The main feature of the esteric group is its ability to accept hydrogen bonds as its oxygen is a typical electron donor group. Probably any other functional group having the same hydrogen bonding accepting capability would display similar behaviour and in effect this could be the case of the 3CN group previously discussed.

It is worth mentioning the hypothesis, supported by crystallographic, quantum-mechanic and structure–activity studies, that BDZs also interact with their receptor mainly via hydrogen bonds and in an essentially planar molecular conformation [16, 17].

Acknowledgements—Work financially supported by C.N.R. (Rome), Progetto Finalizzato Chimica Fine e Secondaria.

Istituto di Farmacologia PIER ANDREA BOREA
Università di Ferrara
44100 Ferrara, Italy

Centro di Strutturistica VALERIA FERRETTI
Diffrattometrica
Università di Ferrara
44100 Ferrara, Italy

REFERENCES

1. G. L. Biagi, A. M. Barbaro, M. C. Guerra, M. Babbini, M. Gaiardi, M. Bartoletti and P. A. Borea, *J. Med. Chem.* **23**, 193 (1980).
2. P. A. Borea, *Arzneim. Forsch.* **33**, 1086 (1983).
3. S. M. Free and J. W. Wilson, *J. Med. Chem.* **7**, 395 (1964).
4. C. Hansch, *Acc. Chem. Res.* **2**, 232 (1969).
5. T. Fujita and T. Ban, *J. Med. Chem.* **14**, 148 (1971).
6. H. Kubinyi and O. K. Kehrahn, *J. Med. Chem.* **19**, 578 (1976).
7. P. A. Borea, V. Bertolasi and G. Gilli, *Proc. 7th International Congress of Pharmacology*, Paris, p. 384 (1978).
8. W. P. Purcell, G. E. Bass and J. M. Clayton, in *Strategy of Drug Design: A Guide to Biological Activity*. Wiley-Interscience, New York (1973).
9. C. Braestrup and M. Nielsen, in *Handbook of Psychopharmacology* (Eds. L. L. Iversen, S. D. Iversen and S. H. Snyder), p. 285. Plenum Press, New York (1983).
10. M. Cain, R. W. Weber, F. Guzman, J. Cook, S. A. Barker, K. C. Rice, J. N. Crawley, S. M. Paul and P. Skolnick, *J. Med. Chem.* **25**, 1081 (1982).
11. G. Loew, J. Nienow, J. A. Lawson, L. Toll and E. T. Uyeno, *Molec. Pharmac.* **28**, 17 (1985).
12. K. P. Lippke, W. G. Schunack, W. Wenning and W. E. Müller, *J. Med. Chem.* **26**, 499 (1983).
13. M. Karobath and P. Supavilai, *Neurosci. Lett.* **31**, 65 (1982).
14. V. Bertolasi, V. Ferretti, G. Gilli and P. A. Borea, *Acta Cryst.* **C40**, 1981 (1984).
15. P. W. Codding, *Can. J. Chem.* **61**, 529 (1983).
16. P. A. Borea and G. Gilli, *Arzneim. Forsch.* **34**, 649 (1984).
17. G. Loew, J. R. Nienow and M. Poulsen, *Molec. Pharmac.* **26**, 19 (1984).

Biochemical Pharmacology, Vol. 35, No. 16, pp. 2839–2841, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Stabilizing action of L-carnitine on the energy-linked processes of mitochondria isolated from perfused rat liver

(*Received* 28 *October* 1985; *accepted* 13 *February* 1986)

L-Carnitine has recently been reported to stabilize rat liver mitochondria exposed to various stressing conditions as well as mitochondria isolated from the liver of L-carnitine treated animals [1]. L-Carnitine has also been found to prevent mitochondrial damage induced in leukaemia cells by the anticarcinogen methylglyoxal bis(guanilhydrazone) [2]. Retrospectively a protective action of L-carnitine on mitochondria might also explain the protection of mice against otherwise toxic doses of ammonium acetate [3], indeed the energy-linked phase of urea cycle takes place within liver mitochondria. In this paper we present evidence that addition of L-carnitine to the perfusion medium significantly improves the energy-linked processes of mitochondria, thereafter isolated from the liver, and their resistance against different damaging factors.

Materials and methods

Materials. Outdated human concentrated erythrocytes were kindly supplied by the Blood Bank of the Padova Hospital (Padova, Italy).

Ketalar® (2-(*O*-chlorophenyl)-2-methylaminocyclo-esanone) was purchased from Parke Davis S.p.A. as a 50 mg/ml solution.

Methods. Male Wistar rats (300–350 g) starved for 24 hr were anaesthetized by intraperitoneal administration of 0.35 ml (17.5 mg) of Ketalar®/100 g of body weight. In addition, 500 U.I. of heparine/100 g of body weight were injected.

