Effects of Tetrazolium Salts on Oxidative Phosphorylation in Rat-Liver Mitochondria

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1. The effects of five different tetrazolium salts on oxidative phosphorylation in rat-liver mitochondria have been investigated. 2. In all cases the mitochondria were uncoupled by very low concentrations of the tetrazolium salts. Further, the transition from a system just exhibiting respiratory control to one in which the mitochondria were totally uncoupled has been shown to occur over very small concentration ranges of the tetrazolium salts. 3. The effectiveness of the five tetrazolium salts as uncoupling agents is discussed in the light of their standard electrode potentials and effectiveness as electron acceptors in dehydrogenase-linked reactions.

The reduction of tetrazolium salts yields intensely coloured, and generally water-insoluble, formazans (see Nineham, 1955). Such reductions occur readily in tissue slices and suspensions and with a wide range of substrates. This property has been extensively used for histochemical and biochemical studies on dehydrogenase reactions (see Pearse, 1960; Cheronis & Stein, 1956).

The interactions of tetrazolium salts with respiratory-chain preparations obtained from mammalian liver have also been extensively studied in recent years (Nachlas, Margulies & Seligman, 1960; Lester & Smith, 1961; Slater, Sawyer & Sträuli, 1963), particularly with succinate as substrate. It is now realized that such interactions are complex and vary with the tetrazolium salt being studied (Slater *et al.* 1963). None of the tetrazolium salts so far tested react with soluble succinate dehydrogenase (EC 1.3.99.1) (Nachlas *et al.* 1960, Lester & Smith, 1961), but instead couple at several sites between the dehydrogenases and the terminal oxidase (EC 1.9.3.1) of the respiratory chain. For example, with succinate as substrate, NT* couples both in the region of ubiquinone and between cytochrome *c* and the cyanide-sensitive region of cytochrome oxidase (Slater *et al.* 1963). With NADH-linked substrates, on the other hand, NT couples in the neighbourhood of the NADH-

flavoprotein (Lester & Smith, 1961; Slater, 1959). Thus NT reacts with rat-liver respiratory-chain preparations at three specific sites that are suggestively similar in sequential position to the three proposed sites of oxidative phosphorylation (see Slater, 1958; Lehninger & Wadkins, 1962). Another tetrazolium salt, NBT, however, reacts appreciably with the respiratory chain, with succinate as substrate, at only one of these sites, namely the one near ubiquinone (Slater *et al.* 1963), whereas TTC reacts (as far as can be detected by colorimetric procedures) exclusively with the terminal region of the chain (Slater *et al.* 1963).

Two important possibilities emerge from these findings and form the basis of the present investigation. First, do tetrazolium salts uncouple oxidative phosphorylation directly and at low concentration? Secondly, if they do uncouple phosphorylation from electron transport, is there any preferential action on any of the sites of oxidative phosphorylation parallel to the preferential sites of coupling described previously for the colorimetric procedures?

METHODS

Animals. Female albino rats, weighing approx. 150 g. and fed *ad lib.* on stock diet, were used.

Chemicals. All reagents were of standard AnalaR grade except where mentioned below. Tris (L. Light and Co. Ltd., Colnbrook, Bucks.) was recrystallized three times from 15% (v/v) methanol. Succinate (disodium salt) was twice recrystallized from aqueous ethanol.

The tetrazolium salt MTT was kindly given by Dr T. Pyl.

* Abbreviations: NT, neotetrazolium chloride; NBT, nitro blue tetrazolium chloride; TTC, triphenyltetrazolium chloride; INT, 3-*p*-iodophenyl-2-*p*-nitrophenyl-5-phenyl-tetrazolium chloride; MTT, *N*'-4,5-dimethylthiazol-2-yl-CN-diphenyltetrazolium bromide.

Other tetrazolium salts were obtained commercially and were recrystallized from water before use.

ADP was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of mitochondria. Rats were killed by cervical dislocation and the livers rapidly removed. Each liver was placed in 0.25 M-sucrose-1 mM-EDTA and washed free of blood. After being chopped finely it was re-washed and homogenized in a Potter-Elvehjem homogenizer (Teflon pestle, clearance 0.015 in.) to give a 1:10 (w/v) suspension.

