Biochimica et Biophysica Acta, 975 (1989) 325–329
Elsevier

BBABIO 43020

Almitrine, a new kind of energy-transduction inhibitor acting on mitochondrial ATP synthase

Michel Rigoulet [1], Rachid Ouhabi [1], Xavier Leverve [2], Florence Putod-Paramelle [2] and Bernard Guérin [1]

[1] *Institut de Biologie Cellulaire et Neurochimie du C.N.R.S., Bordeaux and* [2] *Laboratoire de Thérapeutique et Etudes Métaboliques, Faculté de Médecine de Grenoble, La Tronche (France)*

(Received 22 November 1988)

Key words: Oxidative phosphorylation; Mitochondrial ATPase; Uncoupler; Inhibitor; Almitrine; (Yeast)

At low concentrations, almitrine inhibits yeast cell multiplication by acting on oxidative metabolism. Studies on isolated mitochondria display the following features: (i) almitrine inhibits ATPase activity and decreases ATP/O ratio during oxidative phosphorylation; (ii) no direct effect on respiration can be evidenced; (iii) ATP/O value decreases without any change in the magnitude of Δp; (iv) the higher the ATP synthesis and respiratory fluxes, the larger is the decrease in ATP/O ratio induced by almitrine. These results indicate that almitrine does not act as a classical protonophoric uncoupler nor as previously studied non protonophoric uncouplers (e.g., general anesthetics). Our data show a direct inhibitory effect of almitrine on ATPase-ATP synthase complex. But, in contrast to the classical inhibitors of this complex, almitrine decreases the ATP/O ratio in a flux-dependent manner. Thus, almitrine could induce either an intrinsic uncoupling of H^+-ATPase (i.e., slip in this proton pump) or a change in the mechanistic H^+/ATP stoichiometry at the ATPase level.

Introduction

Almitrine is used for the improvement in blood gases of patients with chronic bronchitis [1]. In addition, possible metabolic effects of this drug have been proposed: namely increase in rate of cerebral metabolic recovery in senescent rat [2]; increase by low almitrine concentration and decrease by high almitrine concentration in coupling between oxidation and phosphorylation in rat brain mitochondria [3]; inhibition of electron transfer at bc_1 complex level in rat liver mitochondria [4]. Moreover, in isolated hepatocytes, we have shown that this drug decreases gluconogenesis and increases glycolysis from dihydroxyacetone as a direct consequence of a drop in both cytosolic and mitochondrial ATP/ADP ratios [5]. These facts, put together, suggested an effect of almitrine on oxidative phosphorylation in different kinds of mammalian mitochondria.

This paper provides evidence that almitrine inhibits yeast cell growth essentially through its effect on oxidative metabolism. The study carried out on isolated mitochondria indicates that almitrine decreases the coupling efficiency of oxidative phosphorylation by a mechanism inconsistent with classical uncoupling (protonophoric effect) and that the direct effect of this drug on ATP synthase differs from those observed with other known inhibitors.

Materials and Methods

Culture conditions and mitochondria preparation. Cells of the diploid wild strain *Saccharomyces cerevisiae* (yeast foam) were grown aerobically at 28°C in a complete medium: 1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate (pH 4.5), supplemented either with 2% sodium lactate or 10% glucose as carbon source.

The cells grown on 2% lactate complete medium were harvested in logarithmic phase. Mitochondria were isolated from protoplasts as in Ref. 6. The protein concentration was estimated by the biuret method using bovine serum albumin as a standard.

Respiration assay and ATP/O measurements. The oxygen consumption rate was measured polarographically at 27°C using a Clark electrode connected to a microcomputer giving an on-line display of rate values. The respiratory medium was as follows: 0.55 M mannitol, 10 mM Tris-maleate (pH 6.7), 0.36 mM EGTA, 0.3% bovine serum albumin, 3 mM Tris-P_i, 10 μM RbCl in the presence of 0.1 μg valinomycin/ml and when

Abbreviations: CCCP, carboxylcyanide *m*-chlorophenylhydrazone; P_i, inorganic phosphate; ΔpH, transmembrane difference of pH; $\Delta\psi$, transmembrane difference of electrical potential; Δp, protonmotive force.

