Sulphate transport by H^+ symport and by the dicarboxylate carrier in mitochondria

Nils-Erik L. SARIS

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, SF-00170 Helsinki 17, Finland

(Received 3 July 1980/Accepted 16 July 1980)

1. Swelling of mitochondria was induced in non-respiring mitochondria by 30 mM or more Na_2SO_4 or by respiration in the presence of K_2SO_4 . Respiration-driven swelling resulted in loss of respiratory control. Sulphate, when present at 10 mM concentration, promoted the release of accumulated Ca^{2+} . 2. Swelling was prevented by *N*-ethylmaleimide and formaldehyde, known inhibitors of the phosphate carrier. Sulphate-induced swelling was more sensitive to the inhibitors than was phosphate-induced swelling. At lower concentrations of sulphate, 5 mM, an alkalinisation of the medium was observed on addition of sulphate, indicating H^+ -sulphate symport. There was competition between sulphate and phosphate for transport by this mechanism. It is suggested that sulphate may be transported, though at a comparatively slow rate, by the phosphate carrier. 3. Uptake of sulphate was stimulated when preceded by energy-dependent accumulation of Ba^{2+} , especially when acetate was also present, indicating precipitation of $BaSO_4$ in the matrix. Using this system the influx of sulphate was studied at lower concentrations, 10 mM or less. The contributions of the H^+ symporter (sensitive to *N*-ethylmaleimide) and the dicarboxylate carrier (sensitive to butylmalonate) could then be studied. The dicarboxylate carrier had a lower K_m and was mainly responsible for sulphate transport at the lower concentration range. At 10 mM-sulphate the transport rates by the two systems appeared to be similar; at still higher concentrations the H^+ symporter may become more important.

Accumulation of sulphate by rat kidney and liver mitochondria was reported by Winters *et al.* (1962) and Rasmussen *et al.* (1964). Chappell & Haarhoff (1967) regarded sulphate as a non-penetrant, since mitochondria did not swell when suspended in iso-osmotic $(NH_4)_2SO_4$ solution; however, no actual data were presented. On the other hand, Mitchell & Moyle (1969) observed some, albeit slight, stimulation of swelling in iso-osmotic K_2SO_4 solution when nigericin or valinomycin plus an uncoupling agent was added. They suggested that sulphate penetrates mitochondrial membranes by an electroneutral mechanism involving H^+ symport (or OH^- antiport) in analogy with phosphate translocation on the phosphate carrier. More recently, Crompton *et al.* (1974) showed that, in non-respiring mitochondria, phosphate at low concentration is translocated on the dicarboxylate carrier.

The present paper reports experiments that show relatively fast swelling of non-respiring mito-

chondria in media containing sulphate as the Na^+ salt. Swelling was also markedly stimulated by respiration, even when K^+ was the cation present. Swelling was sensitive to inhibition by *N*-ethylmaleimide and formaldehyde, indicating sulphate transport by the phosphate carrier or by an essentially similar mechanism. The former appears likely, since there was competition between sulphate and phosphate for transport in mitochondria whose dicarboxylate carrier was blocked. During the course of these studies it was found that the relatively high concentrations of sulphate used in swelling experiments change the permeabilities of mitochondria and cause uncoupling. Transport of sulphate at low concentrations was therefore optimized by trapping transported sulphate as a $BaSO_4$ precipitate.

Experimental

Rat liver mitochondria were prepared by a conventional differential centrifugation method as

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid.

described elsewhere (Wikström & Saris, 1969). Swelling was measured by monitoring absorbance at 520nm with the Aminco—Chance photometer DW 2. Ca^{2+} concentration changes were followed by using the metal indicator murexide at the wavelength pair 540–507nm, and pH was recorded similarly with Neutral Red at 540–583nm. For the measurement of uptake of sulphate and phosphate, the mitochondria were incubated in the presence of 0.1–0.3 μCi of $^{35}\text{SO}_4^{2-}$ or $^{32}\text{P}_i/\text{ml}$ (the total concentration was varied according to experimental needs), the suspension was filtered through Millipore filters (pore size $0.45\mu\text{m}$), the filter was rapidly washed with ice-cold 250mM-sucrose and dried, dissolved in a portion of Aqualuma scintillation fluid (Lumac Systems A.G., Basel, Switzerland) and the radioactivity was measured. The amount of residual medium was checked with $[^{14}\text{C}]$ sucrose, and the appropriate corrections were made. Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; other reagents were of the purest grade obtainable commercially.

