INTERACTION OF AZIDONITROPHENYLAMINOBUTYRYL–ADP, A PHOTOAFFINITY ADP ANALOG, WITH MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE. IDENTIFICATION OF THE LABELED SUBUNITS

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1. Introduction

Two photoactive derivatives of ATP, 8-azido-ATP [1] and azidonitrophenylaminopropionyl–ATP (NAP_3–ATP) [2] have been recently used to label F_1-ATPase by photoaffinity. In the first compound, the azido group is located on the adenine ring of ATP. In the second one, the photosensitive adduct is attached to the ribose portion of ATP. Wagenvoord et al. [1] have reported that upon photoactivation, 8-azido-ATP binds covalently to the β-subunit of F_1-ATPase. Russell et al. [1] have shown that NAP_3-ATP also binds to F_1-ATPase, but there were no data on the nature of the subunit(s) which carry the binding site(s) and no documentation on possible side effects due to the presence of an aryl group in the molecule.

In this paper, we report on the interactions of *N*-4-azido-2-nitrophenylaminobutyryl–ADP (NAP_4–ADP) (fig.1) with isolated F_1-ATPase and on the identification of the subunits which bind NAP_4–ADP. For this purpose NAP_4–ADP was synthesized in radioactive form. When irradiated under visible light in the presence of F_1-ATPase, NAP_4–ADP was found to bind covalently to the α- and β-subunits of F_1-ATPase; this binding was paralleled by a lowering of the hydrolytic activity of F_1-ATPase. Evidence is given, which shows that the ADP moiety of NAP_4–ADP is responsible for the binding specificity of the whole molecule to F_1-ATPase.

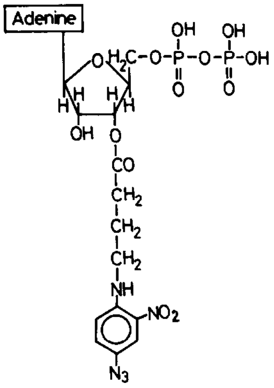

Fig.1. Structure of *N*-4-azido-2-nitrophenylaminobutyryl–ADP (NAP_4–ADP).

2. Materials and methods

2.1. *Materials*

[4-^{3}H]Aminobutyric acid (25 Ci/mmol), *N*-[^{14}C]ethylmaleimide (41 Ci/mol) and 4-chloro-7-nitro-[^{14}C]benzo-2-oxo-1-3-diazole (109 Ci/mol) were obtained from the Commissariat à l'Energie Atomique, Saclay, France. They were diluted before use with the unlabeled compounds to the appropriate specific radioactivity. [γ-^{32}P]ATP was prepared according to Glynn and Chappell [3] and labeled to about 10^9 dpm/μmol.

2.2. *Synthesis of tritiated* N-4-*azido-2-nitrophenylaminobutyric acid ([^3H]NAP_4) and tritiated* N-4-*azido-2-nitrophenylaminobutyryl–ADP ([^3H]NAP_4–ADP)*

Synthesis of [^{3}H]NAP$_4$ was carried out by the method of Fleet et al. [4] applied to [4-^{3}H]aminobutyric acid. [^{3}H]NAP$_4$–ADP was synthesized according to Jeng and Guillory [5]. [4-^{3}H]Aminobutyric acid was used instead of radio-labeled ADP. This choice was dictated by the finding that heat treatment of the NAP$_4$–ADP–F$_1$-ATPase complex, prior to SDS-polyacrylamide gel electrophoresis, damages the ADP moiety of NAP$_4$–ADP, but not the NAP$_4$ portion of the molecule.

2.3. *Biological assays*

F$_1$-ATPase was prepared from beef heart mitochondria according to Knowles and Penefsky [6], and stored at 4°C as an ammonium sulfate precipitate. Prior to treatment with [^{3}H]NAP$_4$–ADP, the suspension was centrifuged and the pellet solubilized in 50 mM Tris–HCl, pH 8, 0.1 M NaCl and 2 mM MgCl$_2$ to a concentration of 1–2 mg/ml. The F$_1$-ATPase solution was dialysed for 3 h at 20°C against two changes of 200 volumes of the same buffer. The activity recovery was more than 95%.

