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INFLUENCE OF DIFFERENT ENERGY DRAINS ON THE INTERRELATIONSHIP BETWEEN THE RATE OF RESPIRATION, PROTON-MOTIVE FORCE AND ADENINE NUCLEOTIDE PATTERNS IN ISOLATED MITOCHONDRIA

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The respiration of rat liver mitochondria was stimulated by three different ways of energy drain: (a) partial uncoupling (equivalent to direct collapse of the proton-motive force), (b) intramitochondrial utilization of ATP for citrulline synthesis, and (c) extramitochondrial utilization of ATP for glucose phosphorylation. At identical rates of respiration, the intramitochondrial ATP : ADP ratios were the same in all three systems. Furthermore, the proton-motive force was the same in partially uncoupled mitochondria and in the presence of hexokinase plus glucose up to a respiration rate amounting to about 60% of that of the fully active state. However, external ATP : ADP ratios were considerably different in various systems at comparable rates of oxygen uptake, being the lowest under conditions when ATP was being utilized externally. On this basis, it is concluded that the respiratory rate is controlled directly by the proton-motive force and the mitochondrial ATP-synthesizing system operates under near-equilibrium conditions with respect to the membrane energy state parameters. However, a disequilibrium exists at the step of the transport of ATP from mitochondria to the external (cytoplasmic) compartment.

Introduction

The rate of respiration of isolated mitochondria can be experimentally varied between the resting state (state 4) and the fully active state (state 3). Earlier studies [1,2], simulating the expenditure of energy preferentially in the extramitochondrial compartment, have shown that the rate of mitochondrial respiration depends on the extramitochondrial ATP : ADP ratio. According to Kunz et al. [3], this dependence is a consequence of the kinetic control of respiration by the adenine nucleotide translocator. The linear relationship between O_2 uptake and atractyloside inhibition in experiments with isolated hepatocytes [4] shows that this control may also be important for the cellular situation.

Wilson and coworkers [5,6] developed the concept of a thermodynamic equilibrium between the extramitochondrial phosphorylation potential and the intramitochondrial redox couples of the respiratory chain between nicotinamide nucleotides and cytochrome *c*. According to these authors, the respiratory control is based on a disequilibrium at the terminal step of the respiratory chain, namely cytochrome oxidase. However, in experiments with an intramitochondrial ADP-regenerating system, we demonstrated that the rate of respiration could be changed independently of the extramitochondrial ATP : ADP ratio [7]. This finding could not be explained in terms of the equilibrium model as proposed by Wilson and

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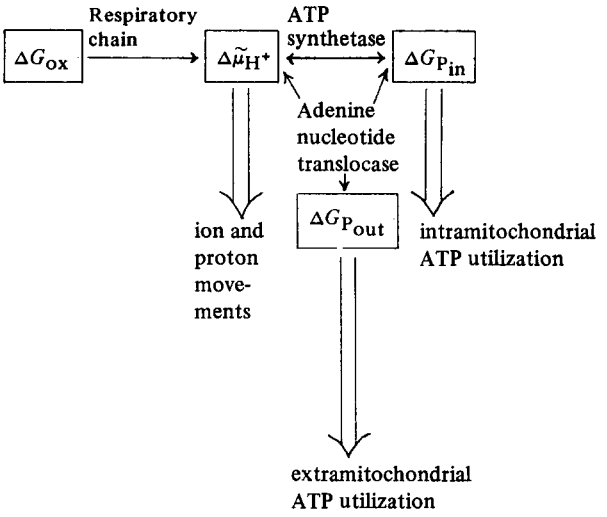
Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

coworkers [5,6]. To cast more light on the problem of respiratory control, in the present study we investigated the relationships between the respiratory rate, intra- and extramitochondrial patterns of adenine nucleotides and the proton-motive force under various steady-state conditions.

Nicholls and Bernson [8] investigated this interrelationship in brown adipose tissue, without regard to the intramitochondrial adenine nucleotides. We applied a new method of simultaneous determination of external and intramitochondrial adenine nucleotides without the separation step [9] and made use of the possibility of significant stimulation of mitochondrial respiration by an intramitochondrial ATP-consuming system [7].

