OSMOTIC EFFECTS ON AMINO ACID OXIDATION IN SKATE LIVER MITOCHONDRIA

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SUMMARY

The mechanism responsible for the osmotic sensitivity of sarcosine oxidation by liver mitochondria from the little skate, *Raja erinacea* Mitchill, is examined. Assay medium tonicity, rather than a solute effect (urea or trimethylamine oxide), is probably responsible for the inverse relationship between osmolarity and the rate of oxidation of sarcosine by these mitochondria. Sarcosine oxidation proceeds through the flavin-linked sarcosine oxidase with the resultant glycine catabolized in the NAD-linked glycine cleavage system. The tonicity-sensitive component of the sarcosine oxidative pathway is not the glycine cleavage system. Sarcosine oxidation in the presence of rotenone is sensitive to medium tonicity. Oxidation of serine, which is also catabolized through the glycine cleavage system, is not as sensitive to tonicity as is sarcosine oxidation. Mitochondrial volume changes also appear to affect the transport of glycine. Although sarcosine does not appear to share the glycine transporter, it is possible that sarcosine transport is similarly sensitive to medium tonicity. The effects of osmolarity on the oxidation of dimethylglycine appear to support this hypothesis. Tonicity effects on sarcosine oxidase cannot yet be eliminated.

INTRODUCTION

Sarcosine oxidation by mitochondria isolated from the liver of the little skate, *Raja erinacea*, has recently been shown to be sensitive to the osmolarity of an assay medium composed primarily of urea, TMAO (trimethylamine oxide) and sucrose. Stimulation of sarcosine oxidation accompanies a reduction of assay medium osmolarity; inhibition of oxidation occurs in response to increased assay medium osmolarity (Ballantyne, Moyes & Moon, 1986). This effect on mitochondrial sarcosine oxidation has been implicated in altering the intracellular concentration of

sarcosine, an important osmolyte in skate tissues (Boyd, Cha, Forster & Goldstein, 1977) during osmotic stress. This study examines the mechanism which may be responsible for the osmotic sensitivity of sarcosine oxidation in skate liver mitochondria.

Hyperosmotically induced inhibition of mitochondrial respiration is well established. Slater & Cleland (1953) and Witter, Watson & Cottone (1955) reported a reduction in oxygen consumption by rat mitochondria incubated in high concentrations of sucrose. Osmotic sensitivity of metabolite oxidation has been demonstrated in mitochondria from mammals (Atsmon & Davis, 1967; Joseph, McGivan & Meijer, 1981), elasmobranchs (Lewiston, Newman, Robin & Holtzman, 1979; Ballantyne et al. 1986) and bivalve molluscs (Ballantyne & Storey, 1983, 1984, 1985; Ballantyne & Moon, 1985; Ellis, Burcham, Paynter & Bishop, 1985). Atsmon & Davis (1967) found that equiosmolar solutions of several impermeable solutes inhibit mitochondrial succinate oxidation equally. Solutions of permeable solutes such as glycine do not inhibit succinate oxidation (Campbell, Raison & Brady, 1975), suggesting that changes in mitochondrial volume occurring in anisotonic media are responsible for the effects on mitochondrial oxidation. Mitochondrial volume changes in response to anisotonic media are well established (e.g. Kovacevic, Bajin & Pavlovic, 1980). The tonicity of a medium (i.e. direction and degree of mitochondrial volume changes) is dependent upon the permeability of the solutes (Tedeschi & Harris, 1955).

Electron micrographs of isolated rat liver mitochondria reveal pronounced volume and structural changes in response to osmotic stress (Holtzman et al. 1978). Alterations in membrane configuration accompanying osmotically induced volume changes have been suggested to cause the osmotic effects on succinate oxidation by rat liver mitochondria (Atsmon & Davis, 1967) and glutamine oxidation in rat liver (Joseph et al. 1981) and kidney (Kovacevic et al. 1980) mitochondria. Campbell et al. (1975), however, proposed that the hyperosmotic inhibition of succinate and glutamate oxidation observed in rat liver mitochondria was not due to tonic effects, but to a direct effect of the solute on oxidative phosphorylation. Such a mechanism would be expected to affect mitochondrial oxidation of several metabolites equally. However, Johnson & Lardy (1958) found that the degree of respiratory inhibition of rat liver mitochondria was dependent on the substrate employed, arguing against an effect on oxidative phosphorylation. Instead, the osmotically sensitive component of the oxidative pathway may be dependent on the metabolite studied.

Atsmon & Davis (1967) suggested that hyperosmotic inhibition of succinate oxidation in rat liver mitochondria was due to reduced transport or access to the appropriate dehydrogenases. Effects on the transport of proline have been suggested to be responsible for the osmotic effects on the oxidation of proline by heart mitochondria of the marine bivalve *Mercenaria mercenaria* (Ballantyne & Storey, 1985). Joseph et al. (1981) suggested stimulation of membrane-bound glutaminase was responsible for enhanced glutamine oxidation in rat liver mitochondria. A similar effect on glutamine oxidation by rat kidney mitochondria was attributed to stimulation of glutamine transport (Kovacevic et al. 1980). Activation of the glycine

cleavage system was suggested to be responsible for the hyposmotic stimulation of glycine oxidation in gill mitochondria of the marine bivalve *Modilus demissus* (Ellis *et al.* 1985), although an effect on the transport of glycine was not considered.

The present study attempts to establish the mechanism by which osmolarity affects skate liver mitochondrial sarcosine oxidation. The effects of medium tonicity (i.e. osmotically induced mitochondrial volume changes) were separated from direct solute effects of urea or TMAO. Sarcosine oxidation in skates is thought to proceed by the same pathway as in mammals (Moyes, Moon & Ballantyne, 1986), but the osmotically sensitive component of the catabolic pathway is not known. Osmotically induced membrane alterations could affect sarcosine oxidase (Hoskins & MacKenzie, 1961), the glycine cleavage system (Hampson, Barron & Olson, 1983) or the sarcosine transporter, as each is associated with the inner mitochondrial membrane.