ATPase activity was assayed at 30°C either by an isotopic method using [^{32}P]ATP as substrate or by a method using a regenerating system. Detailed conditions of incubation are given in Results. In both methods, the incubation was carried for 15 sec and 1 min, and terminated by addition of cold trichloracetic acid. In the isotopic method inorganic phosphate was extracted according to Nielsen and Lehninger [7] and the ^{32}P radioactivity was measured in the extracts. In the other method using the regenerating system, the phosphate released by ATP hydrolysis was estimated in the trichloracetic acid extracts according to Fiske and Subbarow [8].

Protein concentration was estimated according to Lowry et al. [9] with bovine serum albumin as standard.

2.4. *Photolabeling experiments*

About 0.2 mg of F$_1$-ATPase in 0.1–0.2 ml of 50 mM Tris–HCl buffer (pH 8), 0.1 M NaCl and 2 mM MgCl$_2$ was introduced with [^{3}H]NAP$_4$–ADP in a 4 ml tube which was rotated horizontally at 150 rev./min in a water bath at 20°C and illuminated with visible light (10 cm from an Osram lamp 250 W equipped with a filter to cut off radiation below 300 nm).

2.5 *Gel electrophoresis*

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [8] using 10.3% acrylamide gel. Sodium dodecylsulfate and β-mercaptoethanol were added to F$_1$-ATPase to a final concentration of 1% each with traces of Bromophenol Blue as tracking dye; the solution was heated for 10 min at 100°C. The gel tubes had a diameter of 0.6 cm and a length of 12 cm. Electrophoresis was carried out at 5 mA per gel for 16–22 h. Staining with Coommassie Blue and destaining were performed as described in [10]. The following proteins were used as molecular weight standards: bovine serum albumin 68 000, ovalbumin 42 000, triose phosphate isomerase 27 500 and cytochrome *c* 12 400. After scanning in a Chromoscan gel scanner, the gels were sliced in a Gilson slicer. The radioactivity of each slice (0.5 mm) after digestion overnight by 1 ml of 10% H$_2$O at 65°C, was measured by liquid scintillation counting.

3. Results

3.1. *Photoinactivation of F_1-ATPase by NAP_4–ADP and NAP_4*

When F$_1$-ATPase was irradiated in the presence of NAP$_4$–ADP, a marked loss in hydrolytic activity was observed (fig.2A). This loss depended on the concentration of NAP$_4$–ADP. In contrast incubation of F$_1$-ATPase in the dark with NAP$_4$–ADP resulted in a moderate inhibition of the ATPase activity (fig.2A). For example, the rate of ATP hydrolysis was inhibited by 60% when F$_1$-ATPase was incubated for 30 min with 50 μM NAP$_4$–ADP in the light and only by 20% when incubated for the same period of time with the same concentration of NAP$_4$–ADP in the dark. NAP$_4$–ADP behaved as a competitive inhibitor with respect to ATP (fig.3), the K_i value was of the order of 0.6 mM. This is to be compared with the K_i value for ADP which is about 50 μM (not shown).

To evaluate possible unspecific effects due to the NAP$_4$ moiety of NAP$_4$–ADP, the hydrolytic activity

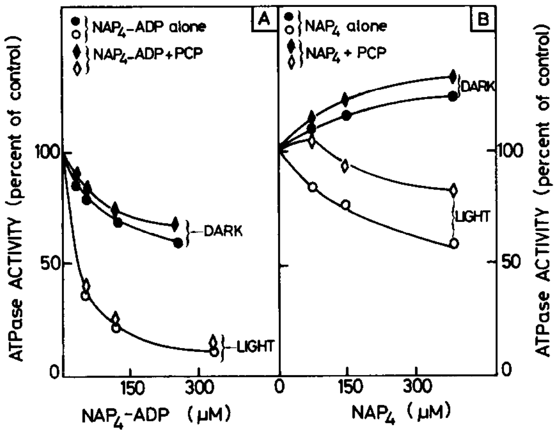

Fig.2. Effect of NAP_4–ADP and NAP_4 on the hydrolytic activity of F_1-ATPase in the dark and in the light. Interaction with pentachlorophenol (PCP). NAP_4–ADP (fig.2A) and NAP_4 (fig.2B) were incubated in the dark or in the light with F_1-ATPase in the presence or absence of pentachlorophenol (PCP). When present, PCP was at the final concentration of 0.3 mM. ATP hydrolysis was induced by addition of an aliquot fraction of the F_1-ATPase solution (10 μg), treated as described above, in a medium made of 4 mM phosphoenolpyruvate, 20 μg pyruvate kinase, 10 mM ATP, 5 mM $MgCl_2$, 40 mM Tris–HCl (pH 8), in a volume of 0.5 ml. Other conditions are given in Materials and methods. The rate of ATP hydrolysis in the control was 80 μmol/min/mg protein.

