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THE EFFECTS OF SOME MITOCHONDRIAL TRANSLOCASE INHIBITORS ON THE ACTIVITY OF RAT LIVER TRANSHYDROGENASE

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Abstract—Non-energy dependent transhydrogenase activity in submitochondrial particles is stimulated at pH 8 by some inhibitors of mitochondrial carboxylate translocases. On solubilising the enzyme or assaying it at pH 6 these inhibitors have little effect. K_m values remain unchanged under all these conditions. Conversely, energy-dependent transhydrogenase activity is inhibited by these substances possibly due to their direct action on proton transport. It is suggested that the observed effects may all be due to changes in membrane conformation induced by the inhibitors.

Metabolically important carboxylate anions (e.g. pyruvate, succinate, citrate) are transferred into the mitochondrial matrix by several different translocases which can be competitively inhibited by various substances structurally related to the substrate carboxylates [1–4]. Although these substances are supposed to be translocase-specific inhibitors, they have recently been shown to prevent the regeneration of glutathione (GSH) after its oxidation to the disulphide (GSSG) within intact mitochondria [5–7]. Such regeneration involves reduction by mitochondrial NADPH and is catalysed by glutathione reductase, the activity of which is not affected by the translocase inhibitors. Since NADPH is produced in mitochondria principally by transhydrogenation between NADH and NADP, as catalysed by a membrane bound transhydrogenase [8], the latter enzyme was considered to be a possible site for the inhibition of the reduction of GSSG. Transhydrogenase activity cannot conveniently be studied in intact mitochondria because of the interference of other dehydrogenase reactions and also the impermeability of the two dinucleotide substrates. However, submitochondrial particles (inside-out inner membrane vesicles [25]) exhibit the enzyme on their outer face and are the usual source for studying its activity [8]. The transhydrogenase activity found on adding NADP and NADH only to such particles (non-energy transhydrogenase) is substantially increased when coupled either to the hydrolysis of added ATP or to the oxidation of added succinate (energy-dependent activity) [9]; the non-energy activity can also be studied by solubilising the enzyme with certain detergents [10].

In this paper, the effect of the translocase inhibitors on these different aspects of the transhydrogenase system is described.

MATERIALS AND METHODS

Butyl-*n*-malonate was prepared as described by Vogel [19], phenylsuccinate and 1,2,3-benzenetricarboxylic acid were purchased from Aldrich Co., Gillingham, 1,2,3-propanetricarboxylic acid (tricarballylic acid) from Koch-Light, Colnbrook, phenylpyruvic acid, type 1 from Sigma Ltd. and quinacrine dihydrochloride from British Drug Houses, Poole, Dorset. Other reagents were of the best available analytic grade.

Transhydrogenase preparations. Rat liver mitochondria (obtained as previously [5]) were sonicated to obtain submitochondrial particles as described by Joshi and Sanadi [11] using a Rapidis 150 sonicator (Ultrasonics Ltd.). Remaining unbroken organelles were removed by centrifugation at 15,000 *g* for 20 min, and the supernatant was then centrifuged at 100,000 *g* for 50 min. The pellet of submitochondrial particles was resuspended in 0.25 M sucrose at a concentration of 15–20 mg protein/ml, and this was used as a source of transhydrogenase activity. Particles were stored for up to 10 days at −70°. They were solubilised when required by treatment with 1 volume of 2% Lubrol PX 12.

Assay of transhydrogenase activity. The standard method [13, 14] was modified to give regeneration of both substrates. Enzyme suspension (50 μl containing 0.1–1.0 mg protein from submitochondrial particles before or after solubilisation) was added to 0.4 ml aliquots of an assay medium prewarmed at 30° and consisting of 0.1 M Tris-HCl, pH 8 or 0.1 M Tris-acetate, pH 6 in 0.5 M sucrose containing $MgCl_2$ (12 mM), NADP (0.1 mM), NADH (0.25 mM), GSSG (1 mM), ethanol (60 mM), rotenone (3.5 μM), glutathione reductase (0.9 IU/ml) and alcohol dehydrogenase (0.9 IU/ml). Where indicated, ATP (1 mM) or succinate (10 mM) plus oligomycin (0.5 μg/ml) were also present. Translocase inhibitors (up to 50 μl) were added from concentrated stock solutions. After incubation for the required time, 50 μl perchloric acid (12%; wt/vol)

