7, respectively.

Triphenyltetrazolium incorporated into the inside of submitochondrial particles, as described in Materials and Methods, inhibited ATP synthesis to about 40% of that of particles without triphenyltetrazolium (data not shown). The rate of this inhibition was constant over 20 min, when examined like that of other anisotropic inhibitors [14,20]. In this experiment triphenyltetrazolium was not present in the suspending medium and energization of the tetrazolium-containing particles with succinate did not cause release of the cation from the particles into the medium.

Therefore, these results indicate that, like anisotropic inhibitors [14–21], triphenyltetrazolium inhibited energy transduction in oxidative phosphorylation by acting on the outer side (C-side) of mitochondrial inner membranes, and that it had no inhibitory activity on the inner side (M-side) of the membranes.

Triphenyltetrazolium also inhibited the reduction of NAD by succinate coupled with oxidation of succinate by O_2 (Fig. 3) and coupled with hydrolysis of ATP (data not shown), in good accord with results on another anisotropic inhibitor [20].

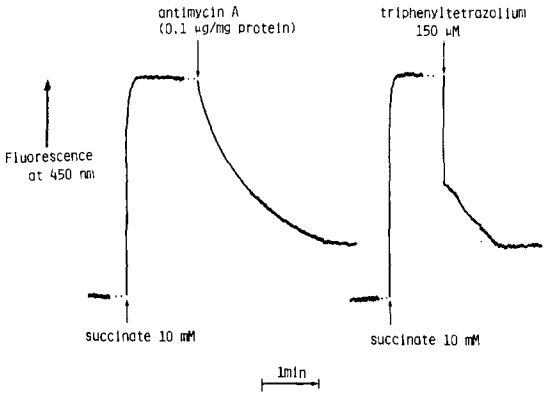


Fig. 3. Inhibition by triphenyltetrazolium of respiration (succinate-O_2)-linked reduction of NAD by succinate in mitochondria. Mitochondria (1 mg protein/ml) were preincubated for 5 min at room temperature in the presence of 3 μg oligomycin/mg protein, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris, in a final volume of 3.0 ml at pH 7.4. Succinate, antimycin A and triphenyltetrazolium were added as indicated. Fluorescence changes of NAD were measured with a Hitachi, model MPF, spectrofluorometer, using the wavelength of 360 nm for excitation and measuring fluorescence at 450 nm. A glass filter was placed in front of the photomultiplier to eliminate the actinic light.

The addition of 150 μM triphenyltetrazolium after addition of antimycin A in Fig. 3 did not cause any change of fluorescence at 450 nm. Triphenyltetrazolium at 100 μM inhibited 83% of the reduction of NAD by succinate coupled with oxidation of succinate by O_2. These inhibitions are clearly different from those of well known inhibitors [43,44], such as oligomycin, dicyclohexylcarbodiimide, and aurovertin. Triphenyltetrazolium at 40 μM also completely inhibited ATP-P_i exchange activity in mitochondria.

Typical plots of the amount of triphenyltetrazolium bound to mitochondria energized with succinate as substrate and nonenergized with antimycin A as functions of the concentration of added triphenyltetrazolium are shown in Fig. 4. The amount of succinate-dependent binding of triphenyltetrazolium (Fig. 4) and ATP-dependent binding of triphenyltetrazolium (Fig. 5) increased to saturation levels at concentrations of about 80 and 60 μM, respectively. The maximum amounts of energy-dependent binding of triphenyltetrazolium to mitochondria energized with succinate and ATP were about 14 and 4 nmol per mg protein, respectively. The difference of bound triphenyltetrazolium in the deenergized states with antimycin A (+succinate) and with oligomycin (+ATP) (Figs. 4 and 5) may be due to partial reduction of triphenyltetrazolium in the presence of succinate. The amount of triphenyltetrazolium

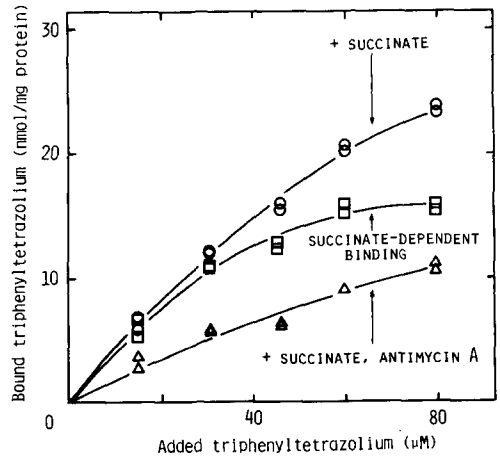


Fig. 4. Binding of triphenyltetrazolium to mitochondria energized with succinate. Conditions were as described in Materials and Methods.