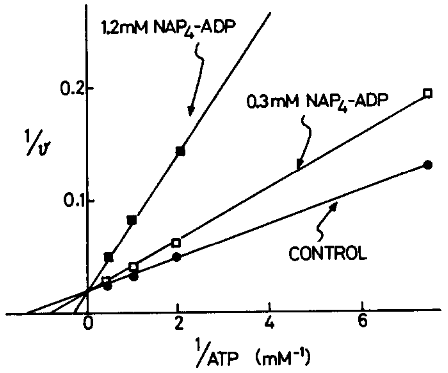

Fig.3. Kinetics of inhibition of ATPase activity by NAP_4–ADP incubated with F_1-ATPase in the dark. The incubation medium was made of 50 mM Tris–HCl (pH 8), 0.1 M NaCl and 2 mM $MgCl_2$. The substrate, $[^{32}P]$ATP, was used at varied concentrations. Other conditions are given in Materials and methods. Rates are given in μmoles of phosphate released per min per mg protein.

of F_1-ATPase has been tested in the presence of NAP_4, in the dark and in the light (fig.2B). NAP_4 in the dark increased the hydrolytic activity of F_1-ATPase; a similar effect has been reported for FNAP [11] and the classical uncouplers 2,4-dinitrophenol [12,13] and pentachlorophenol [12] although NAP_4 at concentrations as high as 10 mM does not uncouple oxidative phosphorylation. On the other hand upon photo-irradiation with NAP_4, the activity of F_1-ATPase was inhibited as already mentioned for NAP_4–ADP. Although F_1-ATPase was photoinactivated by NAP_4–ADP and NAP_4, the extent of inhibition was greater with NAP_4–ADP than with NAP_4. For example, 50 μM NAP_4–ADP caused an inhibition of 60% (fig.2A) and 75 μM NAP_4 an inhibition of only 15% (fig.2B). That NAP_4–ADP is more inhibitory than NAP_4 is probably due to the specific interaction of the ADP moiety of NAP_4–ADP with the ADP/ATP site(s) of F_1-ATPase. This interpretation is substantiated by the finding that pentachlorophenol, a molecule structurally related to NAP_4, interferes with the effect of NAP_4 on F_1-ATPase, but not with that of NAP_4–ADP. For example pentachlorophenol markedly decreased photoinactivation of F_1-ATPase by NAP_4 (fig.2B), but did not affect photoinactivation of F_1-ATPase by NAP_4–ADP (fig.2A).

3.2. *Photolabeling of F_1-ATPase by $[^3H]NAP_4$–ADP and $[^3H]NAP_4$*

Photoinactivation of F_1-ATPase incubated with NAP_4–ADP was accompanied by the covalent binding of $[^3H]NAP_4$–ADP to the enzyme. This is parallel with the report by Russell et al. [2], who used NAP_3–ATP in their experiments. A further step to be described here is the identification of the labeled subunits. After irradiation with $[^3H]NAP_4$–ADP, the F_1-ATPase solution was dialysed to remove the unreacted compound; then it was treated with sodium dodecylsulfate and β-mercaptoethanol as described in Materials and methods, and subjected to gel electrophoresis. As shown in fig.4 the radioactivity was concentrated in a peak corresponding to the α-β-subunits of F_1-ATPase. Subunits γ, δ and ϵ did not retain any significant radioactivity.

For a more precise assignment of the labeling, advantage was made of the rather selective binding of *N*-ethylmaleimide (NEM) to the α-subunit [14] and

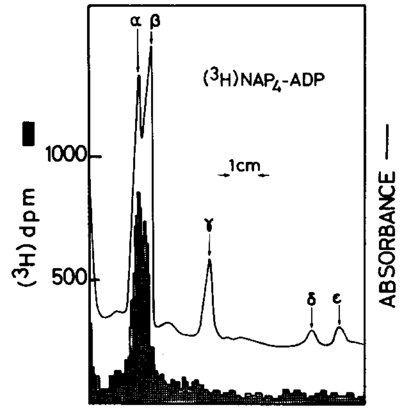

Fig.4. Gel electrophoresis in presence of sodium dodecylsulfate of F_1-ATPase covalently labeled by [^{3}H]NAP_4–ADP. *F_1*-ATPase was incubated in the light at pH 8 with 20 μM [^{3}H]NAP_4–ADP. The figure shows the densitometric tracing of the stained gel and the corresponding radioactivity pattern. Details are given in Materials and methods.