Correspondence: M. Rigoulet, Institut de Biologie Cellulaire et Neurochimie du C.N.R.S., 1 rue Camille Saint-Saëns, 33077 Bordeaux, Cedex, France.

added 1 mM ADP. ATP/O stoichiometries with different respiratory substrates were determined from the average of phosphorylation rates vs. respiratory rates in two different systems: (i) ATP production was monitored either by glucose 6-phosphate formation in the presence of non-limiting amount of hexokinase, 1 mM $MgCl_2$ and 10 mM glucose, or (ii) by labelled P_i incorporation in adenine nucleotides as described in Ref. 7. These two methods gave identical results indicating that neither contaminating ATPase activity nor adenylate kinase activity changed significantly the ATP synthesis rate estimations.

ATPase activity. ATPase activity was measured according to Somlo [8] at 27°C and pH 8.4. Inorganic phosphate was measured as described in Ref. 9.

Measurements of $\Delta\psi$ and ΔpH. A matrix space was determined by the use of ^{3}H-water and inner-membrane-impermeable ^{14}C-mannitol, $\Delta\psi$ and ΔpH by the distribution of ^{86}Rb (in the presence of valinomycin) and ^{3}H-acetate, respectively [10]. Routinely after equilibration (2 min), mitochondria were separated from the medium by rapid centrifugation (20 s) through a silicone oil layer (silicone AR 200 fluid).

Aurovertin D was a gift from Dr. Satre (C.E.N.G. Grenoble, France) and almitrine ((bisallylamino-4,6-*S*-triazinyl-2)-1-(bis-*p*-fluorobenzhydryl)-4-piperazine(bis-methanesulfonate)) was a gift from Servier Laboratory, France.

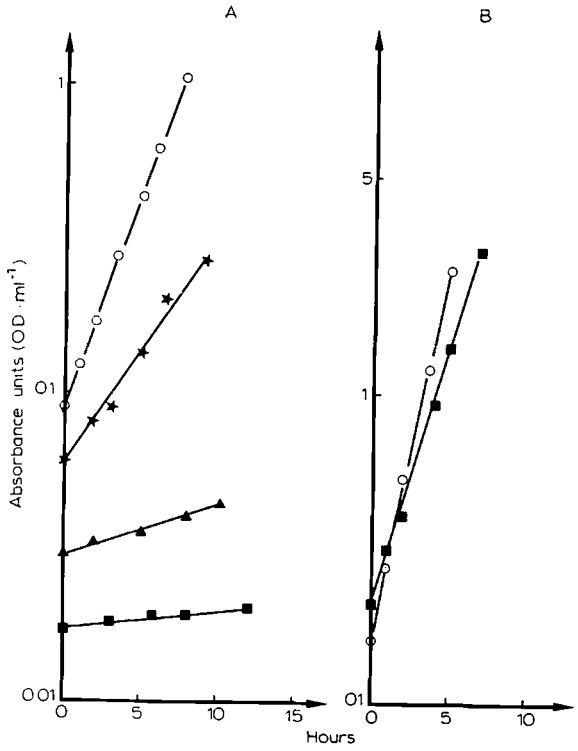

Fig. 1. Almitrine effects on growth rates of yeast. Cells of *Saccharomyces cerevisiae* were inoculated in complete liquid medium supplemented with 2% lactate (A) or 10% glucose (B) as carbon source. Cells were grown at 28°C on a New Brunswick giratory shaker in the absence (○) or in the presence of 10 μM (★), 100 μM (▲) or 200 μM (■) of almitrine.

Results

Growth inhibition

In yeast, energy for growth is supplied from the fermentation or from the aerobic metabolic pathway. As shown on Fig. 1, almitrine inhibits cell multiplication essentially through its effect on oxidative metabolism, since this drug shows an efficient inhibitor effect for non-fermentative medium (Fig. 1A) but only a little effect for fermentative medium at high almitrine concentration (Fig. 1B). A complete cell-growth inhibition was obtained for 200 μM almitrine with lactate as the carbon source.