MATERIALS AND METHODS
Animals

Little skates, *Rana erinacea*, were captured locally by bottom trawl and were distinguished from the closely related species *R. ocellata* by tooth-row count (McEachran & Musick, 1973). Animals (20–50 cm total length; both sexes) were held, without feeding, in continuously flowing seawater ($920 \, \text{mosmol kg}^{-1}$) at seasonal temperatures (8–14°C) for up to 2 weeks before use.

Mitochondrial isolation and oxidation

Liver mitochondria were isolated in a sucrose-based medium as previously described (Moyes *et al.* 1986). This isolation procedure yields mitochondria with high respiratory control ratios (e.g. RCR with isocitrate $= 9.9$). One volume of mitochondrial suspension (0·078–0·115 ml; 10–30 mg protein ml^{-1}) was added to 9 volumes of reaction medium. The standard reaction medium consisted of (in mmol l^{-1}) urea, 370; TMAO, 185; sucrose, 46·25; KCl, 140; KH_2PO_4, 10; Hepes (*N*-2-hydroxy-piperazine-*N*-2-ethanesulphonic acid), 30; and 1 % BSA (essentially fatty acid free bovine serum albumin), pH 7·2 at 20°C. The urea concentration used is similar to that reported *in vivo* (Boyd *et al.* 1977). A 2:1 ratio of urea: methylamines (e.g. TMAO, betaine, sarcosine) may be important in preventing the adverse effects of urea (Yancey *et al.* 1982). TMAO was used exclusively, as it was not oxidized by these mitochondria and, therefore, did not elevate the endogenous rate of oxidation. The osmolarity of the assay mixture was varied by changing the concentration of urea, TMAO and sucrose, keeping the ratio of concentrations constant at 8:4:1, respectively. The concentration of KCl was kept constant at $140 \, \text{mmol l}^{-1}$. Temperature of the cuvette was held at 10°C using a Haake refrigerated water circulator. Oxygen consumption of the assay mixture was monitored using a Clarke-type electrode. Respiratory state 3 is the rate of oxygen consumption in the presence of both ADP and substrate (Chance & Williams, 1956). Endogenous rates of oxidation were determined in the presence of ADP but no added substrate.

Endogenous oxidation rates in skate liver mitochondria remain high, even after 6 weeks of starvation. β-Alanine (BALA) oxidation was determined in the presence of malate ($0 \cdot 1 \text{ mmol l}^{-1}$) to ensure that oxidation of the subsequent acetyl-CoA in the Krebs cycle was not limited by the availability of oxaloacetate. Malate was included in the assay for the endogenous rate when BALA was the substrate. The oxidation rate reported is calculated as the state 3 rate minus the endogenous rate. Substrate concentrations employed were found to be saturating unless stated otherwise. The concentration of sarcosine and BALA was 10 mmol l^{-1}; ADP was $0 \cdot 2 \text{ mmol l}^{-1}$.

Oxidation of [^{14}C] amino acids by mitochondria

The mitochondrial suspension ($0 \cdot 1 \text{ ml}$) was added to a 25 ml serum vial containing $0 \cdot 7 \text{ ml}$ of reaction medium and an ADP regenerating system (20 units hexokinase; 10 mmol l^{-1} glucose). The serum vial was capped with a rubber stopper fitted with a well containing a glass fibre filter disc (Whatman GF/A). After a 10 min pre-incubation at 10°C, $0 \cdot 2 \text{ ml}$ of reaction medium containing ADP ($1 \cdot 0 \text{ mmol l}^{-1}$ final concentration), cold amino acid (5 mmol l^{-1} glycine or 10 mmol l^{-1} L-serine) and [^{14}C] amino acids ($0 \cdot 1 \mu\text{Ci}$ [$^{14}C(U)$]-, [$^{14}C(1)$]- or [$^{14}C(2)$]-glycine or $0 \cdot 02 \mu\text{Ci}$ [$^{14}C(U)$]-L-serine) were injected into the incubation through the cap. In the competition studies, glycine (1 mmol l^{-1} final concentration; $0 \cdot 1 \mu\text{Ci}$ [^{14}C]glycine) was injected into vials preincubated for 10 min with 5 mmol l^{-1} alanine, BALA, sarcosine (with 20 mmol l^{-1} sodium acetate) or dimethylglycine (also with 20 mmol l^{-1} sodium acetate). Acetate (20 mmol l^{-1}), through its effects on sarcosine oxidase (Frisell & MacKenzie, 1955), reduced the oxidation of 5 mmol l^{-1} sarcosine to below detectable levels (data not shown), thus preventing intramitochondrial production of unlabelled glycine from sarcosine and dimethylglycine. Vials were shaken continuously and maintained at 10°C for the 60 min incubation period. Immediately prior to termination, hyamine hydroxide ($150 \mu\text{l}$) was injected through the cap into the well to collect CO_2. The reaction was terminated by the injection of $150 \mu\text{l}$ of 35 % perchloric acid through the cap into the suspension. After a further 90 min of shaking to collect CO_2, the filter discs were removed and counted directly in 5 ml of a scintillation cocktail consisting of 400 ml toluene, 100 ml ethanol (95 %), $1 \cdot 0 \text{ g}$ PPO and $0 \cdot 05 \text{ g}$ POPOP. Background radioactivity was estimated by adding perchloric acid to the mitochondrial incubation prior to the addition of the [^{14}C]amino acid. Radioactivity was at background level in assays performed with only mitochondria omitted. All assays were performed in duplicate.