of 4-chloro-7-nitrobenzo-2-oxo-1,3 diazole (NBD) to the β-subunit [15,16]. In a double labeling experiment F_1-ATPase was incubated first with [^{3}H]NAP_4–ADP in the light and then with either [^{14}C]NEM or [^{14}C]NBD. The radioactivity profiles presented in fig.5 indicate that both the α- and β-subunits are labeled by [^{3}H]NAP_4–ADP.

Preincubation of F_1-ATPase with either ADP, ATP or adenylimidodiphosphate (AMPPNP) before addition of [^{3}H]NAP_4–ADP and photo-irradiation resulted in a marked decrease of the bound radioactivity, which strongly suggests that the same binding site is recognized by NAP_4–ADP, ADP, ATP and AMPPNP (table 1). Quercetin, an inhibitor which mimics the effect of the natural ATPase inhibitor [17,18] inhibited the binding of NAP_4–ADP on both α- and β-subunits of F_1-ATPase. When added to a concentration of 45 μM, quercetin was able to prevent half of the binding of NAP_4–ADP added to a concentration of 54 μM.

No attempt was made to calculate accurately the binding stoichiometry of NAP_4–ADP to F_1-ATPase in the light. However, data in table 1 show that the amount of covalently-bound [^{3}H]NAP_4–ADP increases with the concentration of the added ligand. When [^{3}H]NAP_4–ADP was added at a final concentration of 108 μM, a saturation level was approached; at this concentration the amount of covalently bound [^{3}H]NAP_4–ADP was 0.95 mol/mol of F_1-ATPase (table 1) and 80% of the ATPase activity was lost (fig.2A). It must be noted that loss in ATPase activity following light irradiation may not be entirely due to photo-activated NAP_4–ADP since ATPase activity is already partially inhibited by NAP–ADP in the dark (see fig.1). Therefore the above value of covalently-bound NAP_4–ADP (0.95 mol/mol of F_1-ATPase) may correspond to less than 80% photo-inactivation.

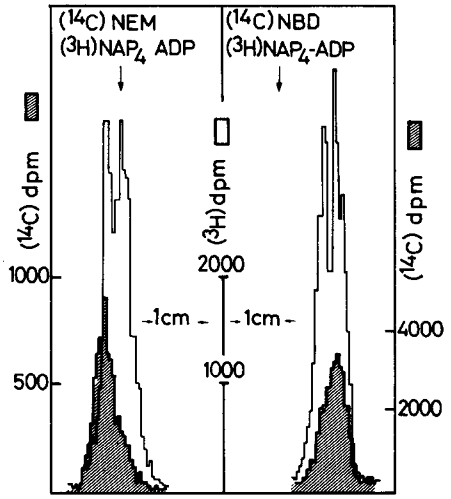

Fig.5. Gel electrophoresis in presence of sodium dodecylsulfate of F_1-ATPase after double covalent labeling by [^{3}H]NAP_4–ADP and by either [^{14}C]NEM or [^{14}C]NBD. (a) *Labeling by [^{3}H]NAP_4–ADP and [^{14}C]NEM (left hand side).* F_1-ATPase was incubated in the light for 30 min at pH 8 with 24 μM [^{3}H]NAP_4–ADP as described in Materials and methods. Then the pH was adjusted to 6.5 by addition to 0.1 N HCl, and [^{14}C]NEM was added to a final concentration of 5 mM and left to react in the dark at 25°C for 2 h. (b) *Labeling by [^{3}H]NAP_4–ADP and [^{14}C]NBD (right hand side).* After photo-irradiation of F_1-ATPase with [^{3}H]NAP_4–ADP as described above, the pH was adjusted to 7, and [^{14}C]NBD was added to the final concentration of 0.25 mM and left to react for 2 h in the dark at 25°C. Then the pH was brought to 9 by addition of 0.1 N NaOH and the sample was incubated in the dark for a further 2 h period.