The rational basis of the experimental approach of the present investigation is shown in Scheme I. It presents four interconnected 'energy pools' which can be separately disturbed experimentally. For example, an increase in proton conductivity of the inner mitochondrial membrane by a low concentration of an uncoupler directly decreases the proton-motive force. Consequently, energy flux through the ATP-synthesizing system and the adenine nucleotide translocator is decreased. On the other hand, under conditions of a high rate of citrulline synthesis, intramitochondrial ATP is utilized and the flux through ATP synthase is high whereas the flux through the adenine nucleotide translocator may be negligible. Finally, when ATP is utilized externally, as in the classical system with the glucose-hexokinase trap, the fluxes through both ATP synthase and the translocator are high.

The results demonstrate that the relationship between the rate of respiration, the protonmotive force and the intramitochondrial ATP : ADP ratio is almost the same in all systems, which means that this relationship does not depend on the site of energy drain. It is therefore suggested that the rate of respiration is controlled directly by the proton-motive force and the ATP-synthesizing complex and that the respiratory chain operates near equilibrium in the sense that there is near-equilibrium state between the respiratory chain (ΔG_{ox}, Scheme I), the proton-motive force ($\Delta p = \Delta\mu_{H^+}/F$) and the intramitochondrial phosphorylation potential ($\Delta G_{P_{in}}$). In contrast, the external phosphorylation potential depends on the site of the energy drain. Under conditions of equal respiratory rates, the external ATP : ADP ratio is lower in the glucose-hexokinase system than in the two other energy drain systems depicted in Scheme I. Therefore, we conclude that the adenine nucleotide translocation is not a part of the near-equilibrium system of oxidative phosphorylation.



Scheme I. Scheme of energy fluxes and its drain.

Materials and Methods

Liver mitochondria were prepared [10] from rats fed either with normal laboratory chow or with a high-protein diet (75 g casein, 25 g starch, 2.5 g sunflower oil and 1.5 g NaCl) ad libitum for 4–6 days. The high-protein diet was used to enhance the activity of carbamoyl-phosphate synthetase I [11]. Mitochondria used to synthesise citrulline were washed and resuspended in 150 mM mannitol. Mitochondria were incubated at 25°C in a closed oxygraphic cell either in a saline medium (50 mM KCl, 20 mM $KHCO_3$, 5 mM KH_2PO_4, 10 mM glutamate, 15 mM glucose, 0.5 mM EDTA, 2 mM $MgCl_2$, 25 mM Tris-HCl and 150–200 μM ATP, pH 7.2, gassed with 95% O_2 and 5% CO_2) or in a sucrose medium (110 mM sucrose, 60 mM KCl, 15 mM glucose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM glutamate, 0.5 mM malate, 60 mM Tris-HCl and 150–200 μM ATP, pH 7.2, gassed with pure O_2). The rate of O_2 uptake was monitored using a rate-meter similar to that described by Sargent and Taylor [12]. Calcula-

tion of O_2 uptake was based on the O_2 concentration in media gassed with pure O_2 and with an O_2/CO_2 mixture (1123 and 1067 μM, respectively) recalculated from the O_2 concentration given in Ref. 13.

For determination of adenine nucleotides, samples were withdrawn and inactivated with $HClO_4$. After neutralization, ATP, ADP and AMP were determined by enzymatic methods as described by Lamprecht and Trautschold [14] and Jaworek et al. [15], respectively. Extra- and intramitochondrial adenine nucleotides were measured without separation of mitochondria from the incubation medium by the procedure recently described [9]. In this method, several samples containing the same medium but various amounts of mitochondria are incubated in parallel. Since the amount of mitochondrial adenine nucleotides is proportional to the amount of mitochondria and the amount of external nucleotides is constant, both sets of values can be obtained from a plot as exemplified in Fig. 1. A crucial point for this method is that all incubations used for calculation must have an identical metabolic state. This was ensured either by the capacity of carbamoylphosphate synthetase I (see linear dependence of the respiratory rate on the amount of mitochondria in Fig. 1A) or by adjusting the incubations with hexokinase or an uncoupler to the same relative rate of respiration (see also Ref. 9).