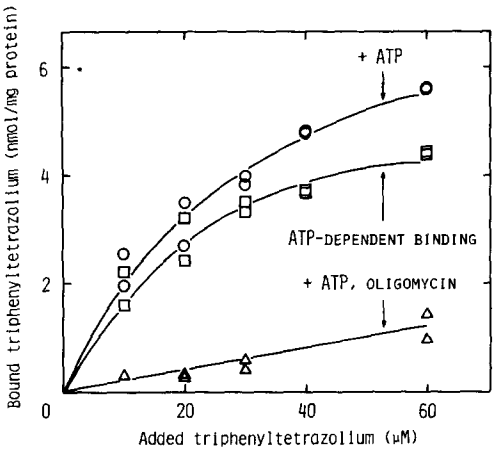


Fig. 5. Binding of triphenyltetrazolium to mitochondria energized with ATP. Conditions were as described in Materials and Methods.

bound to mitochondria energized with both succinate and ATP was the same as that bound to membranes energized with succinate only.

These phenomena cannot be explained by supposing that triphenyltetrazolium is transported electrophoretically down the transmembrane potential, because of the incompatibility of results with the theoretical curve for membrane potential-dependent uptake of the tetrazolium cation based on the membrane-permeant tetrazolium model (Fig. 12 in Ref. 20). However, they can be explained by supposing that triphenyltetrazolium is a membrane-impermeant cation and that succinate generates two kinds of binding sites for the cation on the surface of the C-side of the membranes, whereas ATP generates only one of them [45].

Fig. 6 also shows that, as in the case of the anisotropic inhibitors [18,20], the dose-response curve of the ATP-dependent binding of triphenyltetrazolium to the membranes closely coincides with the dose-response curve for its inhibition of ATP synthesis in the membranes. These results indicate that the energy-dependent binding of triphenyltetrazolium to the membranes causes inhibition of energy transduction in oxidative phosphorylation in good accord with results on other anisotropic inhibitors [20].

The addition of triphenyltetrazolium to mitochondria energized with succinate and ATP also caused H^+ ejection into the medium. Fig. 7 shows that the dose-response curves for triphenyltetrazolium-dependent H^+ ejection from mitochondria energized with succinate and ATP

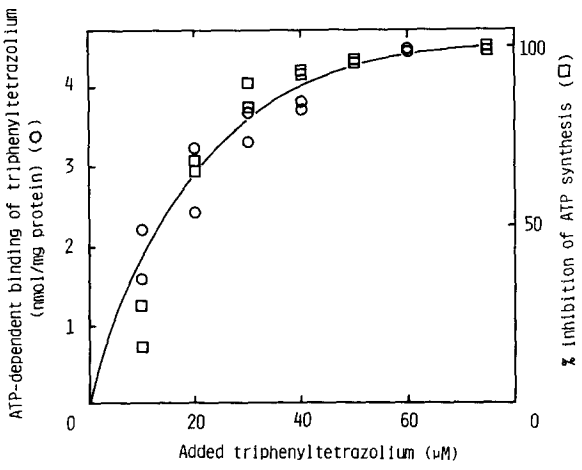


Fig. 6. Dose-response curves of the ATP-dependent binding of triphenyltetrazolium to mitochondria and of inhibition by triphenyltetrazolium of ATP synthesis in the membranes. The amount of ATP-dependent binding of triphenyltetrazolium and the percentage inhibition of ATP synthesis in mitochondria were obtained from Figs. 5 and 2, respectively.

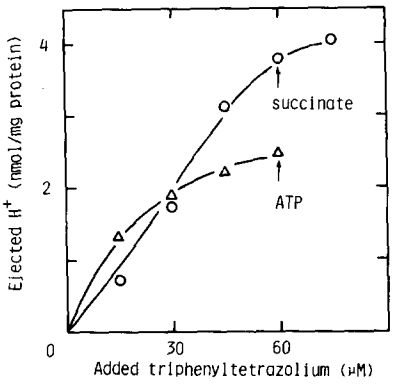


Fig. 7. Effect of triphenyltetrazolium concentration on triphenyltetrazolium-induced H^+ ejection from mitochondria energized with succinate and with ATP. Mitochondria (1 mg protein/ml) were preincubated for 2 min at 25°C in the presence of 2 mM succinate or 2 mM ATP/2 μg rotenone/5 mM $MgCl_2$/2 mM EDTA/20 mM KCl/150 mM sucrose/1 mM Tris in a final volume of 3 ml at pH 7.1. Then, the indicated concentration of triphenyltetrazolium was added. The reaction was followed with a Hitachi-Horiba, Model F-7, expanded scale pH meter equipped with a Horiba pH electrode (6028-10T).

closely coincided with the dose-response curves for energy-dependent binding of triphenyltetrazolium to membranes energized with succinate and ATP, respectively (Figs. 4 and 5). The ratios of the amount of H^+ ejected to the amount of triphenyltetrazolium bound for mitochondria energized with succinate and ATP were 0.3–0.5 and 0.6–0.8, respectively.