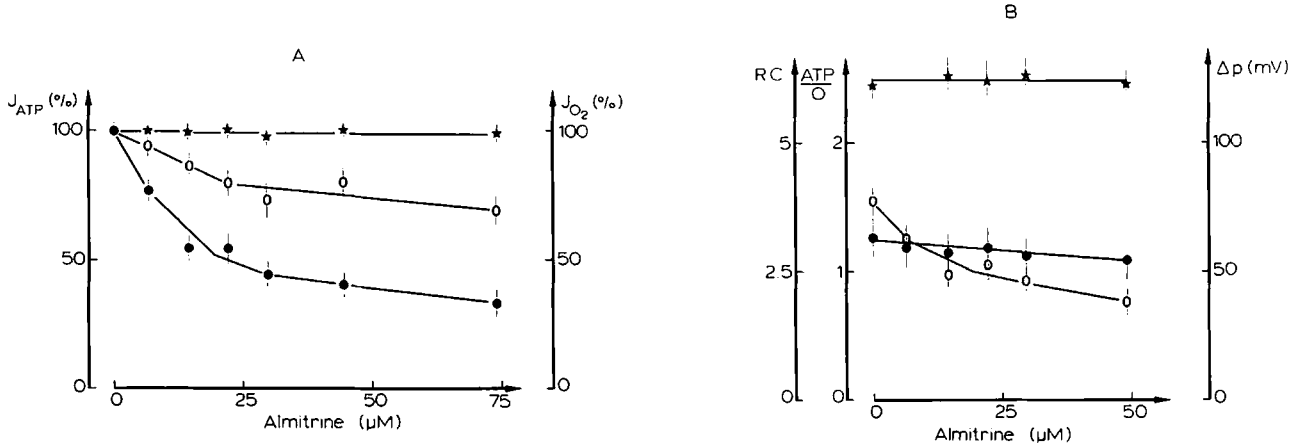

Fig. 2. Dependence of ATP synthesis, respiratory rates, ATP/O ratio, respiratory control and protonmotive force on almitrine concentration. Mitochondria were suspended in 1.5 ml of respiratory medium (see Materials and Methods) supplemented with 0.66% ethanol in the absence or in the presence of various concentrations of almitrine. ATP synthesis (●) and respiratory rates were obtained either with 1 mM ADP addition, state 3 (○) or with 3 μM CCCP, uncoupled respiration (★) in (A). Protonmotive force (★) and ATP/O ratio (○) were obtained during state 3 respiration as indicated above (B). Other experimental conditions are given in the Materials and Methods section. Respiratory control (R.C.) (●) is the slope ratio between state 3 and state 4 respiration (B).

Oxidative phosphorylation efficiency

We have tested, on isolated mitochondria, the effect of almitrine on overall oxidative phosphorylation. As shown on Fig. 2A, almitrine strongly inhibited the ATP synthesis. The respiratory rate in state 3 was only slightly inhibited; the uncoupled respiration was not affected by the drug indicating that respiratory activity itself is not inhibited. Fig. 2B shows that ATP/O ratio decreased largely while the almitrine concentration increased (Fig. 2B). Respiratory control (state 3/state 4 ratio) and protonmotive force measured during state 3 respiration were not significantly modified by almitrine addition indicating that ATP/O ratio decrease is not a consequence of protonophoric effect of the drug.

Table I shows that addition of 22.4 μM almitrine led to a decrease in ATP/O whatever the substrate, except when 2-oxoglutarate is used. It should be noted that, for a given almitrine concentration, the higher the ATP synthesis rate, the larger the effect of almitrine is. Moreover, almitrine does not inhibit uncoupled respiration whatever the substrate used.