Protein was determined by the biuret method [16]. The membrane potential ($\Delta\psi$) and pH difference (ΔpH) between the intramitochondrial compartment and the medium were calculated from the distribution of [^{3}H]triphenylmethylphosphonium [17] and [^{14}C]acetate [18] in the presence of 0.28 M unlabelles acetate, respectively. The concentrations of the labelled compounds were determined after separation of mitochondria from the medium by centrifugation (10 000 $\times g$, 1 min) through silicone oil (prepared by mixing silicone oils DC 200 and DC 550 from William F. Nyke, Inc. New Bedford, MA, U.S.A., in the proportions of 17 : 183) into $HClO_4$. The matrix space was calculated as the difference between the [^{3}H]-H_2O space and the [^{14}C]sucrose space. This measurement was made from parallel incubations adjusted to the same conditions as for determination of $\Delta\psi$ and ΔpH.

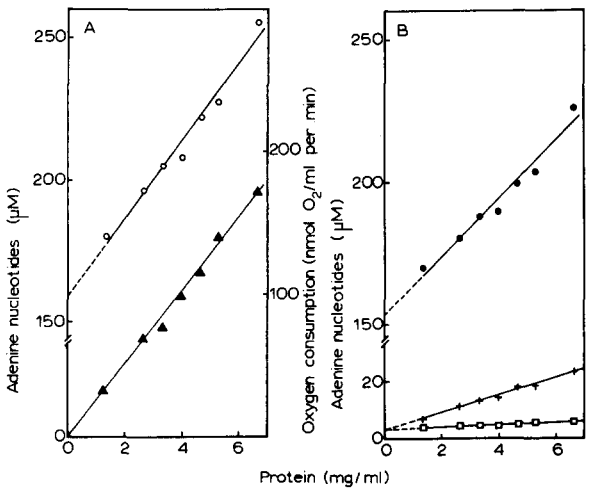


Fig. 1. Rate of O_2 uptake and the content of adenine nucleotides in mitochondria during citrulline synthesis as plotted against protein content. Illustration for the procedure of determining extra- and intramitochondrial content of adenine nucleotides. A, rate of O_2 uptake (▲) and the content of total adenine nucleotides (medium plus mitochondria) (○). B, total content of specific adenine nucleotides (medium plus mitochondria): ATP (●), ADP (+) and AMP (□).

Results

As demonstrated previously [7,19,20], the stimulation of O_2 uptake by ornithine is usually lower than that produced by an excess of hexokinase plus glucose, even in the case of mitochondria from rats kept on a high-protein diet. Therefore, in order to

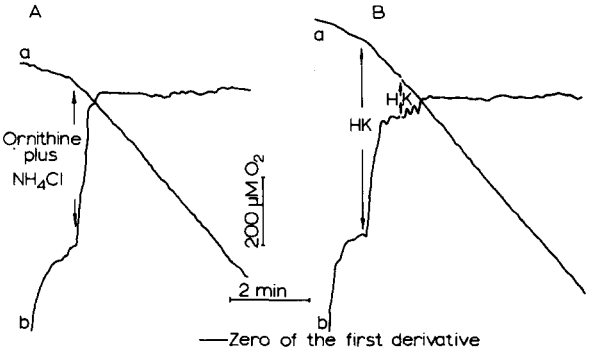


Fig. 2. O_2 uptake of mitochondria during citrulline synthesis (A) and glucose 6-phosphate formation (B). The two traces indicate O_2 concentration (a) and the rate of O_2 uptake (b, i.e., the first derivative of trace a). HK, hexokinase.