Protein determination, chemicals and statistics

The protein concentration of skate mitochondrial suspensions was measured using a modified biuret procedure (Gornall, Bardawill & David, 1949), adding 10 % deoxycholate (pH 13) to solubilize mitochondrial protein. BSA was used as the standard. A biuret assay was also done on the medium used to resuspend the mitochondrial pellet, and this value was subtracted from that of the mitochondrial suspension to determine mitochondrial protein. All biochemicals and hexokinase were from Sigma Chemical Co. (St Louis, Mo); other chemicals and scintillation
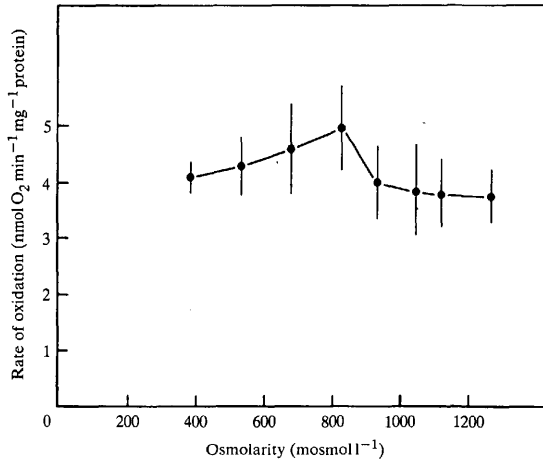
Fig. 1. Oxidation of $10 \, mmol \, l^{-1}$ β-alanine (with $0.1 \, mmol \, l^{-1}$ malate) by *Raja erinacea* liver mitochondria at 10°C in relation to assay medium osmolarity. Results are expressed as mean ± S.E. for five determinations. The KCl concentration was kept constant ($140 \, mmol \, l^{-1}$) and osmolarity was varied as described in Materials and Methods.

cocktails were from Fisher Scientific Co., Canada, and radioisotopes and hyamine hydroxide were from New England Nuclear Canada Ltd (Lachine, Quebec). Statistical comparisons were made using a paired Student's t-test.

RESULTS

The effects of osmolarity on the rate of oxidation of BALA are presented in Fig. 1. With osmolarity varied in the standard manner (urea, TMAO and sucrose varied but kept in constant ratio 8:4:1, respectively, with KCl kept constant), the oxidation of BALA was found to be insensitive to osmolarity over the range of 380–1270 $mosmol \, l^{-1}$ ($P > 0.05$).

Oxidation of sarcosine by isolated mitochondria was inversely related to assay medium osmolarity (Fig. 2A). Decreasing assay medium osmolarity from isosmotic ($920 \, mosmol \, l^{-1}$) to $540 \, mosmol \, l^{-1}$ resulted in a twofold stimulation of oxidation ($P < 0.001$). Increasing osmolarity from isosmotic to $1270 \, mosmol \, l^{-1}$ reduced oxidation to below detectable levels ($P < 0.05$). The effect of osmolarity on sarcosine oxidation in the presence of rotenone is presented in Fig. 2B. Sarcosine oxidation proceeds through FAD-linked sarcosine oxidase, but catabolism of the resulting glycine in the NAD-linked glycine cleavage system would be inhibited by rotenone. Although sarcosine oxidation was reduced by rotenone at each osmolarity, the sensitivity to osmolarity was the same as in uninhibited mitochondria. A twofold stimulation in rotenone-insensitive sarcosine oxidation occurred in response to a

40% reduction in assay osmolarity ($P < 0.05$). Increasing osmolarity by 40% resulted in an apparent, but not statistically significant ($P > 0.05$), reduction in sarcosine oxidation.

An experiment was done to determine whether the osmotic effect on sarcosine oxidation was due to a direct effect of urea or TMAO. Control assays were performed using a medium containing $100 \, \text{mmol} \, l^{-1}$ urea, $50 \, \text{mmol} \, l^{-1}$ TMAO, $12.5 \, \text{mmol} \, l^{-1}$ sucrose, $140 \, \text{mmol} \, l^{-1}$ KCl and the standard concentrations of other reaction medium components. The effects of TMAO and urea on sarcosine oxidation were examined using media with either TMAO or both TMAO and urea omitted, and the sucrose concentration elevated to maintain osmolarity constant at $540 \, \text{mosmol} \, l^{-1}$ (Table 1). When only TMAO was replaced with sucrose, sarcosine oxidation was reduced to 85% of the control rate ($P < 0.02$). When both TMAO and urea were replaced with sucrose, sarcosine oxidation was reduced to 47% of the control ($P < 0.005$).

The effect of osmolarity on the oxidation of dimethylglycine is presented in Fig. 3. Assay medium dilution resulted in a stimulation of dimethylglycine oxidation, as
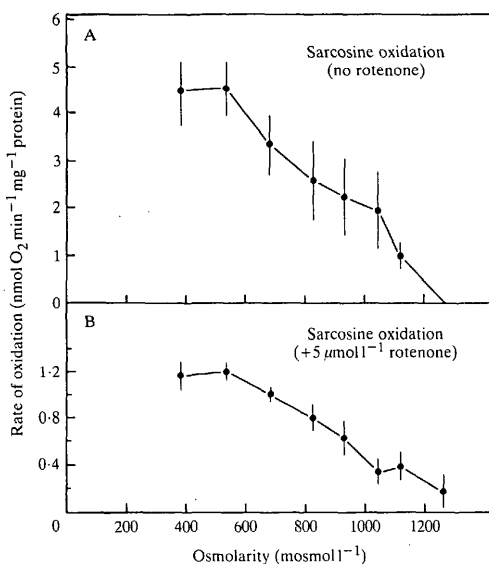

Fig. 2. Oxidation of $10 \, \text{mmol} \, l^{-1}$ sarcosine by *Raja erinacea* liver mitochondria at 10°C in relation to assay medium osmolarity, in the presence and absence of rotenone. (A) Sarcosine oxidation in the absence of rotenone. Mean ± S.E. for six determinations. These data will appear elsewhere (Ballantyne, Moyes & Moon, 1986). (B) Sarcosine oxidation in the presence of $5 \, \mu\text{mol} \, l^{-1}$ rotenone. Mean ± S.E. for five determinations.