After removal of the nuclei and cell debris the mitochondria were sedimented at 80000 g-min. The mitochondrial pellet was freed of the fluffy layer and resuspended in the sucrose-EDTA medium. After centrifuging at 1500 g for 10 min. to remove any traces of cell debris, the mitochondria were resedimented as described above. The mitochondria were washed twice more and the pellet from the final sedimentation was suspended in about 1.5 ml. of 0.25 M-sucrose-1 mM-EDTA to constitute the mitochondrial fraction; 1 ml. of this fraction was equivalent to approx. 5 g. of liver.

Estimation of the P/O ratio. Oxidative phosphorylation was studied by using a Clark-type oxygen electrode (Yellow Springs Instrument Co.) coupled to a recorder (Kent Multilec, Mark 3; 0-2 mv range). The electrode cell was maintained at 27° and the solution it contained was stirred throughout the experiment by a magnetic stirrer. Air was excluded by a tightly fitting seal. The phosphorylating medium used had the following final composition: sodium phosphate (pH 7.4), 5 mM; tris buffer (pH 7.4), 10 mM; KCl, 10 mM; MgSO₄, 5 mM; EDTA (tetrasodium salt), 0.5 mM; sucrose, 200 mM; substrate (disodium succinate or sodium β -hydroxybutyrate, both pH 7.4), 4 mM; tetrazolium salt in aqueous solution (for concentrations see the Tables) added in a volume of 0.02-0.10 ml.; water to make up a final volume of 2 ml. A 0.05 ml. sample of the mitochondrial suspension was added by syringe and, after following the endogenous respiration for about 2 min., 0.4 μ mole of ADP (in 0.01 ml.) was added with an Agla micro-syringe. The resultant stimulation of the respiration was recorded from which the P/2e (P/O ratio) was subsequently determined (Chance & Williams, 1955). This procedure was repeated twice more in the ensuing 5-6 min., so that the variation in the P/O ratio with time after adding the tetrazolium salt to the mitochondria could be followed.

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained when the mitochondria respired in the presence of 4 μ M-NT with β -hydroxybutyrate as substrate. The P/O ratio falls gradually as the time-interval between mixing the mitochondria and NT increases. In other experiments mitochondria were preincubated with 1 μ M-NT at 0° for 10 min. before their addition to the reaction mixture. The P/O ratio found subsequently in the presence of 10-20 μ M-NT was decreased by 4-24%. A further aspect of the effects of preincubation is shown in Fig. 2. In this case mitochondria were incubated with NT (final concn. 160 μ M) at 21° in unshaken tubes for various times: (a) 0 min.; (b) 5 min.; (c) 10 min. Thereafter the mitochondria were added to 2 ml. of medium in the

Fig. 1. Effect of NT on the P/O ratio obtained with β -hydroxybutyrate as substrate. NT (final concn. 4 μ M) and all other assay components except mitochondria were placed in the electrode compartment and the reaction was started by the addition of mitochondria at zero time. P/O ratios were determined at successive short intervals by additions of ADP. The results shown are compounded from two separate experiments (○ and ●).

electrode cell (final concn. of NT 4 μ M) and the P/O ratios measured over a further time-interval of 5 min. (see Table 2 for details). The slopes (α , 0.66; β , 0.93; γ , 1.46) show that the rate at which the P/O ratio falls off with time increases appreciably with the length of the preincubation time. However, the time of preincubation did not affect the initial P/O ratio determined after adding the mitochondria to the reaction mixture. Since the initial P/O value was unaffected by the length of the preincubation period it seems that NT can only exert its uncoupling action during periods of active respiration. This indicates that NT interacts only with reduced carrier.

The increased rate of uncoupling resulting from longer preincubation times at room temperature may be due either to the liberation and build-up of an endogenous uncoupling factor during the preincubation period or to an increased concentration of NT at an active site involved in oxidative phosphorylation.

Table 1 shows the effects of five different tetrazolium salts on oxidative phosphorylation with either succinate or β -hydroxybutyrate as substrate. No preincubations were involved in these experiments. All the tetrazolium salts uncoupled at very low concentration with both substrates. Table 1 also includes the concentrations of tetrazolium salts required to produce the state when respiratory control after the stimulation of respiration by ADP was just detectable; for NT the concentration required to produce this latter state was 9 μ M. The decreasing order of effectiveness of the tetrazolium salts as uncoupling agents was: NT; INT; NBT; TTC; MTT. Even MTT, the least efficient of the tetrazolium salts studied, uncoupled the system completely at a final concentration of 130 μ M.