Results and discussion

Swelling of mitochondria suspended in sulphate-containing media

Fig. 1(a) shows swelling of non-respiring mitochondria in the presence of varying concentrations of K_2SO_4 . Swelling was induced by addition of nigericin and was obtained when the concentration of sulphate exceeded 30mM. Thus, the results of Mitchell & Moyle (1969) were confirmed.

On addition of a respiratory substrate, succinate (Fig. 1b), mitochondria began to swell extensively with a rate that was dependent on the concentration of sulphate. However, the rate of swelling was but a fraction of that obtained in the presence of phosphate. An example of this is shown by the discontinuous line in Fig. 1(b). Here 15mM-phosphate was present in addition to the same concentration of sulphate (curve 2). The rate of swelling in the presence of phosphate was initially very high, but strikingly the swelling was retarded after some time, in contrast with the swelling in the presence of sulphate alone. The swelling in the presence of phosphate was therefore probably due to entry of osmotically active phosphate salts, and uptake of sulphate was either inhibited or its swelling-promoting effects were prevented.

Swelling of non-respiring mitochondria in media containing Na_2SO_4 (Fig. 2b) was faster and more extensive than in the presence of K_2SO_4 plus nigericin (Fig. 2c). The rate of swelling was further increased by the addition of an uncoupling agent (results not shown) and was strongly inhibited by

Fig. 1. Swelling of mitochondria in sulphate-containing media

Mitochondria (0.75 mg of protein/ml) were suspended in media that contained 10mM-Hepes, 1mM-EGTA, 8 μM -rotenone and 1 μg of oligomycin/ml, pH 7.2, adjusted at room temperature. In addition K_2SO_4 and/or KCl was present in varying proportions ($[\text{K}^+]$ was kept at 150mM) so that the concentration of sulphate was in curve 1, 0; 2, 15mM; 3, 30mM; 4, 45mM; 5, 60mM; 6, 90mM. Swelling is indicated by a downward deflection. (a) Non-respiring mitochondria, nigericin (Nig; 0.4 $\mu\text{g}/\text{ml}$) was added at the arrow; (b) respiring mitochondria, succinate (Suc; 2mM) was added at the arrow.

Fig. 2. Swelling of mitochondria in media containing Na^+ or K^+

Experimental conditions were those used in Fig. 1(a). (a) 150mM-NaCl present; (b) 30mM-NaCl and 60mM- Na_2SO_4 present; (c) 30mM-KCl and 60mM- K_2SO_4 present. For the discontinuous curves 1mM-N-ethylmaleimide was present.

N-ethylmaleimide (discontinuous curves). The residual swelling obtained in the presence of the inhibitor was probably due to stimulated uptake of chloride (cf. Fig. 2a).

Mechanism of swelling in media containing sulphate

The inhibition of swelling by *N*-ethylmaleimide suggests that the phosphate carrier is involved in the translocation of sulphate. *N*-Ethylmaleimide has been shown to inhibit phosphate transport on the phosphate carrier but will not affect its transport on the dicarboxylate carrier (Meijer *et al.*, 1970). Scheme 1 shows two models for sulphate-induced swelling under the experimental conditions employed. In Scheme 1(a) sulphate enters mitochondria on the phosphate carrier (or by an equivalent mechanism) in symport with H^+ as suggested by Mitchell & Moyle (1969). Swelling is promoted when cations are taken up in exchange with H^+ . Mitochondria seem to possess a native Na^+/H^+ antiporter, whereas non-respiring, intact mitochondria do not show any evidence of K^+/H^+ exchange; see the review by Brierley (1978). Addition of nigericin will induce K^+/H^+ exchange. In respiring mitochondria protons are extruded, and the resulting pH gradient would stimulate the uptake of sulphate. Cations would move electrophoretically in response to the membrane potential that is formed on energisation.

In Scheme 1(b) sulphate is translocated on the dicarboxylate carrier as suggested by the experi-

ments of Crompton *et al.* (1974). However, this can account only for equimolar exchanges, not for any net accumulation of anions. Swelling could result from cycling of phosphate by uptake of the anion on the phosphate carrier coupled to efflux on the dicarboxylate carrier in exchange for sulphate. Sulphate-induced swelling would then also be sensitive to inhibition by *N*-ethylmaleimide, and the experiments above would not differentiate between the two schemes.