TABLE I

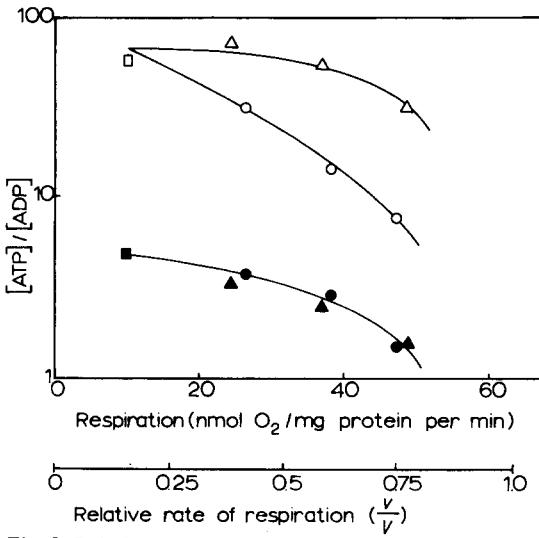
ADENINE NUCLEOTIDE PATTERNS IN MITOCHONDRIA DURING RESPIRATION STIMULATED BY CITRULLINE SYNTHESIS (A) AND BY PHOSPHORYLATION OF GLUCOSE (B)

The values are means ± S.D. (n = 7). The respiration rate amounted to 43 and 44.5% of the fully active state in A and B, respectively.

Nucleotide	Amount or concentration	
	Intramitochondrial (nmol/mg protein)	External (μM)
(A) Citrulline synthesis		
ATP	10.2 ± 0.9	153 ± 4
ADP	3.1 ± 0.2	2.9 ± 0.7
AMP	0.4 ± 0.1	3.3 ± 0.3
Total	13.6 ± 1.0	159 ± 4
ATP : ADP	3.3 ± 0.5	53 ± 14
(B) Hexokinase-glucose system		
ATP	9.6 ± 0.6	151 ± 3
ADP	3.0 ± 0.4	6.6 ± 1.6
AMP	0.4 ± 0.1	3.7 ± 0.2
Total	13 ± 0.7	161 ± 3
ATP : ADP	3.2 ± 0.6	23 ± 6

compare adenine nucleotide patterns in the two ATP-utilizing systems, it was necessary to keep the respiration rate in both systems at the same level. This was performed by adjusting the amount of hexokinase, as illustrated in Fig. 2. To measure adenine nucleotide patterns, the procedure described under Materials and Methods and shown in Fig. 1 was followed with both systems.

As shown in Table I, for mitochondria respiring at the same rate in the ornithine system and the hexokinase-glucose system, the intramitochondria ATP : ADP ratios were identical. There was, however, a large difference in the external ATP : ADP ratio, namely it was much lower in the system utilizing ATP extramitochondrially (hexokinase plus glucose) than in the case where ATP was utilized intramitochondrially (synthesis of citrulline). Additionally, it was recently shown for both systems [21] that at identical respiratory rates, the membrane potential $\Delta\psi$ and ΔpH were the same. This finding does not fit to the postulated [22] near-equilibrium operation mode of the adenine nucleotide translocator.



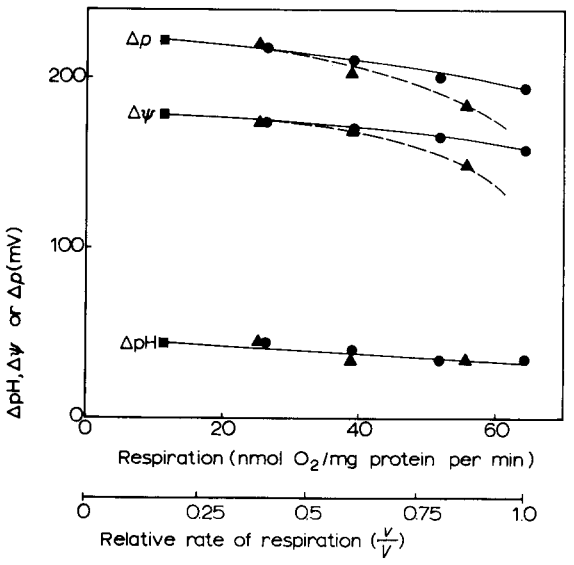


Fig. 3. Relationship between the rate of respiration and the intra- and extramitochondrial ATP : ADP ratios. Mitochondria (11.4–28.2 mg protein) were incubated in the sucrose medium and respiration was stimulated to preset rates by the addition of either hexokinase (○, ●) or FCCP (△, ▲). Open symbols represent external and full symbols internal ATP : ADP ratios. The resting state is represented by squares (□, ■).