3.3. *Effect of pentachlorophenol on photolabeling of F_1-ATPase with [^{3}H]NAP_4–ADP and [^{3}H]NAP_4*

As shown in table 2, pentachlorophenol did not interfere with the photocovalent labeling of F_1-ATPase by [^{3}H]NAP_4–ADP, but it markedly decreased the

Table 1
Photo-dependent covalent binding of [^{3}H]NAP$_4$–ADP to F$_1$-ATPase. Dependence on the concentration of [^{3}H]NAP$_4$–ADP. Effect of added adenine-nucleotides

Additions	Bound [^{3}H]NAP$_4$–ADP nmol/nmol F$_1$
[^{3}H]NAP$_4$–ADP (15 μM)	0.61
[^{3}H]NAP$_4$–ADP (15 μM) + ADP (45 μM)	0.40
[^{3}H]NAP$_4$–ADP (15 μM) + ADP (125 μM)	0.17
[^{3}H]NAP$_4$–ADP (15 μM) + ADP (1 mM)	0.05
[^{3}H]NAP$_4$–ADP (15 μM) + ADP (5 mM)	0.01
[^{3}H]NAP$_4$–ADP (15 μM) + ATP (2 mM)	0.01
[^{3}H]NAP$_4$–ADP (54 μM)	0.75
[^{3}H]NAP$_4$–ADP (54 μM) + AMPPNP (2 μM)	0.35
[^{3}H]NAP$_4$–ADP (54 μM) + AMPPNP (45 μM)	0.20
[^{3}H]NAP$_4$–ADP (54 μM) + AMPPNP (160 μM)	0.06
[^{3}H]NAP$_4$–ADP (108 μM)	0.95

Experimental conditions for photo-labeling were as described in Materials and methods. ADP, ATP and AMPPNP were added together with [^{3}H]NAP$_4$–ADP prior to photo-irradiation. Following photo-irradion, F$_1$-ATPase was submitted to sodium dodecylsulfate acrylamide gel electrophoresis. Bound [^{3}H]NAP$_4$–ADP was calculated from the radioactivity present in the α–β-peaks of F$_1$-ATPase

Table 2
Effect of pentachlorophenol (PCP) on the photo-dependent covalent binding of [^{3}H]NAP$_4$–ADP and of [^{3}H]NAP$_4$

Additions	Bound [^{3}H]NAP$_4$ or [^{3}H]NAP$_4$–ADP nmol/nmol F$_1$-ATPase
[^{3}H]NAP$_4$–ADP	0.65
[^{3}H]NAP$_4$–ADP + 150 μM PCP	0.62
[^{3}H]NAP$_4$–ADP + 800 μM PCP	0.53
[^{3}H]NAP$_4$	0.10
[^{3}H]NAP$_4$ + 110 μM PCP	0.04
[^{3}H]NAP$_4$ + 550 μM PCP	0.02

Conditions are as described in table 1. [^{3}H]NAP$_4$–ADP and [^{3}H]NAP$_4$ were present at a final concentration of 15 μM and 40 μM respectively. PCP was added together with [^{3}H]NAP$_4$ or [^{3}H]NAP$_4$–ADP prior to photo-irradiation

amount of covalently bound [^{3}H]NAP$_4$. These results are in agreement with the data of fig.1, in which it was shown that pentachlorophenol did not affect photo-inactivation of F$_1$-ATPase by NAP$_4$–ADP, and in contrast prevented photo-inactivation of F$_1$-ATPase by NAP$_4$.

4. Discussion

This paper describes the preparation and use of tritiated NAP$_4$–ADP as a photo-affinity reagent for isolated F$_1$-ATPase, and the identification of the two labeled subunits from the ATPase–NAP$_4$–ADP complex after photo-irradiation. The fact that photo-activation of NAP$_4$–ADP can be achieved under visible light could be regarded as an advantageous feature of the NAP$_4$–ADP molecule because visible light, in contrast to UV irradiation, does not damage proteins. However, the hydrolytic activity of F$_1$-ATPase is readily sensitive to UV irradiation only below 280 nm (unpublished data).