Table 1. *Sarcosine oxidation by skate liver mitochondria in equiosmolar media of variable tonicity*

TMAO (mmol l⁻¹)	Urea (mmol l⁻¹)	Experimental rate / Control rate	P-value
50	100	1	
0	100	0.85 ± 0.04	<0.02
0	0	0.47 ± 0.05	<0.005

Mean $\pm$ s.e. for five separate preparations in media with sucrose elevated in each case to maintain osmolarity constant at 540 mosmol l⁻¹.
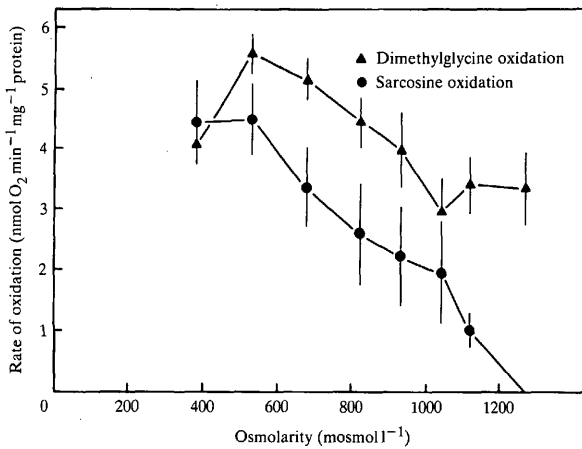TMAO, trimethylamine oxide.

Fig. 3. Oxidation of 2·5 mmol l⁻¹ dimethylglycine by *Raja erinacea* liver mitochondria at 10°C in relation to assay medium osmolarity. Mean $\pm$ s.e. for six determinations. Sarcosine oxidase from Fig. 2A is included for reference.

with sarcosine. Unlike the situation with sarcosine, oxidation of dimethylglycine was not affected by increasing assay medium osmolarity above isosmotic ($P > 0.05$).

The effects of osmolarity on the oxidation of serine and glycine were examined in an attempt to establish the component of the sarcosine oxidative pathway that is sensitive to the osmolarity changes. Oxidation of serine is not inhibited by malonate (Moyes *et al.* 1986) but, in the present study, $^{14}CO_2$ production from [^{14}C]serine was completely inhibited by sodium arsenite ($N = 3$; data not shown), demonstrating that serine catabolism in these mitochondria occurs through the glycine cleavage system, not the Krebs cycle. Serine oxidation was slightly stimulated when osmolarity was reduced below isosmotic and slightly decreased with increased osmolarity

(Fig. 4). An equivalent reduction in assay osmolarity (40%) increased sarcosine oxidation by 100% (Fig. 2A) and serine oxidation by only 35% ($P < 0.02$) (Fig. 4). Increasing osmolarity by 40% resulted in a 20% reduction in serine oxidation ($P < 0.05$) (Fig. 4), whereas sarcosine oxidation was reduced to below detectable limits (Fig. 2A).

Arsenite completely inhibited production of $^{14}CO_2$ from [^{14}C]glycine ($N = 3$; data not shown), confirming that glycine oxidation occurs through the glycine cleavage system. Production of $^{14}CO_2$ from [$^{14}C(U)$]glycine was stimulated at low osmolarities (Fig. 5). As was the case with sarcosine, a twofold increase in oxidation of glycine occurred in response to a reduction of osmolarity from isosmotic to 540 mosmol l^{-1} ($P < 0.05$). A decrease in the rate of glycine oxidation was observed with hyperosmotic media (Fig. 5), although the relative decrease does not appear to be as pronounced as with sarcosine (Fig. 2). Oxidation of sarcosine decreased by 100% in response to a 40% increase in assay osmolarity, but glycine oxidation decreased by only 50% ($P < 0.02$). The response to media of variable tonicity but constant osmolarity is summarized in Table 2. As was the case with sarcosine, the maximum rate of oxidation of glycine occurred in the standard media containing urea and TMAO. Replacement of both urea and TMAO with sucrose caused marked reductions in the rate of glycine oxidation, particularly at the highest osmolarity tested.

The relative rates of $^{14}CO_2$ production from [$^{14}C(1)$]- and [$^{14}C(2)$]glycine (Table 3) reveal the existence of an active pathway for production of CO_2 from methylenetetrahydrofolate in these mitochondria. Production of CO_2 from the C(2) position of the glycine molecule is 75–80% of that from the C(1) position and this ratio was not affected by osmolarity ($P > 0.05$). If it is assumed that the fate of methylenetetrahydrofolate is the same regardless of whether it is generated in the
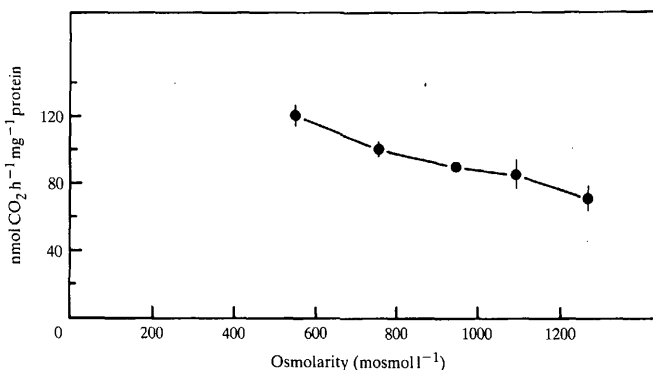

Fig. 4. Oxidation of 10 mmol l^{-1} serine by *Raja erinacea* liver mitochondria at 10°C (0.002 μCi [$^{14}C(U)$]serine μmol^{-1}) in relation to assay medium osmolarity. Mean ± S.E. for five determinations.