Fig. 4. Relationship between the rate of respiration and the proton-motive force (Δp), the membrane potential ($\Delta\psi$) and −59 ΔpH gradient. ■, resting state; ●, respiration stimulated by increasing amounts of hexokinase; ▲, respiration stimulated by increasing amounts of FCCP.

To investigate the relationship between the proton-motive force and the intramitochondrial adenine nucleotide pattern, we compared two systems characterized by different fluxes through ATP synthase at the same rate of respiration, namely the glucose-hexokinase system and a partially uncoupled system (see Scheme I). Fig. 3 shows external and intramitochondrial ATP : ADP ratios under such conditions as plotted against the rate of respiration. It is evident that in both systems the intramitochondrial ATP : ADP ratio is identical and decreases in the same way with increasing respiration. In contrast, external ATP : ADP ratios are quite different in both systems, the ratio in partially uncoupled mitochondria being always higher than that in mitochondria incubated in the presence of hexokinase plus glucose and respiring at the same rate.

As shown in Fig. 4 for the same preparations of mitochondria, the proton-motive force decreased with increasing O_2 uptake. For lower rates of respiration, the values of Δp and its component $\Delta\psi$ and ΔpH were very similar in both systems. However, at higher respiration rates, Δp was somewhat lower for the uncoupler-stimulated respiration, which is in good agreement with observations of other authors [23–25].

The data presented in Figs. 3 and 4 can be further used for evaluation of the intramitochondrial ATP : ADP ratio in the fully active state (state 3). The estimation of this value by the method used in this investigation [9] is not possible because a prerequisite for this method is a definite relationship between the respiration rate and the distribution of adenine nucleotides. This is, however, not ensured in the maximum active state where the extramitochondrial ATP : ADP ratio can be lowered by an excess of hexokinase without a reflection in the respiratory rate [2]. Therefore, we plotted the intramitochondrial ATP : ADP ratios (taken from Fig. 3) against the proton-motive force (taken from Fig. 4).

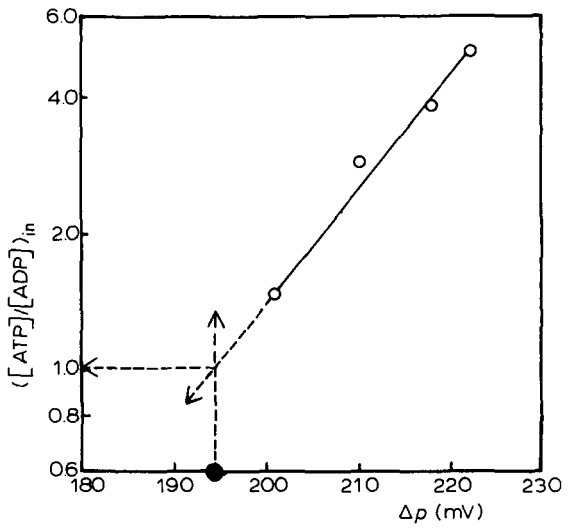


Fig. 5. Dependence between intramitochondrial ATP : ADP ratio and the proton-motive force. Data taken from Figs. 3 and 4. (•) Measurement of Δp in the fully active state.

The plot shown in Fig. 5 gives a straight line. It is therefore reasonable to extrapolate the plot up to the value of Δp measured for the fully active state, viz., 195 mV. The intercept (dashed lines in Fig. 5) gives a value for the intramitochondrial ATP : ADP ratio very close to unity.

Discussion

For the elucidation of the regulation of oxidative phosphorylation we attempted to see whether or not the steps, viz., the synthesis of ATP and its export from mitochondria to the external compartment, are near-equilibrium. The experimental approach was based on measuring adenine nucleotides and membrane energy state parameters at different stationary fluxes (Scheme I). The rationale for this approach was as follows. If in different mitochondrial systems respiring at the same rate and having the same proton-motive force but characterized by different fluxes through ATP synthase the intramitochondrial adenine nucleotide patterns are identical, then that is the demonstration of a near-equilibrium state of the reaction catalyzed by the synthase. If, in addition, fluxes through the ATP translocator were different but the external adenine nucleotide ratios remained the same, then the near-equilibrium state would also extend over the translocator. On the contrary, a change of adenine nucleotide patterns with changing the flux is an indication of a disequilibrium.