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was added to stop the reaction and precipitate the proteins. GSH was then assayed on the neutralised supernatant with 5,5′-dithiobis-(2-nitrobenzoic acid) [15]. The small amount forming when NADP was omitted from the incubations was subtracted and the value halved to convert to NADPH produced. Under these assay conditions, the rate of reduction of GSSG by added NADPH in the presence of glutathione reductase was unaffected by the translocase inhibitors.

Other assays. Protein was determined by a modified Biuret method [16]. Adenosine triphosphatase activity was assayed in submitochondrial particles using pyruvate kinase and phosphoenol pyruvate [17].

RESULTS

The effect of some inhibitors of mitochondrial translocases on the activity of transhydrogenase in rat liver submitochondrial particles has been investigated. The compounds studied were phenylpyruvate (a monocarboxylase translocase inhibitor [4]), butylmalonate and phenylsuccinate (dicarboxylate translocase inhibitors [1]), propanetricarboxylate and benzenetricarboxylate (tricarboxylate translocase inhibitors [2]). (A further monocarboxylate inhibitor, α-cyano-4-hydroxycinnamic acid [3], could not be used because it combines directly with the GSH produced during the assay.) The inhibitors were added at concentrations generally used to inhibit transmembrane transport of the carboxylates. At pH 8, 0.1–10 mM phenylpyruvate inhibited slightly the non-energy transhydrogenase activity of the particles whereas the other substances all induced a considerable stimulation (up to 3-fold). In the case of the tricarboxylic translocase inhibitors, the stimulation was maximal at 5 mM and declined at higher concentrations but with increasing amounts of the dicarboxylate inhibitors, stimulation of transhydrogenase activity increased continuously (Fig. 1A). Non-energy transhydrogenase activity can be solubilised from the particles by treatment with detergents. However the activity of the enzyme preparations obtained by this technique was unaffected by any of these substances (Fig. 1B). It has been reported that beef heart transhydrogenase can be stimulated by decreasing the pH of the assay medium from pH 8 to pH 6 [18] and in agreement we have obtained a similar increase (5-fold) with the rat liver enzyme, whether membrane bound or soluble (results not shown). Moreover at this lower pH the enzyme was not significantly affected by the translocase inhibitors. The effect of various amounts of these inhibitors on the kinetics of non-energy dependent transhydrogenase was studied by measuring rates with varying concentrations of either NADH or NADP. Linear Eadie-Hofstee plots (s/v vs s) of these data were obtained in each case (Fig. 2). K_m values for NADH or NADP were unaffected as shown by the convergence of the lines to the same point on the abscissa. It is, thus, clear that the translocase inhibitors are not competitive with either of the substrates for transhydrogenase. The same