The triphenyltetrazolium-induced H^+ ejection could be explained by supposing that triphenyltetrazolium penetrates the mitochondrial inner membranes and increases H^+ ejection due to transition from the transmembrane potential to ΔpH [37]. However, the maximum amounts of the triphenyltetrazolium-induced H^+ ejection from membranes energized with succinate and ATP were only 4 and 2.4 nmol H^+ per mg protein (Fig. 7), respectively. These amounts are much less than the maximum amounts of valinomycin (KCl)-induced H^+ ejection from membranes energized with succinate and ATP (78 and 97 nmol H^+ per mg protein, respectively). If triphenyltetrazolium is transported electrophoretically down the transmembrane potential [37–41], as in the valinomycin-K^+ system, the maximum amount of triphenyltetrazolium-induced H^+ ejection should be similar as that of valinomycin (KCl)-induced H^+ ejection.

Figs. 8 and 9 show that triphenyltetrazolium also decreased the transmembrane potential in mitochondria energized with succinate or ATP up to about half the control value. This phenomenon is typical of anisotropic inhibitors (Higuti, T. and Rottenberg, H., unpublished observation). These figures also show that triphenyltetrazolium caused shrinkage of mitochondria energized with succinate and ATP. This result is consistent with a previous finding [20] that tetraphenylarsonium, which is an anisotropic inhibitor, caused shrinkage of the membranes in an energy-dependent fashion.

If triphenyltetrazolium is transported electrophoretically down the transmembrane potential [37–41], the cation should cause swelling, rather than shrinkage of the mitochondria and also should decrease the transmembrane potential almost completely, as with the valinomycin-K^+ system.

It should be noted that triphenyltetrazolium is a strong base and is freely soluble in water (it was soluble in water at a concentration of 1 M), but the inside of the membranes is composed of hydrophobic components. Therefore, triphenyltetrazolium is a membrane-impermeant cation [45].

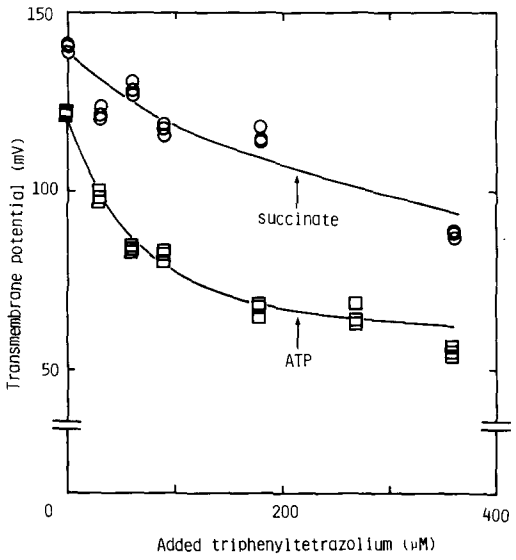


Fig. 8. Triphenyltetrazolium-induced decrease of the transmembrane potential in mitochondria energized with succinate and with ATP. Conditions were as described in Materials and Methods.

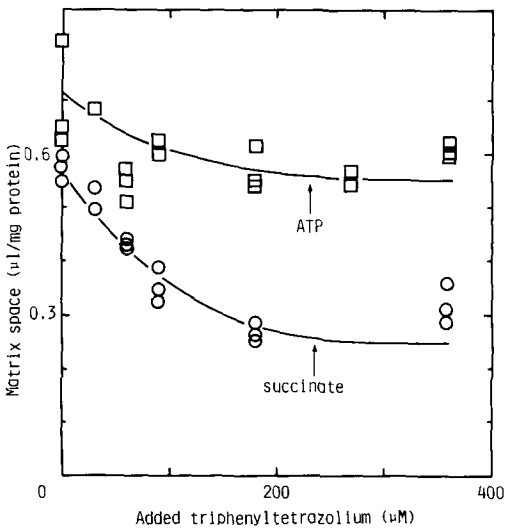


Fig. 9. Triphenyltetrazolium-induced shrinkage of mitochondria energized with succinate and with ATP. Conditions were as described in Materials and Methods.