Fig. 5. Oxidation of $5 \, \text{mmol} \, l^{-1}$ glycine by *Raja erinacea* liver mitochondria at 10°C ($0·01 \, \mu\text{Ci} \, [^{14}\text{C(U)}]$glycine μmol^{-1}) in relation to assay medium osmolarity. Mean ± S.E. for five determinations.
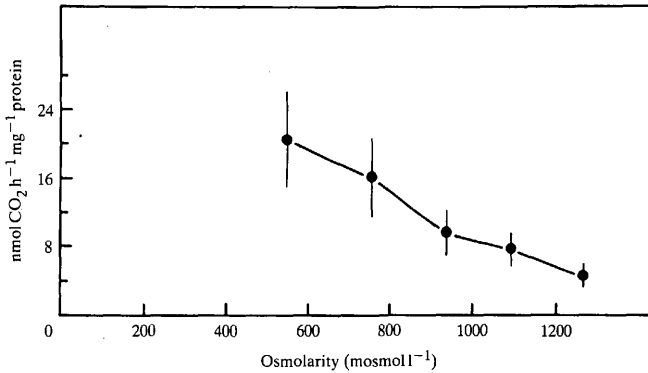
Table 2. *Glycine oxidation in skate liver mitochondria in equiosmolar media of variable tonicity*

TMAO ($\text{mmol} \, l^{-1}$)	Urea ($\text{mmol} \, l^{-1}$)	Osmolarity ($\text{mosmol} \, l^{-1}$)	Experimental rate ——————— Control rate	P-value
0	100	540	0·76 ± 0·09	<0·05
0	0	540	0·53 ± 0·09	<0·005
0	370	920	0·28 ± 0·05	<0·001
0	0	920	0·05 ± 0·01	<0·001
0	600	1260	0·14 ± 0·02	<0·001
0	0	1260	0·10 ± 0·02	<0·001

Mean ± S.E. for three separate preparations in media with sucrose elevated to maintain osmolarity.
Control rate is the osmolarity-specific rate, with 8:4:1 urea:TMAO:sucrose.
TMAO, trimethylamine oxide.

Table 3. $^{14}CO_2$ *production by skate mitochondria given* $[^{14}C(1)]$- *and* $[^{14}C(2)]$gly-cine *at three osmolarities*

Osmolarity ($\text{mosmol} \, l^{-1}$)	Urea ($\text{mmol} \, l^{-1}$)	$\dfrac{[^{14}\text{C(2)}]\text{CO}_2}{[^{14}\text{C(1)}]\text{CO}_2}$
380	100	0·77 ± 0·07
940	370	0·80 ± 0·06
1270	600	0·75 ± 0·02

Mean ± S.E. for three separate preparations at $920 \, \text{mosmol} \, l^{-1}$ in the standard medium.
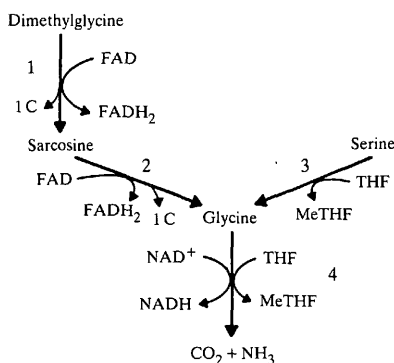Values are not significantly different at $P > 0·05$.

Fig. 6. Oxidative pathway proposed for sarcosine, dimethylglycine, serine and glycine in skate liver mitochondria. 1, dimethylglycine oxidase; 2, sarcosine oxidase; 3, serine hydroxymethyltransferase; 4, glycine cleavage system; THF, tetrahydrofolate; MeTHF, methylenetetrahydrofolate.

glycine cleavage system or through serine hydroxymethyltransferase (Fig. 6), any effects of osmolarity on serine oxidation can be assumed to be effects on these enzymes, rather than on methylenetetrahydrofolate metabolism.

The results of the competition studies are summarized in Table 4. Oxidation of $1 \, mmol \, l^{-1}$ glycine was not significantly affected ($P > 0.05$) by $5 \, mmol \, l^{-1}$ L-alanine, $5 \, mmol \, l^{-1}$ BALA or $5 \, mmol \, l^{-1}$ dimethylglycine (with $20 \, mmol \, l^{-1}$ acetate). Addition of $5 \, mmol \, l^{-1}$ sarcosine in the presence of $20 \, mmol \, l^{-1}$ acetate caused a slight decrease in the oxidation of $1 \, mmol \, l^{-1}$ glycine ($P < 0.05$).

DISCUSSION

The rate of oxidation of sarcosine by liver mitochondria was found to be inversely related to assay medium osmolarity (Fig. 2A). Reduction of osmolarity enhanced the

Table 4. *Glycine oxidation by skate liver mitochondria as affected by other amino acids*

Conditions	Experimental rate / Control rate	P-value
$+5 \, mmol \, l^{-1}$ alanine	0.97 ± 0.06	NS
$+5 \, mmol \, l^{-1}$ β-alanine	0.99 ± 0.03	NS
$+20 \, mmol \, l^{-1}$ acetate only	1.01 ± 0.04	NS
$+5 \, mmol \, l^{-1}$ DMG $+ 20 \, mmol \, l^{-1}$ acetate	0.92 ± 0.05	NS
$+5 \, mmol \, l^{-1}$ sarcosine $+ 20 \, mmol \, l^{-1}$ acetate	0.94 ± 0.02	<0.05

Mean $\pm$ S.E. for four separate determinations in standard media at $920 \, mosmol \, l^{-1}$.
Control rate for dimethylglycine (DMG) and sarcosine determined with $20 \, mmol \, l^{-1}$ acetate.
NS, not significant ($P > 0.05$).