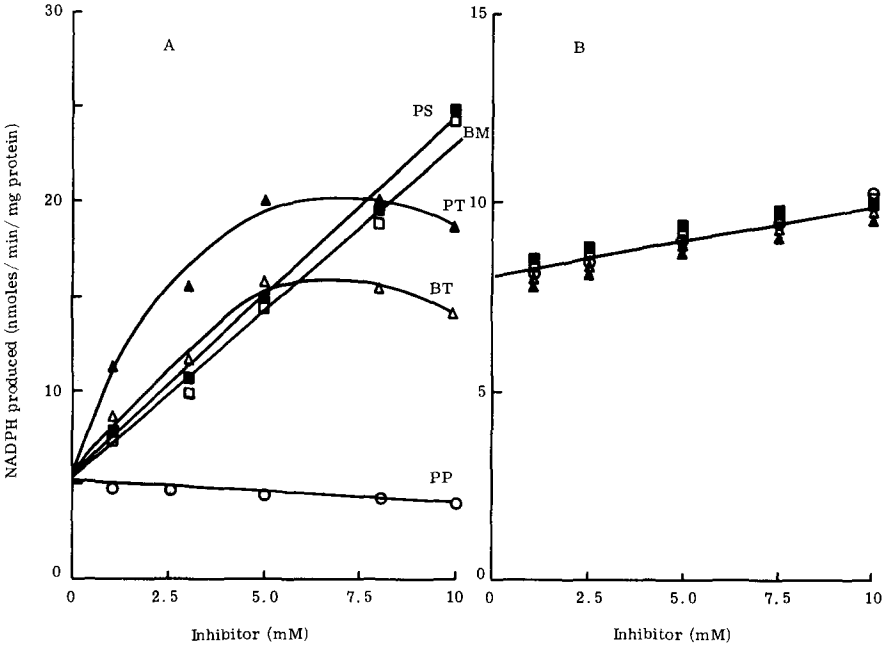

Fig. 1. Effect of the non-energy dependent transhydrogenase activity of submitochondrial particles (A) or solubilised submitochondrial fraction (B) of adding varying amounts of (○) phenylpyruvate (PP), (□) butylmalonate (BM), (■) phenylsuccinate (PS), (△) benzenetricarboxylate (BT) or (▲) propanetricarboxylate (PT). Varying amounts of the inhibitor were mixed at pH 8 with separate aliquots of the transhydrogenase assay medium and the solution preincubated at 37° for 5 min. A suspension (50 μl) of the submitochondrial particles (10 mg protein per ml) or solubilised fraction (6 mg per ml) was added to start the reaction which was terminated 2 min later with perchloric acid. For details of the assay and preparation of the enzyme fractions see the Methods section.