ATPase activity

Although protonophoric uncouplers stimulate the respiration of isolated yeast mitochondria and inhibit some energy-linked reactions [11,12], the ATPase activity assayed with added ATP, is only observed at alkaline pH [8,13] as a consequence of a kinetic limitation in ATP influx [14]. Oligomycin-sensitive ATPase activity measured at pH 8.4 is inhibited by almitrine (Fig. 3A) with 50% inhibition for 30 μM almitrine. This value is higher than the concentration for half inhibition of phosphorylation (see Fig. 2). This difference in sensitivity essentially seems due to the difference of pH, since the sensitivity of partial reaction catalyzed by oligomycin-sensitive ATPase (ATP-P_i exchange) which can be observed at more physiological pH (7.4) is highly sensitive to almitrine (not shown). The inhibition of ATPase is uncompetitive as shown in Fig. 3B.

Comparison between almitrine and other mitochondrial ATPase inhibitors

In order to compare the action of almitrine with that of the well-known inhibitors of ATPase activity, the titration of ATP synthesis and respiratory rates has been performed with oligomycin (acting on F_0 part) or aurovertin D (acting on F_1) [15]. Fig. 4 shows that almitrine decreases the ATP/O ratio value even though the respiratory rate is slightly inhibited in contrast to oligomycin and aurovertin D effects.

We have observed that the decrease in ATP/O ratio induced by a constant concentration of almitrine seems dependent on the rate of ATP synthesis (see Table I). However, it is not greatly informative to compare the ATP/O value obtained with different respiratory substrates even as % of control. Therefore, for a given respiratory substrate, we measured ATP synthesis and respiratory rates when these fluxes were modulated by antimycin, in the presence or in the absence of a given almitrine concentration (Fig. 5). As previously observed in mammalian mitochondria [16–18], antimycin addition to yeast mitochondria leads to a decrease of both fluxes without significant change in ATP/O value. But, in the presence of 22.4 μM of almitrine, the decrease in ATP production and respiratory rate, induced by various concentrations of antimycin, is such that the ATP/O

TABLE I

Almitrine effects on oxidative phosphorylation supported by different substrates

Experimental conditions were as in Fig. 2.

Respiratory substrates and additions	Respiratory rate (natom O per min per mg protein)		ATP synthesis rate (nmol ATP per min per mg protein)	ATP/O	Respiratory control
	state 3	uncoupled			
Succinate (5 mM)	471 ± 29	493 ± 19	730 ± 72	1.55 ± 0.12	2.3 ± 0.3
+ almitrine (22.4 μM)	429 ± 36	472 ± 34	403 ± 56	0.94 ± 0.07	2.3 ± 0.4
NADH (5 mM)	756 ± 88	873 ± 102	953 ± 101	1.26 ± 0.06	3.4 ± 0.6
+ almitrine (22.4 μM)	459 ± 39	918 ± 93	325 ± 38	0.71 ± 0.08	2.3 ± 0.2
Glycerol-3-phosphate (5 mM)	482 ± 62	506 ± 39	636 ± 84	1.32 ± 0.1	3.2 ± 0.3
+ almitrine (22.4 μM)	490 ± 53	493 ± 47	475 ± 39	0.97 ± 0.1	4.0 ± 0.5
2-oxoglutarate (5 mM)	183 ± 24	208 ± 31	441 ± 46	2.4 ± 0.2	3.3 ± 0.7
+ almitrine (22.4 μM)	164 ± 27	216 ± 36	400 ± 38	2.4 ± 0.3	2.7 ± 0.5