rate of oxidation; however, as this involved proportionate reductions in the concentrations of urea, TMAO and sucrose, the nature of the effector was not clear. As urea and TMAO can affect enzyme function directly (Yancey *et al.* 1982), the possibility was examined that the effect of osmolarity on mitochondrial sarcosine oxidation was a direct effect of urea or TMAO. When the TMAO of a hyposmotic medium was replaced with sucrose, without altering osmolarity, a reduction in sarcosine oxidation was observed (Table 1). Clearly, the osmotic response of these mitochondria in the standard medium (Fig. 2A) is not due to the change in TMAO concentration. As TMAO has been reported to counteract the deleterious effects of urea on biochemical systems (Yancey *et al.* 1982), this experiment could be taken as evidence for a urea-sensitive pathway. However, when both urea and TMAO were replaced with sucrose, a much more pronounced reduction in oxidation was observed (Table 2). If urea exerts a direct inhibitory effect on sarcosine oxidation, its omission from the assay medium should have stimulated oxidation.

Instead of a direct effect of urea or TMAO, it is apparent that the mitochondrial response to reduced assay medium osmolarity is dependent on the permeability of the solutes involved. Urea is freely permeable to skate mitochondria (Ballantyne & Moon, 1986), whereas sucrose is thought to be impermeable. It has been suggested that rat mitochondria become increasingly permeable to sucrose with centrifugation (Sambasivarao & Sitaramam, 1983). However, skate liver mitochondrial matrix volume is constant for at least 40 min when [^{14}C]sucrose is used to determine the volume of the extramitochondrial space (J. S. Ballantyne, unpublished data), suggesting low sucrose permeability under these incubation conditions. Although the media used in the present study were of equal osmolarities, it may be inferred that they were of different tonicities. Incubation of mitochondria in a hyposmotic medium where the major solute is permeable (e.g. urea) causes a different degree of swelling from incubation in media with less permeable solutes (e.g. sucrose) (Tedeschi & Harris, 1955). The permeability of TMAO to the mitochondrial membrane has not been established. Ballantyne & Moon (1986) have suggested, on the basis of osmotic effects on glutamate oxidation, that TMAO is slowly permeable or impermeable to skate liver mitochondria. However, the oxidation of both sarcosine (Table 1) and glycine (Table 2) in the present study was reduced when assay medium TMAO was replaced with sucrose, which suggests that TMAO is permeable to the mitochondrial membrane. If TMAO was impermeable and was replaced with sucrose (another impermeable solute), no effect on oxidation would have been expected. The reasons for the difference between the results of the present study and those of Ballantyne & Moon (1986) are not immediately clear.

Atsmon & Davis (1967), Joseph *et al.* (1981) and Kovacevic *et al.* (1980) suggested that osmotically induced changes in the mitochondrial membrane were responsible for the changes in metabolite oxidation in rat liver mitochondria incubated in anisotonic media. Membrane changes could arise directly, through swelling, or indirectly, by the dilution of intramitochondrial Mg^{2+} (Joseph *et al.* 1981). The kinetics of rat liver glutaminase are particularly sensitive to the nature of the lipid environment (McGivan & Bradford, 1983). It is possible that the effect of

mitochondrial volume changes on sarcosine oxidation in skates are also due to membrane modifications. Changes in the intramitochondrial concentration of some unknown enzyme regulator, however, cannot be eliminated.

While it is likely that membrane alterations are responsible for the effects of osmolarity on the oxidation of various metabolites in mitochondria (see Introduction), the osmotically sensitive component of the oxidative pathways may be dependent on the nature of the substrate. In the present study, the osmotically sensitive component in the mitochondrial oxidation of sarcosine is clearly not some aspect of oxidative phosphorylation, as suggested by Campbell *et al.* (1975) for rat mitochondria, or the transporters for phosphate or ADP. Perturbation of any of these processes would have affected the mitochondrial oxidation of BALA (Fig. 1) as well as sarcosine (Fig. 2A). Medium tonicity must, therefore, affect either transport of sarcosine into the mitochondria or the catabolic enzymes, sarcosine oxidase and the glycine cleavage system. The enzymes sarcosine oxidase (Hoskins & MacKenzie, 1961) and the glycine cleavage system (Hampson *et al.* 1983), and the sarcosine transporter are each associated with the inner mitochondrial membrane and could be sensitive to changes in membrane configuration.

The effects of osmolarity on sarcosine oxidation in the presence and absence of rotenone (Fig. 2) suggest that the glycine cleavage system is not involved in producing the effects on sarcosine oxidation. Sarcosine oxidation in the presence of rotenone proceeds through sarcosine oxidase; however, the oxidation of the resultant glycine by the glycine cleavage system is inhibited. Although the absolute rates of oxygen consumption are lower than in the uninhibited mitochondria, the sensitivity to medium tonicity remains (Fig. 2B). The effect of osmolarity on the oxidation of serine (Fig. 4) is further evidence that the glycine cleavage system is not the osmotically sensitive component of the sarcosine pathway. Exogenous serine and sarcosine both generate glycine intramitochondrially (Fig. 6), and under isosmotic conditions these amino acids are oxidized at similar rates (Moyes *et al.* 1986). If flux through the glycine cleavage system limits oxidation of sarcosine, an effect of similar magnitude would be expected with serine as the substrate. A slight stimulation of serine oxidation at low osmolarities did occur; it was not, however, as pronounced as with sarcosine oxidation.