Sensitivities of phosphate- and sulphate-induced swelling to inhibitors of the phosphate carrier

The phosphate carrier is inhibited not only by *N*-ethylmaleimide but also by formaldehyde (Tyler, 1968, 1969). Table 1 shows that sulphate translocation was more sensitive to the inhibitors than was phosphate-induced swelling. This was especially marked in the case of *N*-ethylmaleimide. The finding that sulphate-induced swelling was the more sensitive one makes it appear unlikely that sulphate uptake is mediated by cycling of phosphate according to Scheme 1(b). Rather, the data suggest that sulphate transport on the phosphate carrier, or by a similar mechanism, is inhibited. The reason for the difference in sensitivity to inhibitors remains obscure. Since phosphate-induced swelling was several-fold faster than sulphate-induced swelling, the former would have been expected to be the more sensitive one.

Uncoupling effects of high concentrations of sulphate

During the course of these studies it was observed that relatively high concentrations of sulphate, above 30 mM, had some uncoupling effects upon mitochondria. Fig. 3 shows that State-2 respiration was stimulated by sulphate and State-3 respiration was inhibited. There was thus a loss of respiratory control. The increasing rate of State-2 respiration with time indicates progressive loss of coupling due to increased ion permeabilities in the presence of sulphate. [See also the increasing rate of swelling in the presence of sulphate (Fig. 1b).] Above 30 mM-sulphate the phenomenon of 'reversed respiratory control' was observed (Fig. 3, curves 3 and 4); addition of ADP lowered the rate of respiration. ADP (or ATP) may thus bring about this effect by decreasing the permeability of the inner membrane to various ions and thereby decreasing the energy drain due to ion transport.

An effect of sulphate upon the retention of accumulated Ca^{2+} can also be demonstrated (Fig. 4). Sulphate at a concentration of 10 mM will cause a release of accumulated Ca^{2+} and will prevent a retention if present at the time of addition of Ca^{2+} . This effect is similar to that of phosphate under corresponding conditions (Saris, 1963). It is evident

Scheme 1. *Sulphate transport in swelling mitochondria* In (a) sulphate is taken up by H^+ symport alone; in (b) it is taken up by exchange with phosphate on the dicarboxylate carrier and re-uptake of phosphate on the phosphate carrier. To swell, mitochondria must extrude H^+ and take up cations. In resting mitochondria Na^+/H^+ exchange accomplishes this, or K^+/H^+ exchange in the presence of nigericin (Nig). In respiring mitochondria H^+ is extruded by H^+ pumps and cations move in response to the membrane potential.

Table 1. *Inhibition by N-ethylmaleimide and formaldehyde of mitochondrial swelling in the presence of phosphate or sulphate*

The medium contained 60mM- Na_2SO_4 or $\text{-NaH}_2\text{PO}_4$, 10mM-Hepes, 6 μM -rotenone, 2 μg of oligomycin/ml, and 1mM-EGTA; the pH was adjusted to 7.2 at 23°C, which was the incubation temperature. Mitochondria were preincubated at 0°C in 250mM-sucrose with the inhibitor for 5 min, and 75 μl of the suspension was added to 2.5 ml of medium in the cuvette. The inhibition was calculated from the maximal rates of swelling. The concentration of mitochondrial protein was 1.2 mg/ml.

Inhibitor	Concentration (mM)	Inhibition of swelling (%) in presence of:	
		Phosphate	Sulphate
<i>N</i> -Ethylmaleimide	0.025	0	35
	0.04	0	62
	0.05	0	78
	0.10	8	94
	0.20	22	—
	0.40	71	—
	1.0	91	—
Formaldehyde	0.045	9	13
	0.10	10	38
	0.24	35	63
	0.45	61	78
	1.0	80	95
	1.7	96	—

Fig. 3. *Effect of sulphate upon respiration of mitochondria*

The medium contained 30mM-Hepes, 4mM-phosphate, 2mM- MgCl_2 , 8 μM -rotenone, 4mM-Tris/succinate, pH 7.2, and 0.8mg of mitochondrial protein/ml. At the arrow approx. 100 μM -ADP was added. In addition the medium contained: in 1, 120mM-KCl; 2, 30mM- K_2SO_4 ; 3, 3.45mM- K_2SO_4 ; 4, 60mM- K_2SO_4 . The osmolality was kept constant in 2–4 with sucrose.