The perfusion medium (modified Hanks' medium) contained 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 10 mM Na–Hepes* (pH 7.4), 0.6 mM $MgSO_4$ and 1 μM Ca^{2+}.

Prior to being added to the perfusion medium the outdated human erythrocytes were washed twice in 10 vol. of 125 mM NaCl, 30 mM Tris HCl (pH 7.2), and the hematocrit of the resulting concentrated suspension (80–90%) was determined.

Reversed liver perfusion was achieved by cannulation of the caval vein (inlet) via the right atrium and the portal vein (outlet). A ligature was put around the inferior caval vein, just above the right renal vein, the hepatic vein and artery. During the perfusion, which was performed at a flow rate of 13 ml/min, the liver was left *in situ* and temperature (35°) and surface wetting were carefully controlled. The perfusion medium was constantly oxygenated with an oxygen:carbon dioxide (19:1) mixture, and the perfusate leaving the portal catether was discarded.

At the end of the perfusion the liver was excised, immediately immersed in ice-cold 0.25 M sucrose, 5 mM Na–Hepes (pH 7.4), washed thoroughly and then homogenized in 50 ml of 0.25 M sucrose, 5 mM Na–Hepes (pH 7.4) using a Potter homogenizer with Teflon pestle driven at 900 rev/min. Mitochondria were then isolated by differential centrifugation in the same buffered solution.

The protein content of mitochondrial suspensions was assayed according to Gornall *et al.* [4] using bovine serum albumin as the standard.

Mitochondrial oxygen consumption rates were measured at 20° with a Clark oxygen electrode (Yellow Spring Ind.) in

* Abbreviations used: Hepes: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

2.5 ml of a respiration mixture containing 100 mM sucrose, 50 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM Tris–HCl (pH 7.4) (medium A) or 0.2 M NaCl, 20 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris HCl (pH 7.4) (medium B) in the presence of 5 mM succinate, and 1.25 μM rotenone. The oxygen traces were started by addition of 3 mg of mitochondrial proteins; state 3 respiration was initiated by additions of 150 μM ADP.

The mitochondrial transmembrane potential was measured by incubating at 20° 1 mg of mitochondrial protein/ml in a medium containing 200 mM sucrose, 10 mM Na–Hepes (pH 6.8), 5 mM succinate, 1.25 μM rotenone, 20 μM $CaCl_2$ and 2 mM NaH_2PO_4 and by monitoring the distribution of tetraphenylphosphonium across the mitochondrial membrane with a tetraphenylphosphonium-selective electrode prepared in our laboratory according to Kamo *et al.* [5] using a calomel electrode (Radiometer K401) as the reference electrode. The electrode potential was linear with respect to the logarithm of tetraphenylphosphonium concentration with a slope of 59 mV, in agreement with Nernst equation.

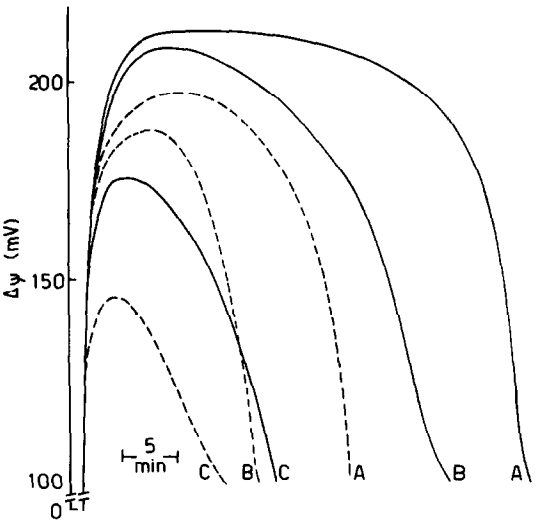

Fig. 1. Protective action of L-carnitine on transmembrane potential of mitochondria isolated from perfused rat livers. Mitochondria were isolated from rat livers after 10 min perfusion in the absence (dotted lines) or in the presence (full lines) of 0.75 mM L-carnitine. Mitochondria were kept in 0.25 M sucrose, 5 mM Na–Hepes (pH 7.4) at 0° as a concentrate suspension and transmembrane potential ($\Delta\psi$) was measured 0 (A), 6 (B) and 20 (C) hr after mitochondria isolation. The transmembrane potential traces represent a typical set of experiments.