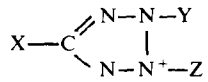
Structure-activity relationships of tetrazolium salts determined from Hammett's sigma constants

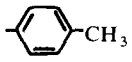
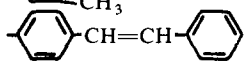
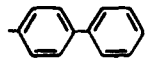
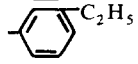
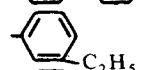
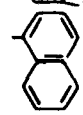
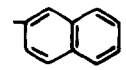
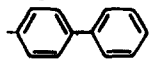
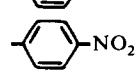
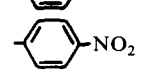
The effects of 23 different tetrazolium salts on oxidative phosphorylation in mitochondria were examined. Ten of these salts inhibited energy transduction in oxidative phosphorylation in mitochondria, like anisotropic inhibitors [14,15,17,20].

TABLE I

INHIBITIONS OF ENERGY TRANSDUCTION BY TETRAZOLIUM SALTS

Bound inhibitor: the amount of ATP-dependent binding of inhibitor to mitochondria. Compound Nos.: 1, 2,3,5-triphenyltetrazolium chloride; 2, *p*-tolyltetrazolium red [2,5-diphenyl-3-(*p*-tolyl)tetrazolium chloride]; 3, *m*-tolyltetrazolium red [2,5-diphenyl-3-(*m*-tolyl)tetrazolium chloride]; 4, 2,5-diphenyl-3-(4-strylphenyl)tetrazolium chloride; 5, 2,5-diphenyl-3-(*p*-diphenyl)tetrazolium chloride; 6, 2,3-bis-(3-ethylphenyl)-5-phenyltetrazolium chloride; 7, tetrazolium violet [2,5-diphenyl-3-(α-naphthyl)tetrazolium chloride]; 8, 2,5-diphenyl-3-(β-naphthyl)tetrazolium chloride; 9, 2,3-diphenyl-5-(*p*-diphenyl)tetrazolium chloride; 10, 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride; 11, 2,3-diphenyl-5-aminotetrazolium chloride; 12,2,3-bis(*p*-nitrophenyl)-5-phenyltetrazolium chloride. –, not determined.



No.	X	Y	Z	Concentration (μM) for inhibition of State 3		Bound inhibitor (nmol/mg protein)	Hammett's σ constant
				50% inhibition	100% inhibition		
1				8.0	40	4.2	0
2				7.0	30	2.5	−0.17
3				3.4	10	1.8	−0.07
4				4.2	12	–	−0.07
5				1.6	8.0	1.5	−0.02
6				2.8	10	–	−0.07
7				6.5	20	–	
8				4.1	12	–	
9				14	45	–	
10				10	50	–	
11	$-NH_2$			no inhibition		–	
12				uncoupling activity		–	
				19	60		+0.78

Hammett's sigma constant (σ) for substituents was applied to triphenyltetrazolium derivatives with various substituents on the 3-benzene ring. Hammett's sigma constants were obtained from the literature [42]. The concentrations of various substituted triphenyltetrazolium compounds required for 50% inhibition of State 3 respiration decreased with increase in the Hammett's sigma constant, except in the case of triphenyltetrazolium, as shown in Table I. These results suggest that lower concentrations of triphenyltetrazolium derivatives with a high electron-withdrawing capacity in their 3-benzene ring were required to inhibit energy transduction in oxidative phosphorylation in mitochondria.

Table I also shows that 2,5-diphenyl-3-(*m*-tolyl)tetrazolium chloride (compound No. 3 in Table I) inhibited energy transduction at lower concentration than 2,5-diphenyl-3-(*p*-tolyl)tetrazolium chloride (compound No. 2 in Table I). The values of *p*- and *m*-substituents are -0.17 and -0.07, respectively [42]. In general in aromatic compounds the hydrophobicities of *p*- and *m*-substituents on the benzene ring are the same. Thus, the difference in the inhibitory activities of the two compounds could be due to a difference in their positive charges. A similar explanation could apply to the difference between the α- and β-naphthyl derivatives (compound Nos. 7 and 8 in Table I), since an α-naphthyl group is more electronegative than a β-naphthyl group. The same concentration of formazan of triphenyltetrazolium, which has no positive charge, did not inhibit oxidative phosphorylation. Therefore, the intensity of the positive charge of triphenyltetrazolium and its derivatives is one of important factors in determining their inhibition of energy transduction.

We conclude from this work that triphenyltetrazolium and its derivatives act as anisotropic inhibitors of energy transduction by binding to negative charges created on the outer side (C-side) of energized mitochondria, and that the positive charge of these inhibitors is one of important factors for their inhibitory activity. These negative charges may be an essential part of the H^+ pump, as described in detail in Ref. 45.

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