THE EFFECT OF TRIETHYLTIN ON MITOCHONDRIAL ION ACCUMULATION

J.R.MANGER
Biochemistry Department, University College London, Gower Street, London W.C.1. England

Received 13 October 1969

1. Introduction

Aldridge and Rose [1] have recently summarised the inhibition of the processes of oxidative phosphorylation and the connected activities of the P_i-ATP exchange and the ATPase reactions by triethyltin (TET) and its analogues. They draw conclusions about the protein structure involved in the phosphorylation reaction from their observation that when the above reactions are 100% inhibited only 15% of the TET binding sites are occupied. Further measurements presented here describe the effect of TET on respiring mitochondria and their respiratory control. Different substrates show different sensitivities to the inhibition by TET when being oxidised in the presence of P_i and ADP. Measurements of intramitochondrial substrate accumulation show that TET is potent agent for the discharging of anion gradients across the mitochondrial membrane.

2. Materials and methods

Mitochondria were isolated from rats' livers by initial homogenization in 0.25 M sucrose, 0.5 mM EGTA, 0.05% BSA. The three subsequent washes and resuspensions were in 0.2 M sucrose, 35 mM KCl, 0.5 mM EGTA, 0.05% BSA. Mitochondrial protein in the final suspension, which was determined by the biuret method, was about 50 mg/ml. The triethyltin was the gift of Dr. J.E.Cremer; non-labelled substrates were obtained from the Sigma Chemical Co., London and the labelled substrates from the Radiochemical Centre, Amersham, Bucks., England. Experiments on substrate accumulation were made by the centrifugation through silicone technique [2] and pyruvate and malate were assayed enzymatically as described in Bergmeyer [3]. Succinate accumulation was measured using ^{14}C-labelled succinate. A Packard Tri-Carb scintillation counter was used for radioactive experiments. Mitochondrial respiration was measured using a Clarke-type oxygen electrode modified to accomodate a light-scattering detector. Mitochondrial cation contents, e.g. K, Ca and Mg were measured using an atomic absorption spectrophotometer (Model AA--100, Techtron Pty., Melbourne, Australia).

3. Results

3.1. *Effect of TET on mitochondrial substrate accumulation*

The accumulation by mitochondria of pyruvate, malate and succinate was tested in the presence of TET and TET plus ATP. The experiments were performed by either adding TET at 1 μM to mitochondria in the presence of substrate and measuring the decrease in the quantity accumulated or by titrating the substrate into the mitochondrial suspension containing TET. The results of a series of experiments of the first type are seen in table 1. At an applied concentration of 0.55 mM malate the intramitochondrial malate in the presence of rotenone was 12.35 μmole/g of protein. Addition of TET at 1 μM caused a decrease in the internal quantity which could be restored to the value obtained with 0.55 mM by a further addition to about 4 mM malate. The results of a similar series of experiments on accumulation on pyruvate (+ 0.5 mM arsenite) and succinate (+ thenoyltrifluoroacetone to inhibit succinate oxidation, see [8]) can also be seen in table 1.

Table 1

Addition	Internal anion (μmole/g protein)	Q_i:C_e
a) 0.55 mM Malate + rotenone (1.6 μg/g protein)	12.35	22.4
0.5 μM TET	1.6	2.5
2 mM Malate	8.9	3.3
4 mM Malate	20.0	5.7
b) 0.57 mM Pyruvate + arsenite (0.5 mM)	6.5	11.2
0.5 μM TET	0.8	0.8
2 mM Pyruvate	7.3	3.2
4 mM Pyruvate	16.1	4.7
c) 0.50 mM Succinate + TTB*	10.6	21.0
0.5 μM TET	4.0	7.8
2 mM Succinate	18.6	9.1
4 mM Succinate	39.6	10.4

The effect of TET on accumulated substrates in mitochondria. The measured internal anion was malate in a), pyruvate in b) and succinate in c). Q_i = [internal anion] expressed as μmole/g mitochondrial protein, C_e = the [external anion] expressed as mM. The medium used 0.25 M sucrose, 5 mM KCl, 20 mM tris chloride, pH 7.4. The temperature was 20°C. The additions shown in the order in which they occurred during the experiment. In tables 1 and 2, the internal anion is corrected for anion carried in the sucrose-accessible space.

* Thenoyltrifluoroacetone. (8)

The smaller effect of TET on succinate accumulation may be due in part to dual compartmentation of succinate described by Harris and Manger [4].

Fig. 1 illustrates the results of a series of experiments in which the substrate anion was titrated into the mitochondrial suspension and the internal and external concentrations measured. The results are shown as double reciprocal plots for accumulation as described by Harris and Manger [5]. In each case, curve (a) represents the titration of the anion into untreated mitochondria, curve (b) titration in the presence of TET and curve (c) titration in the presence of TET plus ATP. It is evident from these results that ATP addition gives only a 10–20% increase in the concentration ratio of the anion across the mitochondrial membrane observed in the presence of TET.