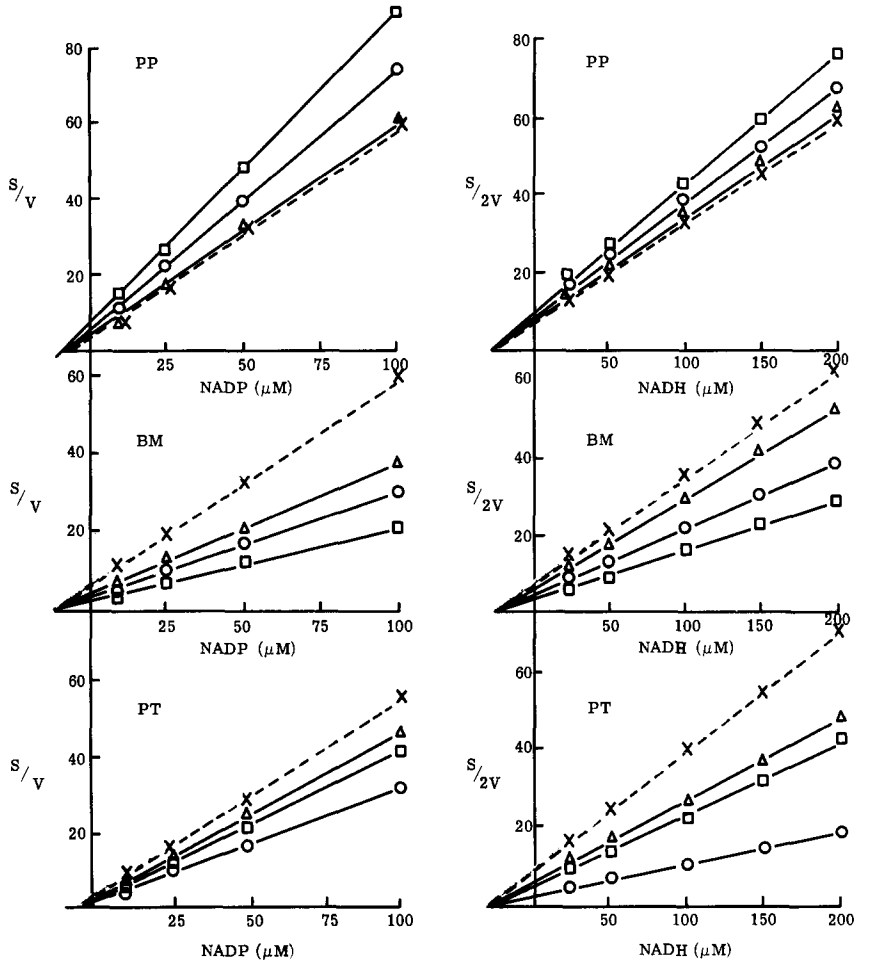

Fig. 2. Effect of translocase inhibitors on the transhydrogenase activity of submitochondrial particles at different fixed concentrations of NADP and NADH. Assays were performed at pH 8 as described in Fig. 1 except that the reaction time was extended to 5 min. The assay mixtures differed only in the concentration of the substrate varied. The inhibitors used were phenylpyruvate (PP) butylmalonate (BM) and propanetricarboxylate (PT). Their concentrations in the assay medium were (×) nil (dotted line), (△) 2.5 mM, (○) 5 mM and (□) 10 mM.

unchanged K_m values were also obtained on repeating these kinetic experiments at pH 6 with or without an inhibitor present. Provision of an energy source (ATP hydrolysis or succinate oxidation) at pH 8 is well known to increase the V_{max} of the transhydrogenase reaction in submitochondrial particles by up to tenfold [9] and our data confirm this. Driven by either of these processes, the augmented activity of the enzyme was inhibited by up to 50% by the translocase inhibitors at concentrations above 2.5 mM (Fig. 3). Kinetic analysis (performed in the same way as shown in Fig. 2) showed that K_m values were still unaffected and were not significantly different from those of the non-energy dependent reaction. Inhibition of the energy-dependent transhydrogenase activity by the dicarboxylate and tricarboxylate translocase inhibitors can be reconciled with their stimulation of the non-energy dependent reaction since in the former case they could additionally be acting on the systems concerned with energy coupling.

ATP hydrolysis and succinate oxidation both induce proton influx into submitochondrial particles, and it is the establishment of a transmembrane proton gradient which drives energy-dependent transhydrogenase activity. The magnitudes of such a gradient can be studied using the quinacrine fluorescence quenching technique [20]. Using this method, development of the proton gradient induced by either succinate or ATP was found to be impaired by each of the translocase inhibitors, the least effective of which was phenylpyruvate (Table 1). The inhibitors could act to reduce the steady-state value of the proton gradient by either increasing its rate of dissipation, or decreasing its rate of formation. We have obtained some evidence to support the latter alternative, at least in the case of ATP-driven transhydrogenase. As illustrated by Fig. 4, the rate of ATP hydrolysis was reduced by the translocase inhibitors; the effect of propanetricarboxylate was greatest (inhibition up to 50%) and phenylpyruvate was again the least effective.

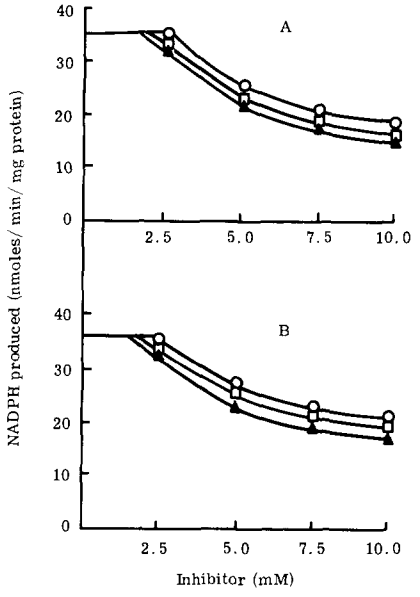

Fig. 3. Action of inhibitors on energy-dependent transhydrogenase driven by (A) adenosine triphosphate or (B) succinate. To separate aliquots of the reaction mixture at pH 8 containing ATP or succinate plus oligomycin (see Methods section) were added varying amounts as shown of (○) phenylpyruvate (PP) (□) butylmalonate (BM) or (▲) propanetricarboxylate (PT). The reaction and assays were carried out with submitochondrial particles as described in Fig. 1.