Fig. 4. *Effect of sulphate on the retention of accumulated Ca^{2+}*

Mitochondria (1.5 mg of protein/ml) were suspended in a medium containing, in addition to oligomycin, rotenone and succinate as in Fig. 1(b), 120mM-KCl, 30mM-Hepes and 30 μM -murexide, pH 7.2. At the arrow 250 μM - CaCl_2 was added. (a) Control; (b) 10mM- K_2SO_4 added at the arrow after Ca^{2+} had been taken up; (c) sulphate was present from the beginning.

that sulphate will promote cycling of Ca^{2+} in mitochondria (i.e. loss and reuptake of Ca^{2+}). It may be concluded from the data presented that caution should be exercised in judging data obtained in the presence of relatively high concentrations of

sulphate, above 10mM. The permeabilities of the inner membrane may be substantially changed, making possible the translocation of ions not transported in mitochondria suspended in media of more physiological composition.

Demonstration of H⁺-sulphate symport

The H⁺ accompanying sulphate transport by the symporter should cause a shift of the pH in the more alkaline direction. This was indeed observed (Fig. 5). In the presence of an uncoupling agent (Fig. 5, trace *a*) only a small signal was observed on addition of sulphate; this was due to slight differences in pH between the solutions. Since uncoupling agents increase the permeability of membranes towards H⁺, no gradients of H⁺ can be formed in their presence.

Stimulation of sulphate uptake by Ba²⁺ transport

Mitochondria were allowed to accumulate 50 μM of transportable bivalent cations before the addition of 1 mM-sulphate. Table 2 shows that sulphate uptake was not appreciably influenced by a prior accumulation of Ca²⁺ or Sr²⁺. However, transported Ba²⁺ clearly stimulated the uptake of sulphate, presumably by trapping it as precipitated BaSO₄ in the matrix space. In the presence of acetate the uptake of sulphate was further stimulated, indicating that under these conditions more of the Ba²⁺ was free and available for precipitation. Mitochondria preloaded with Ba²⁺ in the presence of acetate thus constitute a suitable system for the study of the properties of sulphate influx at relatively low concentrations.

Properties of the mitochondrial sulphate-transporting systems

The kinetics of sulphate transport could be

conveniently studied by using Ba²⁺ to precipitate intramatrix sulphate. Mitochondria were allowed to swell by respiration in the presence of Ba²⁺ and acetate. On addition of sulphate, the mitochondria contracted, due to lowering of the osmotic activity of the matrix space (Fig. 6). An analogous system has

Table 2. Effect of transport of bivalent cations upon accumulation of sulphate

Mitochondria, 2.2 mg of protein/ml, were incubated in a medium containing 250 mM-sucrose, 10 mM-Hepes, 2 mM-succinate, 6 μM-rotenone, 2 μg of oligomycin/ml, and, when indicated, 5 mM-potassium, acetate; the pH was adjusted to 6.8. Mitochondria were allowed to accumulate the added metal chloride (50 μM) and 1 mM-K₂³⁵SO₄ was added. Results are expressed as nmol of sulphate taken up/mg of protein.

Cation added	Mitochondrial sulphate uptake after:	
	1 min	3 min
None	1.3	2.9
Ca ²⁺	1.7	2.85
Sr ²⁺	2.0	3.05
Ba ²⁺	8.0	13.6
Ba ²⁺ + acetate	12.0	24.4

Fig. 5. Alkalization of the medium on addition of sulphate

Mitochondria (4.6 mg of protein/ml) were suspended in a medium containing 250 mM-sucrose, 2 mM-Hepes, 8 μM-rotenone, 2 μg of oligomycin/ml, 2 mM-succinate and 70 μM-Neutral Red, pH 7.2. At the arrow 5 mμ-K₂SO₄ was added. A downward deflection indicates alkalization. In (a) 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present.