As depicted in Scheme I, a comparison of the partially uncoupled system with the hexokinase-glucose system could offer a suitable approach to the evaluation of the equilibrium state of the reaction catalyzed by mitochondrial ATP synthease. In both systems, the flux through ATP synthase was quite different

and this difference increased with increasing respiratory rate. Nevertheless, the proton-motive force declined in both systems in an identical way up to 60% of the maximum respiratory rate (Fig. 4). Simultaneously, the intramitochondrial ATP : ADP ratio also decreased (Fig. 3). The reason for the difference in Δp in the range of fast respiration (Fig. 4) is still unclear. We assume that this phenomenon is a methodological problem as discussed by Azzone et al. [25]. However, on the basis of the chemiosmotic theory, we conclude from the identity of the intramitochondrial ATP : ADP ratios at the same rate of respiration over the whole range (Fig. 3) that the mediator Δp should also be identical. The interrelationship between the rate of respiration and the intramitochondrial ATP : ADP ratio corresponds to the 'control characteristic' reported for the extramitochondrial one [2]. In the present investigation, intramitochondrial P_i was not measured. However, since it can be assumed that it does not change considerably, changes of the ATP : ADP ratio could be taken as a reflection of changes in the intramitochondrial phosphorylation potential ($\Delta G_{P_{in}}$). It follows from the results shown in Figs. 3 and 4 that $\Delta\mu_{H^+}$ must represent the most important factor in the control of the rate of respiration, as suggested by Nicholls and Bernson [8], since respiratory rate and proton-motive force changed in both systems in an identical way up to 60% of the maximum respiratory rate. This correlation was independent of the site of energy drain. In addition, a close relationship seems to exist between the proton-motive force and $\Delta G_{P_{in}}$. This means that no measurable driving force was needed to increase the flux through the ATP synthase. Therefore, a near-equilibrium operation of the ATP synthase can be concluded to occur.

A comparison of the partially uncoupled system with the hexokinase-glucose system shows that not only the flux through ATP synthase, but also that through the adenine nucleotide translocator is different, being higher in the glucose-hexokinase system than in partially uncoupled mitochondria. The action of this electrogenic [26] transport system is controlled by three factors: the electrical membrane potential, and the intramitochondrial and extramitochondrial ATP : ADP ratios. In the case of mitochondria respiring at the same rate in the uncoupler-stimulated system and the hexokinase-glucose system, two of these factors, namely the membrane potential and the internal ATP : ADP ratio, are identical. However, the external ATP : ADP ratios are different and this difference increases with increasing respiration rate (Fig. 3). For mitochondria respiring at higher rates, the differences in corresponding fluxes through the adenine nucleotide translocator also increase. It seems, therefore, that a significant part of ΔG is utilized to increase the flux through the adenine nucleotide translocator. It can be concluded, therefore, that the translocator does not operate near equilibrium under our conditions, since a driving force is needed to enhance the flux.

Further support to this postulate was provided by comparison of the hexokinase-glucose system with mitochondria synthesizing citrulline. In the latter system, ATP is utilized intramitochondrially and the flux through the adenine nucleotide translocator is negligible. When respiring at the same rate, mitochondria in both systems are characterized by an identical proton-motive force and identical internal ATP : ADP ratios (Table I). There is, however, a 2-fold difference in the external ATP : ADP ratio. This is again an indication of a disequilibrium of the translocation step, since an increased flux through the carrier must be connected with an increase in ΔG. The most likely explanation of this disequilibrium is a competitive inhibition of the inward transport of ADP by external ATP [27–30]. As shown with the use of a mathematical model [31], this inhibition would be sufficient to explain the observed disequilibrium. In conclusion, we postulate that the respiratory rate is controlled by $\Delta\mu_{H^+}$ (in the small range between about 195 and 225 mV) or by the internal phosphorylation potential because of the near-equilibrium operation of the ATP synthase. Many regulatory functions of the adenylate system may make it advantageous for the cell to change to one adenine nucleotide pool quite independently of the other. As shown by the experiments presented here, this relative independence of adenine nucleotide patterns can result due to the disequilibrium at the adenine nucleotide translocator.