Although the rates of both serine oxidation and rotenone-insensitive sarcosine oxidation suggest that osmolarity does not affect the glycine cleavage system, the oxidation of glycine was strongly affected by osmolarity. Based on the response to media of constant osmolarity, but of variable tonicity (Table 3), oxidation of glycine is dependent on medium tonicity, as is the oxidation of sarcosine. As both serine and glycine are catabolized through the glycine cleavage system in skate mitochondria, the relative rates of oxidation (compare Fig. 4 with 5) suggest that the transport of glycine is rate-limiting to its oxidation (Moyes *et al.* 1986). Metabolite oxidation reflects the rate of transport into the mitochondria if it can be shown that transport is the rate-limiting step in oxidation (LaNoue & Schoolwerth, 1979). As this is clearly the situation with mitochondrial glycine oxidation, osmotic effects on oxidation reflect changes in the rate of glycine transport into the mitochondria. The increased

oxidation of glycine at low osmolarities indicates a stimulation of the mitochondrial transporter, while at high osmolarities a reduction in the rate of transport occurs. A similar mechanism was proposed to account for the osmotic effects on metabolite oxidation in rat liver mitochondria oxidizing succinate (Atsmon & Davis, 1967) and bivalve heart mitochondria oxidizing proline (Ballantyne & Storey, 1985). Ellis *et al.* (1985) suggested that the hyposmotic stimulation of glycine oxidation by bivalve mollusc mitochondria is due to regulatory effects on the glycine cleavage system. In view of the effects of tonicity on the skate liver mitochondrial glycine transporter, it is possible that the effects on bivalve mollusc mitochondria (Ellis *et al.* 1985) are also due to changes in the rate of glycine transport, which were not examined in their study.

The effects on the oxidation of glycine demonstrate that mitochondrial volume changes induced by anisotonic media are capable of stimulating or inhibiting the transport of at least glycine. Glycine, L-serine and several other amino acids are thought to be transported on a single neutral amino acid transporter in rat liver mitochondria (Cybulski & Fisher, 1977). The mitochondrial transporter for sarcosine has not been established. Cybulski & Fisher (1977) found that sarcosine was unable to protect against *p*-mercurobenzoate inactivation of the transporter for glycine, L-serine and other neutral amino acids, suggesting that sarcosine does not share the glycine transporter in rat liver mitochondria. It was proposed that methylation of the primary amine group of sarcosine prevented its binding to the carrier. As with sarcosine, both BALA and proline were unable to prevent *p*-mercurobenzoate inactivation of the neutral amino acid transporter, suggesting different transporters. In the reciprocal experiment, neutral amino acids did protect against *p*-mercurobenzoate inactivation of BALA and proline transport, implying that these amino acids are transported on the neutral amino acid carrier. As the reciprocal experiment was not done with sarcosine, it is possible that sarcosine is, in fact, transported on the same carrier as glycine in rat liver. In skate liver mitochondria, the lack of substantial inhibition of glycine transport by a fivefold greater concentration of sarcosine or dimethylglycine (with sarcosine oxidase-mediated conversion to glycine inhibited by $20\,\text{mmol}\,\text{l}^{-1}$ acetate) as well as BALA and L-alanine (Table 4) suggest that these amino acids do not share the glycine transporter. While it is clear that the effects on the glycine transporter are not responsible for the effects on mitochondrial sarcosine oxidation, it is possible that the transporter for sarcosine responds similarly to anisotonic media.

Dimethylglycine is converted to sarcosine in rat mitochondria by the enzyme dimethylglycine oxidase (Fig. 6) (Frisell & MacKenzie, 1962). It has been suggested that dimethylglycine oxidase and sarcosine oxidase of rat liver mitochondria are the same protein (Sato, Ohishi & Yagi, 1979). If these enzymes exist as a single protein in skate liver mitochondria, differences in the osmotic response to dimethylglycine and sarcosine, particularly at high osmolarities (Fig. 3), suggest that tonicity affects sarcosine transport. Transport is the only component of the sarcosine oxidative pathway not shared with dimethylglycine. However, Frisell & MacKenzie (1962) have reported that dimethylglycine oxidase and sarcosine oxidase of rat liver can be

separated chromatographically. Different degrees of inhibition of the purified enzyme of methoxyacetate and acetate (Frisell & MacKenzie, 1955) support the existence of separate proteins. Until the nature of these enzymes in skate liver mitochondria is established, the significance of the differences between the effects of osmolarity on sarcosine and dimethylglycine oxidation will remain uncertain.

Osmotic effects on the rate of sarcosine oxidation by skate liver mitochondria are probably due to volume changes (i.e. medium tonicity) rather than a direct effect of urea or TMAO. The membrane-bound glycine cleavage system is not particularly sensitive to osmolarity in this tissue. The glycine transporter of skate liver mitochondria appears to be sensitive to mitochondrial volume changes. Although sarcosine apparently does not share the glycine transporter, similar effects on the sarcosine transporter may be responsible for the osmotic sensitivity of sarcosine oxidation. Effects of tonicity on sarcosine oxidase cannot be eliminated at this point.

REFERENCES

ATSMON, A. & DAVIS, R. P. (1967). Mitochondrial respiration under conditions of varying osmolarity. *Biochim. biophys. Acta* **131**, 221–233.

BALLANTYNE, J. S. & MOON, T. W. (1985). Hepatopancreas mitochondria from *Mytilus edulis*: substrate preferences and effects of pH and osmolarity. *Mar. Biol.* **87**, 239–244.

BALLANTYNE, J. S. & MOON, T. W. (1986). Solute effects on mitochondria from an elasmobranch (*Raja erinacea*) and a teleost (*Pseudopleuronectes americanus*). *J. exp. Zool.* (in press).

BALLANTYNE, J. S., MOYES, C. D. & MOON, T. W. (1986). Osmolarity affects oxidation of organic solutes by isolated hepatocytes and mitochondria from a euryhaline elasmobranch. *J. exp. Zool.* (in press).

BALLANTYNE, J. S. & STOREY, K. B. (1983). Mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*: substrate preferences and effects of pH and salt on proline oxidation. *Comp. Biochem. Physiol.* **76B**, 133–138.