3.2. *The effect of TET on respiration and phosphorylation*

Each of the anions tested with mitochondria in an oxygen electrode, with P_i at 2 mM and ADP (added as 70 μM aliquots), showed a different response to TET at 0.5 μM. 2 mM glutamate-malate was the least affected by TET phosphorylation cycles were obtained with up to 5 μM TET but with a reduced respiratory control ratio due to an increased state 4 rate of respiration. With β-hydroxybutyrate as substrate at 2 mM, the mitochondria were almost completely without respiratory control at low levels of TET (0.5 μM), but

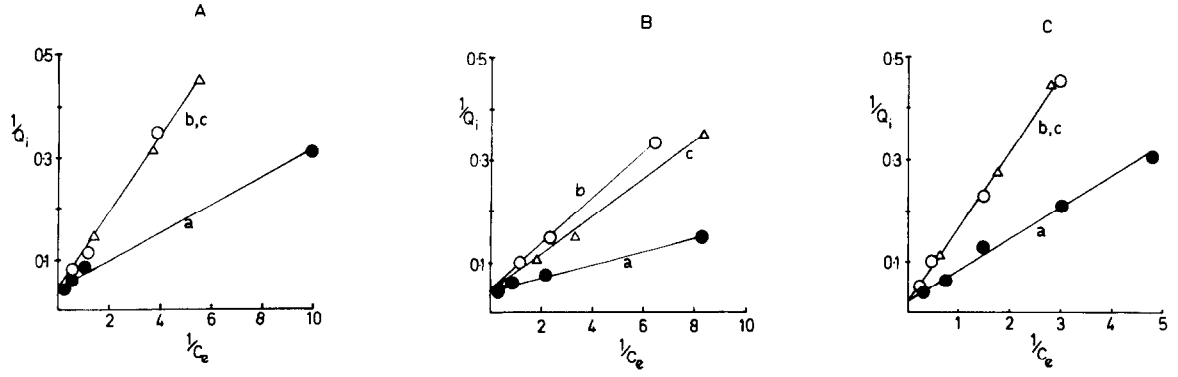

Fig. 1. Double reciprocal plots relating external anion concentration (C_e) to anion accumulated in the sucrose-impermeable space (Q_i). a) ● = titration of anion into untreated mitochondria; b) ○ = titration of anion into mitochondria in the presence of 1.0 μM TET; c) △ = the same as b) but with ATP at 1.3 mM. In fig. 1b, pyruvate was the anion tested, succinate in fig. 1B and malate in fig. 1C. The medium used was 0.25 M sucrose, 20 mM tris chloride, 5 mM KCl, pH 7.4. The temperature was 20°C.

did not exhibit the high oxygen uptake rates associated with an uncoupled respiratory assembly. The sensitivity of succinate oxidation to TET fell between that of malate and β-hydroxybutyrate. At 0.5 μM TET, a slight degree of uncoupling was observed, in common with β-hydroxybutyrate, but phosphorylation cycles with ADP were still obtained. Increasing the succinate concentratior partially restored the respiratory control ratio, but the state 4 rate remained high. TET at 1.5 μM reduced the respiratory control ratio and the mitochondria lost their response to ADP.

Pyruvate oxidation was blocked by 0.5 μM TET, the inhibition being relieved by a further addition of malate to 1 mM; unlike malate, with succinate and β-hydroxubutyrate as substrate, the effect of TET could not be reduced by increasing the external pyruvate concentration. No uncoupling of pyruvate oxidation occurred and in this respect also pyruvate differed from the other substrates tested. These results are summaristed in table 2.

Table 2

Addition	Respiratory control ratio
a) 2 mM Glutamate and malate	5.0
1.3 μM TET	2.8
2.6 μM TET	2.0
4.0 μM TET	1.6
b) 1 mM Succinate	4.7
0.5 μM TET	2.0
2 mM Succinate	3.0
4 mM Succinate	3.6
c) 2 mM β-Hydroxybutyrate	3.8
0.5 μM TET	1.0
2 mM β-Hydroxybutyrate	2.0
3 mM β-Hydroxybutyrate	2.7
d) 1.5 mM Pyruvate	3.7
0.5 μM TET	1.0
2 mM Pyruvate	1.0
1 mM Malate	2.7
1.3 μM TET	1.6

The effect of TET on mitochondrial respiratory control with different substrates. A value of 1.0 in the right-hand column indicates no response to added ADP. The medium used was 120 mM KCl, 20 mM tris chloride, pH 7.4. The temperature was 20°C. The additions are shown in the order in which they occurred during the experiment.