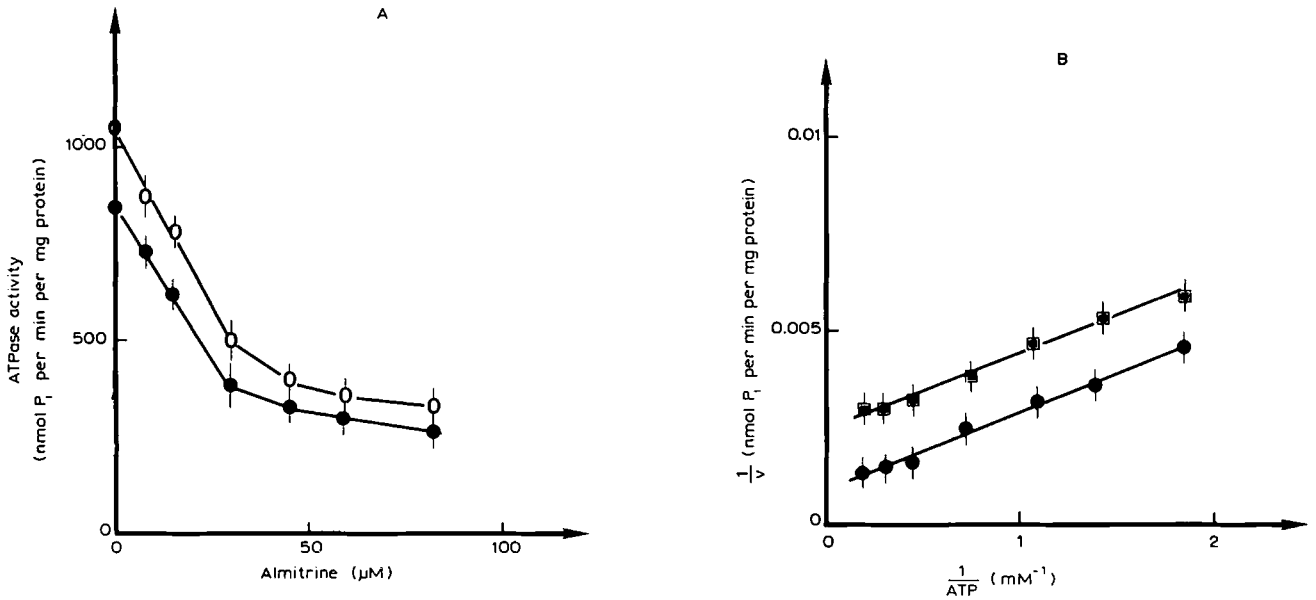

Fig. 3. Effect of almitrine on mitochondrial ATPase. Mitochondria (1 mg protein) were incubated in 2 ml of the following medium: 0.2 M KCl, 5 mM MgCl, 10 mM Tris-HCl (pH 8.4). (A) total ATPase activity (○) and oligomycin sensitive ATPase activity (●) as a dependence of almitrine concentration; 5 mM ATP and when used 20 μg per ml oligomycin. (B) Dependence on ATP concentration of oligomycin sensitive ATPase activity in the absence (●) or in the presence (▣) of 22.4 μM almitrine.

value increases when the fluxes decrease and reaches a value similar to that of the control at low fluxes.

Discussion

Almitrine inhibits yeast cell multiplication essentially by acting on oxidative metabolism. Its action on isolated mitochondria can be summarized as follows: (i) almitrine inhibits ATP synthesis and ATPase activities. The observation that ATPase activity measured at pH 8.4 is sensitive to almitrine excludes that the adenine nucleotide translocator was involved in this process. Indeed, at high pH value, ATP enters into mitochondria by a way insensible to atractyloside [12,13]; (ii) almitrine inhibits also state 3 respiration but in a lower extent than the ATP synthesis. This leads to a decrease in ATP/O ratio by almitrine; (iii) this drug inhibits only slightly the state 4 respiration but does not inhibit the respiration stimulated by a protonophore (CCCP); (iv) finally, it does not decrease the protonmotive force.

Moreover, it is worth noting that the effect of almitrine on ATP/O ratio depends on the respiratory and ATP synthesis flux values; the larger the fluxes the lower the ATP/O value. This feature could explain the lack of ATP/O decrease by almitrine when 2-oxoglutarate is used as respiratory substrate (see Table I). Indeed, by substrating participation of the substrate level phosphorylation [19], the oligomycin-sensitive ATPsynthesis is only about 250 nmol ATP per min per mg protein and uncoupling, induced by almitrine, is undetectable for such a low flux when ethanol is used as substrate.

Almitrine appears to act essentially on the ATP synthase complex. But its effect is very different from that of classical ATP synthase inhibitors, such as oligomycin or aurovertin D, which decrease ATP synthesis and respiratory rates in such a manner that ATP/O ratio remains constant. Recently a new type of uncoupler of oxidative phosphorylation has been described in the literature. For instance, the general anesthetics, such as chloroform or halothane, inhibit ATP synthesis, stimulate mitochondrial ATPase activity and state 4 respiration like pure protonophoric uncouplers but have no measurable effect on the magnitude of Δp [20]. But almitrine acts differently, since it does not stimulate state 4 respiration and ATPase activity as general anesthetics do.