BALLANTYNE, J. S. & STOREY, K. B. (1984). Mitochondria from the hepatopancreas of the marine clam, *Mercenaria mercenaria*: substrate preferences and salt and pH effects on the oxidation of palmitoyl-L-carnitine and succinate. *J. exp. Zool.* **230**, 165–174.

BALLANTYNE, J. S. & STOREY, K. B. (1985). Solute effects on mitochondrial respiration: the kinetics of proline oxidation by mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*. *Comp. Biochem. Physiol.* **81B**, 777–780.

BOYD, T. A., CHA, C.-J., FORSTER, R. P. & GOLDSTEIN, L. (1977). Free amino acids in tissues of the skate *Raja erinacea* and the stingray *Dasyatis sabine*: effects of environmental dilution. *J. exp. Zool.* **199**, 435–442.

CAMPBELL, L. C., RAISON, J. K. & BRADY, C. J. (1975). Factors limiting mitochondrial respiration in media of high solute content. *J. Bioenerg.* **7**, 189–200.

CHANCE, G. & WILLIAMS, G. R. (1956). The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **17**, 65–134.

CYBULSKI, R. L. & FISHER, R. R. (1977). Mitochondrial neutral amino acid transport: evidence for a carrier mediated mechanism. *Biochemistry, N.Y.* **16**, 5116–5120.

ELLIS, L. L., BURCHAM, J. M., PAYNTER, K. T. & BISHOP, S. H. (1985). Amino acid metabolism in euryhaline bivalves; regulation of glycine accumulation in ribbed mussel gills. *J. exp. Zool.* **233**, 347–358.

FRISELL, W. R. & MACKENZIE, C. G. (1955). The binding sites of sarcosine oxidase. *J. biol. Chem.* **217**, 275–285.

FRISELL, W. R. & MACKENZIE, C. G. (1962). Separation and purification of sarcosine dehydrogenase and dimethylglycine dehydrogenase. *J. biol. Chem.* **237**, 94–98.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751–766.

HAMPSON, R. K., BARRON, L. L. & OLSON, M. S. (1983). Regulation of the glycine cleavage system in isolated rat liver mitochondria. *J. biol. Chem.* **258**, 2993–2999.

HOLTZMAN, D., LEWISTON, N., HERMAN, M. M., DESAUTEL, M., BREWER, E. & ROBIN, E. (1978). Effects of osmolar changes on isolated mitochondria of brain and liver. *J. Neurochem.* **30**, 1409–1419.

HOSKINS, D. D. & MACKENZIE, C. G. (1961). Solubilization and electron transfer flavoprotein requirement of mitochondrial sarcosine dehydrogenase and dimethylglycine dehydrogenase. *J. biol. Chem.* **236**, 177–183.

JOHNSON, D. & LARDY, H. (1958). Substrate-selective inhibition of mitochondrial oxidations by enhanced tonicity. *Nature, Lond.* **181**, 701–702.

JOSEPH, S. K., MCGIVAN, J. D. & MEIJER, A. J. (1981). The stimulation of glutamine hydrolysis in isolated rat liver mitochondria by Mg^{2+} depletion and hypo-osmotic incubation conditions. *Biochem. J.* **194**, 35–41.

KOVACEVIC, Z., BAJIN, K. & PAVLOVIC, M. (1980). Volume changes of rat kidney mitochondria, transport of glutamine and its inhibition by mersalyl. *Int. J. Biochem.* **12**, 139–143.

LANOUE, K. F. & SCHOOLWERTH, A. C. (1979). Metabolite transport in mitochondria. *A. Rev. Biochem.* **48**, 871–922.

LEWISTON, N., NEWMAN, A., ROBIN, E. & HOLTZMAN, D. (1979). Shark heart mitochondria: effects of external osmolarity on respiration. *Science* **206**, 75–76.

MCEACHRAN, J. D. & MUSICK, J. A. (1973). Characters for distinguishing between immature specimens of the sibling species, *Raja erinacea* and *Raja ocellata* (Pisces: Rajidae). *Copeia* **1973**, 238–250.

MCGIVAN, J. D. & BRADFORD, N. M. (1983). Influence of phospholipids on the activity of phosphate-dependent glutaminase in extracts of rat liver mitochondria. *Biochem. J.* **214**, 649–652.

MOYES, C. D., BALLANTYNE, J. S. & MOON, T. W. (1986). Oxidation of amino acids, Krebs cycle intermediates, fatty acids and ketone bodies by *Raja erinacea* liver mitochondria. *J. exp. Zool.* **273**, 119–128.

SAMBASIVARAO, V. & SITARAMAN, M. K. J. (1983). Studies on the non-linear osmotic pressure–volume relationships in mitochondria and entry of sucrose into the matrix space during centrifugation. *Biochim. biophys. Acta* **722**, 256–270.

SATO, M., OHISHI, N. & YAGI, K. (1979). Identification of a covalently bound flavoprotein in rat liver mitochondria with sarcosine dehydrogenase. *Biochem. biophys. Res. Commun.* **87**, 706–711.

SLATER, E. C. & CLELAND, K. W. (1953). The effect of tonicity of the medium on the respiratory and phosphorylative activity of heart-muscle sarcosomes. *Biochem. J.* **53**, 557–567.

TEDESCHI, H. & HARRIS, D. L. (1955). The osmotic behavior and permeability to non-electrolytes of mitochondria. *Archs Biochem. Biophys.* **58**, 52–67.

WITTER, R. F., WATSON, M. L. & COTTONE, M. A. (1955). Morphology and ATP-ase activity of isolated mitochondria. *J. biophys. biochem. Cytol.* **1**, 127–138.

YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMERO, G. N. (1982). Living with water stress: evolution of osmolyte system. *Science* **217**, 1214–1222.