Results

A necessary prerequisite for the validity of the experiments here described was the isolation from perfused livers of mitochondria having acceptable respiratory control ratios. In preliminary experiments in which rat livers were perfused for 30 min with the modified Hanks' medium, the subsequently isolated mitochondria exhibited respiratory control ratios slightly higher than 2.0 in medium A and just above 1.0 in medium B. Assuming that this poor coupling was due, as pointed out by Hulsmann and Kuzpershoek-Davidov [6], to heterogeneity of oxygen supply in the perfused liver, the experiments were then repeated with the addition of 4% (v/v) of human erythrocytes to the perfusion solution. Under these conditions the mitochondria isolated from perfused livers, although less coupled than those prepared from fresh livers, constantly had respiratory control ratios in the range 4.5–5.5 in medium A and 3.0–4.0 in the NaCl-rich medium B (Fig. 2, curves 1, 2). Therefore erythrocyte addition to the perfusion medium was adopted in all subsequent experiments.

Figure 1 shows the time course of the transmembrane potential, relative to a typical experiment, of mitochondria isolated from rat livers perfused for 15 min either with or without L-carnitine, stored in 0.25 M sucrose, 5 mM Na–Hepes (pH 7.4) at 0° for different lengths of time and then incubated in a medium containing 20 μM Ca^{2+} and 2 mM phosphate. It was previously demonstrated that the energy-linked processes of liver mitochondria incubated in the presence of these two ions undergo a rapid decay [7]. It may be observed that mitochondria obtained from L-carnitine-perfused livers constantly reached transmembrane potentials significantly higher than control mitochondria, with increasing difference as the storage period at 0° was shifted from 0 to 20 hr. The difference results are even more striking if the maintenance of transmembrane potential is considered. Indeed, immediately after isolation, mitochondria isolated from L-carnitine-perfused livers maintained a transmembrane potential higher than 200 mV for about 30 min, while control mitochondria had a lower transmembrane potential and maintained it no longer than 10 min. Upon storage at 0° for 6 or 20 hr the maintenance of transmembrane potential was significantly shortened, but its decay was constantly more rapid in control mitochondria.

The oxygen traces and respiratory control ratios of mitochondrial preparations obtained from perfused livers (Fig. 2) confirm that the addition of L-carnitine to the perfusion medium allowed the isolation of mitochondria more resistant to the impairing action of medium B and to storage at 0°. On the contrary no significant difference between mitochondria isolated from livers perfused with or without L-carnitine was observed in medium A.

On the basis of both parameters (respiratory control ratio and transmembrane potential) adopted for monitoring mitochondrial energy-linked processes it may be deduced that perfusion of the liver with L-carnitine allows the isolation of mitochondria less susceptible to the damaging action of Ca^{2+} and phosphate and of high concentrations of NaCl, and more resistant to aging than control mitochondria.

Discussion

The reported results clearly indicate that L-carnitine acts as a stabilizing factor of mitochondrial energy-linked processes not only when added directly to isolated liver mitochondria, as previously demonstrated [1], but also when present in the perfusion medium of livers from which mitochondria have been isolated. The protective action of L-carnitine appears to be a consequence of modifications induced during liver perfusion rather than the result of a direct interaction with mitochondrial membranes (L-carnitine added to the perfusion medium is completely or largely removed during the isolation of mitochondria).

The higher and longer lasting transmembrane potentials observed in mitochondria obtained from L-carnitine-treated livers may be attributed to the removal of long-chain acyl CoA by the L-carnitine present in the perfusion medium. It has to be taken into account that experiments have been carried out in 24-hr-starved rats. Since under this condition an increased level of free fatty acids and acyl CoA conceivably occurs, such a removal might be relevant for the cell integrity.

It is in fact well known that the accumulation of long-chain acyl CoA implies many adverse effects (for a review see [8]) among which are the inhibition of adenylate translocase [9] and dicarboxylate transport [10]. Furthermore by an aspecific interpolation into the lipid bilayer these metabolites may be able to deform mitochondrial membranes [8] which consequently become more susceptible to the