Fig. 6. Inhibition of sulphate transport by butylmalonate and *N*-ethylmaleimide

Mitochondria (1.5 mg of protein/ml) were suspended in a medium containing 250 mM-sucrose, 5 mM-potassium acetate, 10 mM-Hepes, pH adjusted to 6.5. In addition, rotenone, oligomycin and succinate were present as in Fig. 1(b). BaCl₂ (50 μM) was added and, after the swelling due to Ba²⁺ uptake was complete (trace not shown), 1 mM-K₂SO₄ was added at the arrow. (a) Control; (b) 200 μM-*N*-ethylmaleimide present; (c) 1 mM-butylmalonate present. At the arrow 200 μM-*N*-ethylmaleimide (MalNEt) was added.

been used by Fonyo *et al.* (1974) to study phosphate influx. Influx of sulphate at 1 mM concentration was only slightly inhibited by 200 μ M-*N*-ethylmaleimide (Fig. 6). Butylmalonate, an inhibitor of the dicarboxylate carrier (Chappell & Robinson, 1968, referred to by Palmieri *et al.*, 1971), was a potent inhibitor, and uptake was completely inhibited when both inhibitors were present. These data thus confirm the conclusion of Crompton *et al.* (1974) that sulphate at low concentrations is mainly translocated by the dicarboxylate carrier.

The kinetic properties of the two sulphate-translocating systems may be differentiated by using the two inhibitors *N*-ethylmaleimide (phosphate carrier) and butylmalonate (dicarboxylate carrier). Fig. 7 shows that the affinity of the dicarboxylate carrier for sulphate is substantially higher than that of the *N*-ethylmaleimide-sensitive carrier (the apparent K_m values were 2 and 8 mM respectively). In other experimental conditions Crompton *et al.* (1975) report a K_m of 0.3 mM for sulphate transport on the

Fig. 7. Concentration-dependence of sulphate transport

The experimental conditions were as described in the legend to Fig. 6, but different amounts of sulphate were added after the Ba^{2+} -induced swelling had occurred. Open circles, 200 μ M-*N*-ethylmaleimide was present to inhibit the H^+ symporter; filled circles, 1 mM-butylmalonate was present to inhibit sulphate transport by the dicarboxylate carrier. At the highest concentration of sulphate, 2 mM-butylmalonate did not further increase inhibition, which indicates that 1 mM was sufficient. Succinate was omitted; respiration used 2 mM-ascorbate in the presence of 0.12 mM-tetramethylphenylenediamine.

dicarboxylate carrier. At 10 mM-sulphate the rates of transport by the two systems are similar, and at still higher concentrations transport by the phosphate carrier may exceed that by the dicarboxylate carrier (Fig. 7). The apparently conflicting results of Mitchell & Moyle (1969), which were interpreted as sulphate- H^+ symport, and those of Crompton *et al.* (1974), which seem to exclude the operation of such a mechanism, may thus be resolved. On the other hand, Mitchell & Moyle (1969) have used high concentrations of sulphate that, according to our data, have profound uncoupling effects upon mitochondria. We have been able to confirm sulphate transport by a H^+ symporter by using lower, well tolerated concentrations. It is of interest that swelling of mitochondria in sulphate-containing solutions is strongly stimulated by conditions that tend to make the matrix compartment more alkaline [e.g. respiration, or the presence of Na^+ (Na^+/H^+ exchange)].

Competition between sulphate and phosphate for transport by the H^+ symporter

An indication of competition between the two anions for transport by the H^+ symporter was the ability of phosphate to prevent the excessive swelling of mitochondria in the presence of sulphate (Fig. 1b). An attempt was made to study the competition more specifically by blocking the dicarboxylate carrier with the aid of the competitive inhibitor, butylmalonate. Naturally, only comparatively low concentrations of the anions can be used under these conditions. Table 3 shows that the uptake of sulphate was strongly inhibited by phosphate at the lowest concentration of sulphate employed, 200 μ M. At higher concentrations of sulphate, phosphate was less potent. The inhibition of phosphate transport by sulphate was less marked, as would be expected from the considerable differences in the rate of transport of the two anions. The experimental conditions employed were somewhat different and the rates cannot be compared directly. Since only low substrate and inhibitor concentrations could be used to keep the dicarboxylate carrier inactive, a more detailed analysis was not possible.

These data strengthen the interpretation that sulphate may be transported on the phosphate carrier, since there is mutual inhibition of phosphate and sulphate transport and the inhibition is overcome by increasing the substrate concentration.