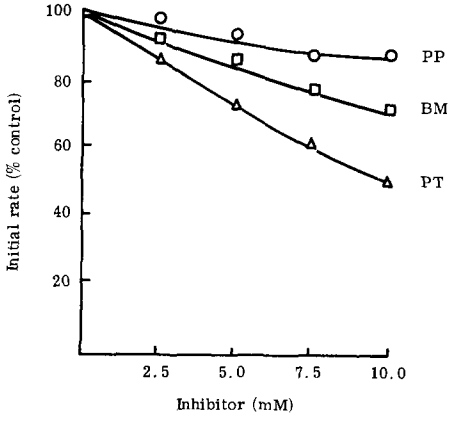

Fig. 4. Mitochondrial adenosine triphosphatase activity in the presence of (○) phenylpyruvate (PP), (□) butylmalonate (BM), and (△) propanetricarboxylate (PT). To the reaction mixture (0.9 ml) consisting of NADH (0.2 mM), phosphoenolpyruvate (10 mM) and inhibitor (5 mM) in Tris-sucrose-HCl buffer, pH 8 containing $MgCl_2$ (1 mM), a mixture (20 μl) of pyruvate kinase (1 IU) and lactate dehydrogenase (1 IU) was added followed at once by a submitochondrial particle suspension (80 μl containing 0.8 mg protein). The reaction was followed at 340 nm. Data plotted are means from two assays.

Table 1. Effect of translocase inhibitors on quinacrine fluorescence quenching induced by ATP or succinate*

Inhibitor	Fluorescence quenching With ATP	With succinate
Nil	100	100
Phenylpyruvate	83	87
Butylmalonate	13	31
Propanetricarboxylate	13	11

* ATP (1 mM) or succinate (10 mM) were added to mixtures at 20° of submitochondrial particles (0.8 mg protein), KSCN (4 mM) rotenone (3.4 μM) and quinacrine dihydrochloride (3 μM) in Tris-sucrose-HCl buffer, pH 8 (3 ml) containing the inhibitor (5 mM). The fall in fluorescence intensity (excitation, 420 nm; emission, 500 nm) (arbitrary units) was determined after 15 sec. Fluorescence quenching [20] is given as a percentage of the quenching obtained with no inhibitor present. Results are means of two experiments (max. difference from mean, 4 arbitrary units).

DISCUSSION

The inhibitors used in this study are supposedly specific for blocking carboxylate translocases but we have now shown that in the same concentration range they also affect the activity of mitochondrial transhydrogenase. The dicarboxylate and tricarboxylate inhibitors stimulate the non-energy dependent reaction and inhibit the energy-dependent reaction while the monocarboxylate inhibitor, phenylpyruvate, inhibits in both cases. Transhydrogenase activity may be affected by conformational modifications to the enzyme. These have been suggested to be brought about by pH changes [21], substrate-binding [10, 23] and energy-generation [18, 24]. It is possible that translocase inhibitors might also affect enzyme conformation. Thus, the effect of the dicarboxylate and tricarboxylate translocase inhibitors upon non-energy dependent transhydrogenase was not additional to the stimulation of this activity brought about by decreasing the pH from 8 to 6. This result may indicate that stimulation in both cases occurs through the same mechanism, which in the case of the pH changes was suggested to affect the pK of an essential bound phosphoryl group [21].

Solubilised transhydrogenase was not significantly affected by the inhibitors, so they may influence the enzyme indirectly, for example by inducing primary conformational changes in other proteins such as the translocase for which they are supposedly specific. Such changes may secondarily affect the conformation of adjacent transhydrogenase molecules. Superimposed upon these phenoma is the effect of the translocase inhibitors upon the proton gradient, in part due to their inhibition of the proton-translocating ATPase (F_1) as measured by ATP hydrolysis. Whether conformational changes are also involved here is unclear, but such a mechanism could explain why one enzyme (transhydrogenase) is activated whilst others (involved in proton transfer) may be inhibited.