Fig. 2. Effect of preincubation of mitochondria with NT on P/O ratios with β -hydroxybutyrate as substrate. (a) NT (final concn. $4\mu\text{M}$) was added to the electrode compartment and the P/O ratio measured at successive time-intervals. The slope (fall in P/O/min.) was 0.12. (b) Mitochondria were preincubated for 5 min. at 21° with NT (final concn. $160\mu\text{M}$); after this the mixture was transferred to the electrode compartment and P/O ratios were determined over the next 5 min. (final NT concn. $4.0\mu\text{M}$). The slope (fall in P/O/min.) was 0.18. (c) Mitochondria and NT ($160\mu\text{M}$) were preincubated for 10 min. at 21° ; other details were as given for (b). The slope (fall in P/O/min.) was 0.29.

Table 1. *Effect of tetrazolium salts on oxidative phosphorylation*

Assay conditions are given in the text. The lowest concentration of each tetrazolium salt that was found completely to uncouple the phosphorylation reactions in the presence of either succinate or β -hydroxybutyrate is shown in column 3. Column 4 gives the concentration of tetrazolium salts found to inhibit the oxidative phosphorylation system such that respiratory control with ADP was just detectable.

Substrate	Tetrazolium salt	Final concn. (μM) to produce effect	
		Complete uncoupling	Just coupled
Succinate	NT	18	9
	INT	28	13
	NBT	38	—
	TTC	51	34
	MTT	130	81
β -Hydroxybutyrate	NT	18	9
	INT	28	13
	NBT	38	26
	TTC	51	34
	MTT	130	81

Tetrazolium salts show a very sharp concentration effect so far as their action on oxidative phosphorylation is concerned. For instance, the P/O ratios obtained for succinate and β -hydroxybutyrate in the presence of $16\mu\text{M}$ -NT were 1.75 and 2.15 respectively, but in the presence of $20\mu\text{M}$ -NT both systems were completely uncoupled (Fig. 3). The

very narrow range of concentration producing extensive changes in the P/O ratio makes it difficult to decide whether preferential uncoupling of single sites of phosphorylation is occurring.

However, Table 2, which shows results for NBT, suggests that at a concentration of $20\mu\text{M}$ the step occurring at the NADH-flavoprotein level is

Fig. 3. Effects of NT on the P/O ratio obtained with β -hydroxybutyrate ($\circ$) or succinate ($\bullet$) as substrate. Various concentrations of NT were added to the electrode compartment and the P/O ratios determined for two successive additions of ADP. The points shown are the means of these pairs of estimations. Other details were as given in the text.

almost completely uncoupled, since with succinate the P/O ratio is 1.98 and with β -hydroxybutyrate it is 2.03. An analogous situation occurs with NT at a concentration of $16\mu\text{M}$ (Fig. 3). Thus both tetrazolium salts at these particular concentrations appear to affect preferentially the same phosphorylation site, although at higher concentrations all three sites are affected. No evidence is available to decide whether the greater susceptibility of the NADH-flavoprotein site to NT and NBT is due to a relatively higher susceptibility to deterioration or to a preferential uncoupling. However, because of the very sharp concentration effects and doubtful specificity, the tetrazolium salts studied in the present work do not appear to be suitable agents for use as preferential uncoupling agents.

One peculiar aspect of these results is the relative effectiveness of the tetrazolium salts as uncoupling agents. This order is very different from the effi-

Table 2. Effect of NBT on oxidative phosphorylation with either succinate or β -hydroxybutyrate as substrate

Details of the isolation of the mitochondrial suspension and of the assay procedure are given in the text.

Final concn. of NBT (μM)	P/O ratio observed	
	With β -hydroxybutyrate	With succinate
0	2.73	2.08
20	2.03	1.98
28	1.50	1.89
40	Uncoupled	Uncoupled

ciency of tetrazolium salts (measured in μg . of formazan produced/mg. of tissue) as acceptors of electrons in colorimetric assays of dehydrogenase-linked reactions (Slater *et al.* 1963), where MTT is the most efficient, followed by INT, NBT, NT and TTC respectively. Further, neither series shows any correlation with the reported standard electrode potentials (E'_0 at pH 7.0): NBT, -50mv ; INT, -90mv ; MTT, -120mv ; NT, -170mv ; TTC, -460mv (Karmarkan, Pearse & Seligman, 1960).

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