Therefore, by comparing the effect of almitrine to that of different kinds of inhibitors and uncouplers, it clearly appears that almitrine corresponds to a new type of mitochondrial energy-transduction inhibitor. Its main characteristic is that almitrine appears to decrease the efficiency of oxidative phosphorylation by acting essentially on ATP synthase complex. It shall be noted that almitrine does not inhibit ATPase activity of the isolated F1 moiety nor the ATPase activity of mitochondria, which are devoided of functional F_0 moiety (not shown) suggesting that the almitrine target is the membranal F_0 sector or F_1 as part of the entire complex. The fact that almitrine produces a parallel shift of the double reciprocal plot of ATPase activity in dependence with ATP concentration cannot be clearly interpreted. Indeed, such a behaviour, classified as uncompetitive inhibition, could occur for an enzyme when the inhibitor acts on the enzyme-substrate complex, but

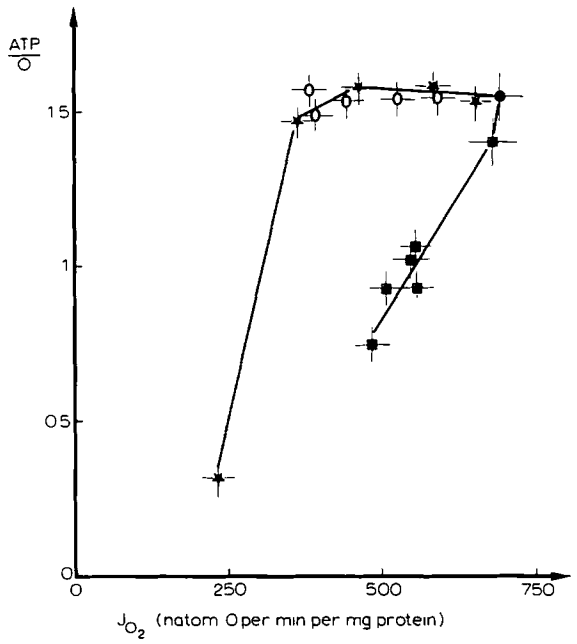

Fig. 4. Effects of aurovertin D, oligomycin or almitrine on the relation between ATP/O value and respiratory rate. Mitochondria (0.6 mg protein) were suspended in 1.5 ml of respiratory medium supplemented with 0.66% ethanol, 1 mM ADP and various concentrations of aurovertin D (○), oligomycin (★) or almitrine (▣).

also for a multi-enzyme system when the inhibitor reacts with a controlling enzymatic step intervening beyond the enzyme for which the kinetics is done [21], for instance when ATP hydrolysis is mainly controlled by H^+ flux through F_0.

Since almitrine does not decrease Δp, its effect on the ATP/O ratio may be due to either a decrease in the H^+/e or an increase in the H^+/ATP ratios. However,

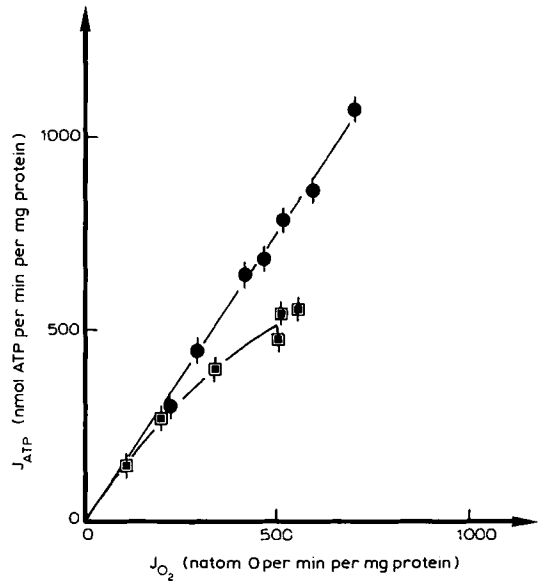

Fig. 5. Measurement of ATP/O by antimycin titration in the absence or in the presence of almitrine. Mitochondria (0.6 mg protein) were suspended in 1.5 ml of respiratory medium supplemented with 0.66% ethanol, 1 mM ADP, various concentrations of antimycin and without (●) or with (□) 22.4 μM almitrine.