Conclusions

Rat liver mitochondria contain two systems able to translocate sulphate. One is the dicarboxylate carrier, which is the main one operating at low sulphate concentrations (1 mM or less) in the presence of exchangeable anions. The other one shares the properties of the phosphate carrier, being

Table 3. *Competition between sulphate and phosphate for transport by the N-ethylmaleimide-sensitive pathway*

The medium was as described in the legend to Fig. 7; the concentration of mitochondrial protein 1.4 mg/ml. Mitochondria were allowed to accumulate bivalent cations to stimulate transport; then 2 mM-butylmalonate was added, followed after 25 s by a mixture of sulphate and phosphate, labelled with ^{35}S or ^{32}P as appropriate. For sulphate transport $50\text{ }\mu\text{M-Ba}^{2+}$ was used and incubation was for 10 s at 23°C ; for phosphate transport $200\text{ }\mu\text{M-Sr}^{2+}$ was used and incubation was for 3 s at 0°C . The times were short enough to give initial rates.

Transported anion	Concentration (mM)	Competing anion	Concentration (mM)	Uptake ($\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$)	Inhibition (%)
Sulphate	0.2	Phosphate	0	0.226	
	0.2		0.2	0.097	58
	0.2		1.0	0.071	68
	0.5		0	0.306	
	0.5		0.2	0.276	10
	0.5		1.0	0.222	27
	2.0		0	1.06	
	2.0		0.2	0.98	8
	2.0		1.0	0.74	30
Phosphate	0.025	Sulphate	0	0.74	
	0.025		1.0	0.62	16
	0.025		3.0	0.55	26
	0.055		0	1.55	
	0.05		1.0	1.05	32
	0.05		3.0	1.03	33
	0.10		0	2.36	
	0.10		1.0	1.96	17
	0.10		3.0	1.98	16

inhibited by *N*-ethylmaleimide and causing H^+ -sulphate symport. It has a higher K_m for sulphate than has the dicarboxylate carrier and may become significant at higher sulphate concentrations and when a pH gradient is formed over the inner membrane. This system may thus be responsible for the swelling of mitochondria suspended in media containing Na_2SO_4 or under respiring conditions.

The study was aided by a grant from the Sigrid Jusélius Foundation, Helsinki. The expert technical assistance of Mrs Kaija Niva is gratefully acknowledged.

References

- Brierley, G. P. (1978) in *The Molecular Biology of Membranes* (Fleischer, S., Hatefi, Y., MacLennan, D. H. & Tzagoloff, A., eds.), pp. 295–308, Plenum Press, New York and London
- Chappell, J. B. & Haarhoff, K. M. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z. & Wojtczak, L., eds.), pp. 75–91, Academic Press, London and New York
- Chappell, J. B. & Robinson, B. H. (1968) in *Metabolic Roles of Citrate* (Goodwin, T. W., ed.), pp. 123–129, Academic Press, New York
- Crompton, M., Palmieri, F., Capano, M. & Quagliariello, E. (1974) *Biochem. J.* **142**, 127–137
- Crompton, M., Palmieri, F., Capano, M. & Quagliariello, E. (1975) *Biochem. J.* **146**, 667–673
- Fonyo, A., Palmieri, F., Ritway, J. & Quagliariello, E. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M., Quagliariello, E. & Siliprandi, N., eds.), pp. 283–286, North-Holland, Amsterdam
- Meijer, A. J., Groot, G. S. P. & Tager, J. M. (1970) *FEBS Lett.* **8**, 41–44
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **9**, 149–153
- Palmieri, F., Prezioso, G., Quagliariello, E. & Klingenberg, M. (1971) *Eur. J. Biochem.* **22**, 66–74
- Rasmussen, H., Sallis, J., Fang, A., DeLuca, H. F. & Young, R. (1964) *Endocrinology* **74**, 338–394
- Saris, N.-E. L. (1963) *Commentat. Phys. Math. Soc. Sci. Fenn.* **28**, 1–59
- Tyler, D. D. (1968) *Biochem. J.* **107**, 121–123
- Tyler, D. D. (1969) *Biochem. J.* **111**, 665–678
- Wikström, M. K. F. & Saris, N.-E. L. (1969) *Eur. J. Biochem.* **9**, 160–166
- Winters, R. W., Delluva, A. M., Deyrup, I. J. & Davies, R. E. (1962) *J. Gen. Physiol.* **45**, 757–775