A problem inherent to the use of NAP$_4$–ADP in photo-labeling experiments is that the aryl group may bind to uncoupler binding sites on the ATPase as suggested by the following data obtained with 2-azido-4-nitrophenol (NPA) and 4-fluoro-3-nitrophenylazide (FNAP). NPA, which is an uncoupler of oxidative phosphorylation, binds covalently upon photo-activation to two subunits of the ATPase complex; one of these subunits appears to be the α-subunit of F_1-ATPase, the other one is an uncoupler binding protein [19]. FNAP is also able to bind upon photoactivation at a site which is shared by the uncoupler 2-4-dinitrophenol although FNAP per se does not uncouple the mitochondrial oxidative phosphorylation [11]. NAP_4–ADP, like FNAP and NAP_4, does not uncouple oxidative phosphorylation. Its binding specificity mainly depends on the nucleotide moiety of the NAP_4–ADP molecule, as indicated by the following observations: (1) when F_1-ATPase was pre-incubated in the dark with ADP, ATP or AMPPNP together with $[^3H]NAP_4$–ADP prior to photo-irradiation, the photo-labeling of α- and β-subunits was strongly decreased; (2) when incubated in the light with F_1-ATPase, $[^3H]NAP_4$ was incorporated to a much smaller degree (five to ten times less) than $[^3H]NAP_4$–ADP; (3) the uncoupler pentachlorophenol competed efficiently with $[^3H]NAP_4$ but not with $[^3H]NAP_4$–ADP for binding to F_1-ATPase.

Wagenvoord et al [1] have shown that only the β-subunit of F_1-ATPase is photo-labeled by $[8\text{-}^3H]$azido-ATP. We have found that $[^3H]NAP_4$–ADP binds covalently upon photo-irradiation to both the α- and β-subunits of F_1-ATPase. The apparent contradiction between these two results may be explained by the respective geometry of the two photo-affinity reagents. Because of the proximity of the azido group and ATP in 8-azido-ATP, the nitrene which is photogenerated from 8-azido-ATP is expected to bind covalently to F_1-ATPase just at the site to which the ATP moiety of the molecule is attached, i.e. on the β-subunit. In the case of NAP_4–ADP, the arylazido group is separated from the ribose portion of ADP by a bridge of four carbon groups. Assuming that the ADP moiety of NAP_4–ADP binds to the β-subunit of F_1-ATPase like the ATP moiety of 8-azido-ATP, it is inferred that the arylnitrene group of NAP_4–ADP, because of the length of the carbon bridge, interacts with and binds covalently not only with the β-subunit, but also with the α-subunit of F_1-ATPase. This would require that the ADP binding site on the β-subunit is close to the α-subunit. This postulate agrees with the results of cross linking experiments [20] which have shown that α- and β-subunits in isolated ATPase are close to each other. Photo-labeling experiments are presently extended to NAP_n–ADP derivatives where the number n of carbon units between the ribose and the NAP group is varied in the hope of further delineating the topology of adenine nucleotide sites in F_1-ATPase.

Acknowledgements

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References

[1] Wagenvoord, R. J., Van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17–24.

[2] Russell, J., Jeng, S. J. and Guillory, R. J. (1976) Biochem. Biophys. Res. Commun. 70, 1225–1234.

[3] Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149.

[4] Fleet, G. W. J., Knowles, J. R. and Porter, R. R. (1972) Biochem. J. 128, 499–508.

[5] Jeng, S. J. and Guillory, R. J. (1975) J. Supramol. Biol. 3, 448–468.

[6] Knowles, A. F. and Penefsky, H. S. (1972) J. Biol. Chem. 247, 6617–6623.

[7] Nielsen, S. O. and Lehninger, A. L. (1955) J. Biol. Chem. 215, 555–570.

[8] Fiske, C. A. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.

[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.

[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.

[11] Senior, A. E. and Timetsko, A. M. (1975) in: Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. and Siliprandi, N., eds), pp. 155–160, North-Holland, Amsterdam.

[12] Stockdale, M. and Selwyn, M. J. (1971) Eur. J. Biochem. 21, 416–423.

[13] Cantley, L. W. and Hammes, G. G. (1973) J. Biol. Chem. 12, 4900–4904.

[14] Senior, A. E. (1975) Biochemistry 14, 660–664.

[15] Ferguson, S. J., Lloyd, W. J. and Radda, J. K. (1975) Eur. J. Biochem. 54, 127–133.

[16] Deters, D. W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041–1047.

[17] Lang, D. R. and Racker, E. (1974) Biochim. Biophys. Acta 333, 180–186.

[18] Di Pietro, A., Godinot, C., Bouillant, M. L. and Gautheron, D. C. (1975) Biochimie 57, 959–967.

[19] Hanstein, W. G. (1976) Biochim. Biophys. Acta 456, 129–148.

[20] Satre, M., Klein, G. and Vignais, P. V. (1976) Biochim. Biophys. Acta 453, 111–120.