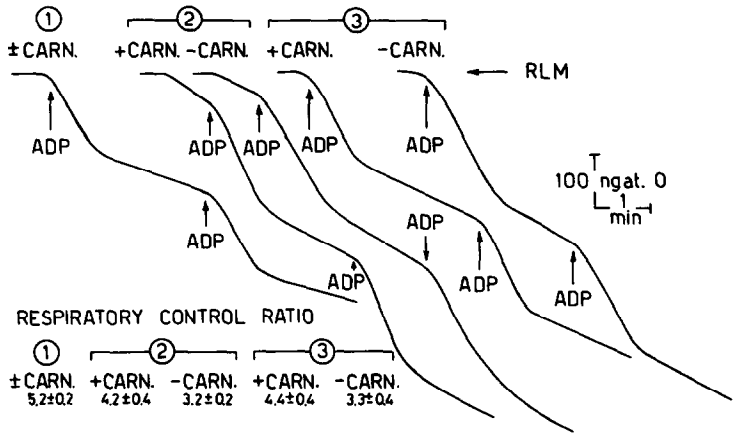

Fig. 2. Oxygen consumption traces of mitochondria isolated from rat livers perfused in the presence and in the absence of L-carnitine. Mitochondria were isolated from rat livers after 10 min perfusion in the presence and in the absence of 0.75 mM L-carnitine. The respiratory rates were measured in medium A (1) and B (2) immediately after the isolation procedure, and in medium B after 6 hr storage at 0° (3). Mitochondria (RLM; 3 mg of mitochondrial proteins) or 150 μM ADP were added when indicated by the arrows. The oxygen traces represent a typical set of experiments and the mean values (±S.D.) of respiratory control ratios of 10 different sets of experiments are also reported (+CARN vs −CARN: $P < 0.01$ for RCR ②, $P < 0.025$ for RCR ③).

damaging action of noxious conditions, like aging. It may be assumed that the observed protective action of L-carnitine is due to the removal, during liver perfusion, of long-chain acyl CoA by conversion to the corresponding long-chain acyl carnitines by the membrane bound carnitine palmitoyl transferases (E.C.2.3.1.21). This removal results in an increased stability of the mitochondrial membrane with higher efficiency of state 3 respiration (Figs 1 and 2). Furthermore the observed accelerated rate of respiration in state 3 might reflect an easier translocation of adenine nucleotides resulting from the activation of adenylate translocase partially inhibited by long-chain fatty acids.

Acknowledgements—The authors thank Sigma-Tau (Roma, Italy) for providing L-carnitine and other chemicals, and Dr M. Luisa Varotto for skilled assistance.

Institute of Biological Chemistry
University of Padova and Centro
Studio Fisiologia Mitocondriale
C.N.R., Padova, Italy

DONATA BRANCA
FABIO DI LISA
GUIDO SCUTARI
ANTONIO TONINELLO
NORIS SILIPRANDI

REFERENCES

1. F. Di Lisa, V. Bobyleva-Guarriero, P. Jocelyn, A. Toninello and N. Siliprandi, *Biochem. biophys. Res. Commun.* **131**, 968 (1985).
2. P. Nikula, H. Ruohola, L. Alhonen-Hougisto and J. Jarme, *Biochem. J.* **228**, 513 (1985).
3. J. E. O'Connor, M. Costell and S. Grisolia, *FEBS Lett.* **166**, 331 (1984).
4. A. G. Gornall, C. J. Bardawill and M. M. David. *J. biol. Chem.* **177**, 751 (1949).
5. N. Kamo, M. Muratsugu, R. Hongoh and Y. Kobatake, *J. Membr. Biol.* **49**, 105 (1979).
6. W. C. Hullsman and R. Kuzpershoek-Davidov, *Biochim. biophys. Acta* **354**, 39 (1974).
7. F. Zoccarato, M. Rugolo, D. Siliprandi and N. Siliprandi, *Eur. J. Biochem.* **114**, 195 (1981).
8. P. Brecher, *Mol. Cell. Biochem.* **57**, 3 (1983).
9. S. V. Pande and M. C. Blanchaer, *J. biol. Chem.* **246**, 402 (1971).
10. N. Siliprandi, F. Di Lisa and L. Sartorelli, in *Membrane and Muscle* (Eds. M. C. Berman, W. Gevers and L. H. Opie), p. 105. ICSU-IRL Press, Oxford (1985).

Biochemical Pharmacology, Vol. 35, No. 16, pp. 2841–2843, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Glibenclamide induces glucokinase in rat pancreatic islets and liver

(*Received* 12 *December* 1985; *accepted* 11 *February* 1986)

Hypoglycemic sulfonylureas such as glibenclamide initiate insulin secretion through a direct stimulatory effect on pancreatic B-cell plasma membrane receptors in the B-cells [1]. The insulin secretory response of isolated pancreatic islets to hypoglycemic sulfonylureas is considerably exaggerated by the concomitant presence of glucose in the incubation medium [1], particularly when islets have been isolated from fed animals [2]. But through their hypoglycemic action the sulfonylureas diminish the insulin secretory responsiveness of the B-cell. Thus another mechanism is required to maintain the long-term hypoglycemic action of the sulfonylureas *in vivo*. Here we show that this mechanism is the induction of pancreatic islet and liver glucokinase.

Glucokinase is a high K_m glucose phosphorylating and rate limiting enzyme [3], which regulates the amount of glucose to be stored in the liver [3–5] and the flux through the glycolytic pathway in the pancreatic B-cells thereby coupling extracellular glucose concentration and insulin secretion [6]. While glucose regulates the rate of glucose