the fact that almitrine does not stimulate the respiration in state 4 shows no modification of the respiratory complex efficiency, but strongly suggests a flux dependent change in the H^+/ATP ratio due to a direct action of almitrine on the ATP synthase. Two explanations can be proposed; (i) almitrine increases intrinsic uncoupling of the H^+/ATPase also called slip [22,23]. Such a mechanism has been proposed for different kinds of uncoupler which act both on ATP synthase and respiratory chain by decreasing the efficiency of proton pumps [24,2]; (ii) almitrine changes the real H^+/ATP ratio of ATP synthase, for instance, by modifying the conformational protein transition during the catalytic cycle. Experiments are underway for testing these possibilities.

Acknowledgements

This work was generously supported by IRIS (Institut de Recherche International Servier) and by grants from the Université de Bordeaux II.

References

1 Arnaud, F., Bertrand, A. and Charpin, J. (1982) Bull. Eur. Physiopath. Resp., Suppl. 4, 373–382.
2 Villa, R.F. and Pastoris, O.(1986) J. Pharmac. 17, 623–633.
3 Lepagnol, J.M. and Weinachter, S.N. (1987) J. Physiol. 396, 92P.
4 Mottershead, J.P., Nye, P.C.G. and Quirk, P.G. (1987) J. Physiol. 396, 91P.
5 Leverve, X., Rigoulet, M., Putod, F. and Paramelle, B. (1989) in Contrôle du Métabolisme Cellulaire (Mazat, J.P. and Reder, C., eds.), in press.
6 Guérin, B., Labbé, P. and Somlo, M. (1979) Methods Enzymol. 55, 149–159.
7 Rigoulet, M. and Guérin, B. (1979) FEBS Lett. 102, 18–22.
8 Somlo, M. (1968) Eur. J. Biochem. 42, 439–445.
9 Sumner, J.B. (1944) Science 100, 413–418.
10 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
11 Ohnishi, T., Kröger, A., Heldt, H.W., Pfaff, E. and Klingenberg, M. (1967) Eur. J. Biochem. 1, 301–311.
12 Kovac, L., Groot, G.S.P. and Racker, E. (1972) Biochim. Biophys. Acta 256, 55–65.
13 Kovac, L., Bednarova, H. and Greshsak, L. (1968) Biochim. Biophys. Acta 153, 32–42.
14 Ezzahid, Z., Rigoulet, M. and Guerin, B. (1986) J. Gen. Microbiol. 132, 1153–1158.
15 Roberton, A.M., Holloway, C.T., Knight, I.G. and Beechey, R.B. (1968) Biochem. J. 108, 445–456.
16 Zoratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. J. Biochem. 126, 443–451.
17 Beavis, A.D. and Lehninger, A.L. (1986) Eur. J. Biochem. 158, 315–322.
18 Stoner ,C.D. (1987) J. Biol. Chem. 262, 10445–10453.
19 Rigoulet, M., Velours, J. and Guerin, G. (1985) Eur. J. Biochem. 153, 601–607.
20 Rottenberg, H. (1983) Proc. Natl. Acad. Sci. USA 80, 3313–3317.
21 Segel, I.H. (1975) Enzyme Kinetics, John Wiley, New York.
22 Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 117, 389–394.
23 Pietrobon, D., Zoratti, M. and Azzone, G.F. (1983) Biochim. Biophys. Acta 723, 317–321.
24 Luvisetto, S., Pietrobon, D. and Azzone, G.F. (1987) Biochemistry 26, 7332–7338.
25 Pietrobon, D., Luvisetto, S. and Azzone, G.F. (1987) Biochemistry 26, 7339–7347.