3.3. *Effect of TET on mitochondrial cation accumulation*

3.3.1. Potassium

Although it gives rise to a considerable swelling, TET showed no effect on the intamitochondrial K. However, with malate as substrate, the net K which had been gained after addition of valinomycin was discharged by TET (see table 3). In this and several other respects, TET resembles nigericin or excess uncoupler in mode of action, e.g. the discharge of intamitochondrial anion as well as K after valinomycin treatment, [6] and restoration of respiration by adding excess substrate [7].

3.3.2. Calcium

The internal level of Ca in mitochondria was unaffected by TET under any of the conditions used.

3.3.3. Magnesium

The mean values from several experiments indicate that a portion of the internal Mg is discharged by 0.5 μM TET; typical values for the internal Mg content of fresh mitochondria were 15–20 μmole/g, addition of TET reduced this value to 10 μmole/g. The addition of up to 4 mM to a mitochondrial suspension gave no protection against inhibition of respiration by TET.

4. Discussion

The observations presented here, which show that with TET at concentrations which inhibit oxidative phosphorylation [1] intramitochondrial anion accumulation is lessened or stopped, imply that the observed inhibition of ADP-stimulated respiration will partly be due to less substrate reaching its dehydrogenase. In conformity with this reasoning a partial restoration is observed if excess substrate is added. The unequal sensitivities of different NAD-linked substrates (see also [9]) to TET inhibition may reflect the accessibility to TET of the individual substrate compartments [4]. The fact that TET does not directly inhibit the isolated mitochondrial dehydrogenases [10,11 and J.Wimhurst, unpublished observations] suggests that each of the substrate compartments may have its own discreet respiratory assembly.

The tendency of oxidative phosphorylation to be stopped before saturation of the binding sites may reflect a cumulative effect initiated by TET, for

Table 3

Addition	$[Malate]_i$ (μmoles/g protein)	$[K]_i$ (μmoles/g protein)	O_2 rate (μatom O/min/g protein)
a) 1.5 mM Glutamate and Malate	10.7	110	19
Valinomycin	15.7	160	70
1.5 μM TET	10.1	100	40
b) 1.5 mM Glutamate and Malate	9.3	105	15
1.5 μM TET	3.6	100	25
Valinomycin	7.0	120	55

The effect to TET on intramitochondrial K. The medium used was 0.25 M sucrose, 5 mM KCl, 20 mM tris chloride, pH 7.4. The te nperature was 20°C. Valinomycin was added at a final concentration of 40 μg/g of mitochondrial protein. The additions are shown in the order in which they occurred during the experiment.

example, de-energisation of the mitochondria will result in less substrate being accumulated which in turn will lead to a more extensive effect on respiration and phosphorylation.

A conclusion could be that TET is a respiratory enzyme poison exhibiting a broad spectrum of observed effects, imitating to some extent the inhibitory effects on mitochondria of oligomycin, nigericin and excess uncoupler It may indeed be bound to histidine groups, precisely as suggested by Aldridge and Rose [1], but it seems premature at this stage to draw conclusions from their data concerning the structure of a respiratory assembly which must of necessity involve a coupling to cation and anion transport. If a OH^-/anion exchange reaction were to be incorporated into section E of the Aldrdge and Rose scheme [1], this could in part explain the findings presented here. Although this does not answer the problem of a minimal discharge of K and Ca with TET, which does not appear to be consistent with the mechanism as proposed, it could make way for an energy-linked ion transporting complex to be involved in a more functionally complete mechanism.

Acknowledgements

The author expresses thanks to the Science Research Council for the award of a studentship. Thanks also are due to Dr. E.J.Harris, Dr. E.M.Chance and Dr. J.E.Cremer for valuable discussion, and the Muscular Dystrophy Association of America Inc. and the Wellcome Trust for grants and assistance for apparatus.

References

[1] W.N.Aldridge and M.S.Rose, FEBS Letters 4 (1969) 61.
[2] E.J.Harris and K.van Dam, Biochem. J. 106 (1968) 759.
[3] H.U.Bergmeyer, Methods of Enzymic Analysis (London Academic Press, 1963).
[4] E.J.Harris and J.R.Manger, Biochem. J. 109 (1968) 239.
[5] E.J.Harris and J.R.Manger, Biochem. J. 113 (1969) 617.
[6] B.C.Pressman, E.J.Harris, W.S.Jagger and J.H.Johnson, Proc. Natl. Acad. Sci. 58 (1967) 1949.
[7] E.J.Harris, K.van Dam and B.C.Pressman, Nature 213 (1967) 1126.
[8] D.D.Tyler, Biochem. J. 106 (1968) 121.
[9] N.Sone and B.Hagihara, J. Biochem. (Japan) 56 (1964) 151.
[10] W.N.Aldridge and J.E.Cremer, Biochem. J. 61 (1955) 406.
[11] W.N.Aldridge and B.W.Street, Biochem. J. 91 (1964) 287.