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References

1 Davis, E.J. and Lumeng, L. (1975) J. Biol. Chem. 250, 2275–2282
2 Küster, U., Bohnensack, R. and Kunz, W. (1976) Biochim. Biophys. Acta 440, 391–402
3 Kunz, W., Bohnensack, R., Küster, U., Letko, G. and Schönfeld, P. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa, S. and Klingenberg, M., eds.), pp. 313–316, Elsevier, Amsterdam
4 Van der Meer, R., Akerboom, T.P.M., Groen, A.K. and Tager, J.M. (1978) Eur. J. Biochem. 84, 421–428
5 Holian, A., Owen, C.S. and Wilson, D.F. (1977) Arch. Biochem. Biophys. 181, 164–171
6 Erecińska, M., Wilson, D.F. and Nishiki, K. (1978) Am. J. Physiol. 234, 82–89
7 Letko, G. and Küster, U. (1979) Acta Biol. Med. Ger. 38, 1379–1385
8 Nicholls, D.G. and Bernson, V.S.M. (1977) Eur. J. Biochem. 75, 601–612
9 Letko, G., Küster, U., Duszyński, J. and Kunz, W. (1980) Biochim. Biophys. Acta 593, 196–203
10 Steinbrecht, I. and Kunz, W. (1970) Acta Biol. Med. Ger. 25, 731–747
11 Schimke, R.T. (1962) J. Biol. Chem. 237, 459–468
12 Sargent, D.F. and Taylor, C.P.S. (1971) Anal. Biochem. 42, 446–454
13 Hinkle, P.C. and Yu, M.L. (1979) J. Biol. Chem. 254, 2450–2455.
14 Lamprecht, W. and Trautschold, J. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 3, pp. 2024–2033, Akademie Verlag, Berlin
15 Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 3, pp. 2051–2055, Akademie Verlag, Berlin
16 Sokolowski, A. and Liese, W. (1973) Z. Med. Labortech. 14, 247–251
17 Azzone, G.F., Bragadin, M., Pozzan, T. and Dell'Antone, P. (1976) Biochim. Biophys. Acta 459, 96–109
18 Nicholls, D. (1974) Eur. J. Biochem. 50, 305–315
19 Letko, G., Markefski, M. and Bohnensack, R. (1979) Acta Biol. Med. Ger. 38, 1081–1090
20 Duszyński, J., Bogucka, M., Letko, H.G., Küster, U. and Wojtczak, L. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa, S. and Klingenberg, M., eds.), pp. 309–312, Elsevier, Amsterdam
21 Duszyński, J., Küster, U., Letko, G., Bogucka, K., Kunz, W. and Wojtczak, L. (1980) Short Reports of the First European Bioenergetics Conference, Sogesta Centre, Urbino, Italy, June 29- July 5, 1980, pp. 309–310, Pàtron Editore, Bologna
22 Stubbs, M., Vignais, P.V. and Krebs, H.A. (1978) Biochem. J. 172, 333–342
23 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484
24 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437.
25 Azzone, G.F., Pozzan, T., Massari, S. and Bragadin, M. (1978) Biochim. Biophys. Acta 501, 296–306
26 La Noue, K., Mizani, S.M. and Klingenberg, M. (1978) J. Biol. Chem. 253, 191–198
27 Souverijn, J.H.M., Huisman, L.A., Rosing, J. and Kemp, A., Jr. (1973) Biochim. Biophys. Acta 305, 185–198
28 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79
29 Duée, E.D. and Vignais, P.V. (1969) J. Biol. Chem. 244, 3920–3931
30 Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1–38
31 Bohnensack, R. (1981) Biochim. Biophys. Acta 634, 203–218