The translocase inhibitors are considered not to be able to penetrate the mitochondrial inner membrane (with the possible exception of benzenetri-

carboxylate [22]). Consequently in submitochondrial particles their effects would be exerted upon the outside face of this membrane. In intact mitochondria, this aspect of the membrane which contains the transhydrogenase activity, would be inaccessible to the inhibitors. The inhibitors therefore could only have the same effects upon transhydrogenase activity in intact mitochondria as they have in the inverted particles if they could influence the enzyme indirectly. Energy-dependent transhydrogenase may be modified by the inhibitors through their inhibition of proton pumping, for which the results section provides some evidence. Their impairment of GSSG reduction (see Introduction) could be brought about by such an inhibition of transhydrogenase activity if sites concerned with proton pumping are sufficiently similar on both sides of the membrane. However, our data indicate that this would only be achieved if energy-dependent transhydrogenase predominated over energy-independent activity during GSH regeneration.

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REFERENCES

1. B. H. Robinson and J. B. Chappell, *Biochem. biophys. Res. Commun.* **28**, 249 (1967).
2. B. H. Robinson and G. R. Williams, *Eur. J. Biochem.* **20**, 65 (1971).
3. A. P. Halestrap and R. M. Denton, *Biochem. J.* **138**, 313 (1974).
4. A. P. Halestrap, M. D. Brand and R. M. Denton, *Biochem. biophys. Acta* **367**, 102 (1974).
5. P. C. Jocelyn, *Biochem. J.* **176**, 649 (1978).
6. P. C. Jocelyn and J. Dickson, *Biochem. biophys. Acta* **590**, 1 (1980).
7. P. C. Jocelyn, *Biochem. Pharmac.* **29**, 331 (1980).
8. J. Rydstrom, *Biochem. biophys. Acta* **463**, 155 (1977).
9. C. P. Lee and L. Ernster, *Biochem. biophys. Acta* **81**, 187 (1974).
10. W. M. Anderson and R. R. Fisher, *Archs Biochem. Biophys.* **187**, 180 (1978).
11. S. Joshi and R. R. Sanadi, *Methods Enzymol.* **55F**, 393 (1979).
12. L. A. Petsch, *Biochem. biophys. Acta* **81**, 229 (1964).
13. A. Texeira da Cruz, J. Rydstrom and L. Ernster, *Eur. J. Biochem.* **23**, 203 (1971).
14. L. Danielson and L. Ernster, *Biochem. biophys. Res. Commun.* **10**, 91 (1963).
15. G. L. Ellman, *Archs. Biochem. Biophys.* **82**, 70 (1958).
16. E. E. Jacobs, M. Jacob, D. R. Sanadi and L. B. Bradley, *J. biol. Chem.* **223**, 147 (1956).
17. P. R. Pougeois, M. Satre and P. V. Vignais, *Biochemistry, N.Y.* **17**, 3018 (1978).
18. S. R. Earle, S. G. O'Neal and R. R. Fisher, *Biochemistry, N.Y.* **17**, 4683 (1978).
19. A. I. Vogel, *Textbook of Practical Organic Chemistry*, 3rd edn, p. 488. Longmans Green, London (1956).
20. C. P. Lee, *Biochemistry, N.Y.* **10**, 4375 (1971).
21. Y. M. Galante, Y. Lee and Y. Hatefi, *J. biol. Chem.* **255**, 9641 (1980).
22. J. W. Stucki, *Eur. J. Biochem.* **78**, 183 (1977).
23. D. C. Phelps and T. Hatefi, *J. biol. Chem.* **256**, 8217 (1981).
24. J. Rydstrom, *J. biol. Chem.* **254**, 8611 (1979).
25. C. P. Lee and L. Ernster, *BBA Library* **7**, 218 (1966).