Journal of Bioenergetics and Biomembranes, Vol. 17, No. 1, 1985

Interaction of Fluorescent Berberine Alkyl Derivatives with Respiratory Chain of Rat Liver Mitochondria

Vladimír Mikeš,[1,2] and Lev S. Yaguzhinskij[3]

Received December 19, 1983; revised May 23, 1984

Abstract

The cationic fluorescent dyes, berberines, have been observed to inhibit NAD-linked respiration in rat liver mitochondria. Low concentrations inhibit electron transport in the NAD-ubiquinone span after penetration into mitochondria. More hydrophobic alkyl derivatives proved to be stronger inhibitors showing more rapid onset of inhibition. The inhibition was totally dependent on the energization of the membrane; however, the addition of a hydrophobic anion stimulated the inhibition effects in uncoupled mitochondria. Substantially higher concentrations of berberines are needed for the inhibition of the oxidation of succinate. The excess of dye interacting with surface dipoles in the energized state can inhibit the energy transduction through the complex bc_1. On the basis of the difference in the rate of fluorescence response when berberines are added to coupled mitochondria and the corresponding inhibition effects, the presence minimally of two binding sites was suggested. The dye bound on the outer surface is highly fluorescent and inhibits the energy transduction if added in excess. The remaining dye interacting with NADH dehydrogenase does not fluoresce. The accumulation of alkylberberine in mitochondria results in additional effects in the region of cytochrome *b* the nature of which is not fully understood.

Key Words: Rat liver mitochondria; NADH dehydrogenase; complex bc_1; inhibition; fluorescent berberine derivatives.

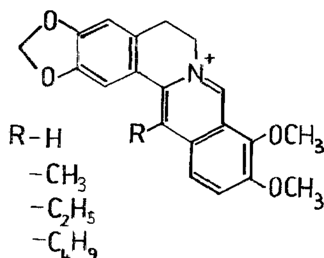
Introduction

Many organic cations are reported to inhibit energy transduction in the respiratory chain of rat liver mitochondria. Thus the interactions of alkylguanidines (Pressman, 1963; Chappel, 1963; Chance and Holunger, 1963; Papa *et al.*, 1975), cyanide dyes (Conover and Schneider, 1981), and

[1]Department of Biochemistry, Purkyně University, 61137 Brno, Czechoslovakia.
[2]To whom correspondence should be addressed.
[3]Belozerskij Institute, Moscow State University, 117234 Moscow, USSR.

0145-479X/85/0200-0023$04.50/0 © 1985 Plenum Publishing Corporation

cetylpyridinium cation (Chavez and Bravo, 1981) with NADH dehydrogenase were investigated. The inhibition is probably caused by the presence of an anionic lipophilic center in site I (Pressman, 1963). Moreover, it was reported that some cationic dyes inhibit nonspecifically the oxidation of succinate in coupled rat liver mitochondria (Higuti *et al.*, 1978, 1980a, b) and can affect respiration through their interaction with negative surface charges (Higuti *et al.*, 1978). The inhibition with cationic dyes seems to be an energy-requiring process and, with increasing lipophilicity of the derivatives, the inhibition becomes more potent (Pressman, 1963). The berberine alkyl derivatives were used as suitable probes for the fluorimetric investigation of



the energized state of mitochondria (Mikeš and Dadák, 1983). Their fluorescence markedly increased after their interaction with the mitochondrial membrane and the membrane of liposomes (Mikeš and Kovář, 1981).

We present here a study of the kinetics of the fluorescence changes and the nature of the possible inhibition sites associated with the binding of berberines to rat liver mitochondria.

Materials and Methods

Mitochondria from rat liver were isolated in a medium containing 0.3 M sucrose, 5 mM Tris-Cl, and 0.25 mM EDTA adjusted to pH 7.7 according to Schneider and Hogeboom (1950). Oxygen consumption was recorded with a Clark-type electrode. The incubation medium contained 0.2 M sucrose, 30 mM Tris-Cl, pH 7.4, 10 mM KH_2PO_4, 5 mM $MgSO_4$, 10 mM KCl, and 0.25 mM EDTA. The absorption difference spectra of cytochromes were measured with an Aminco-Chance DW-2 Spectrophotometer. The redox state of cytochromes *b* and *c* was calculated from $\Delta A_{562-575}$ and $\Delta A_{550-535}$, respectively. The fluorescence was recorded with a Hitachi–Perkin-Elmer fluorescence spectrophotometer. The membrane potential was calculated on the basis of the distribution of the tetraphenylphosphonium[4] cation. The

[4]Abbreviations: FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium cation; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

concentration of TPP^+ in the external medium was measured with the sensitive electrode described by Kamo *et al.* (1979). The alkyl derivatives of berberine were synthesized and characterized as described by Pavelka and Kovář (1976). All other reagents were commercially available products of the highest purity.

Results

The oxidation of substrates involving NADH dehydrogenase was completely inhibited after a sufficient berberine concentration had been added to rat liver mitochondria. Figure 1 presents an oxygen electrode trace of experiments where the influence of the alkyl chain length on the inhibition

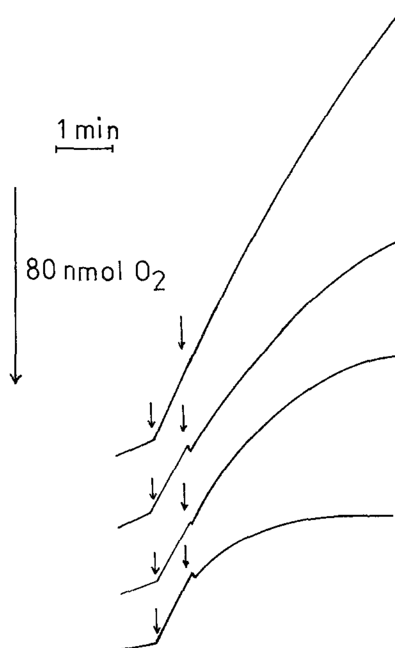


Fig. 1. Effect of alkylberberines on the oxygen consumption of rat liver mitochondria. 4 mM glutamate with 4 mM malate, concentration of derivatives 20 μM, concentration of mitochondria 1.2 mg protein/ml. First addition: 1 mM ADP, second addition: alkylberberine; from upper to lower trace: berberine, methyl-, ethyl-, and butylberberine.

potency of our derivatives was studied. As expected, the increasing hydrophobicity of the derivatives results in a marked increase of the inhibition effects. The inhibition was time dependent. The kinetics of the inhibition was very slow in the case of unsubstituted berberine and relatively fast in the case of butylberberine. After preincubation of mitochondria (15 min) with the berberine derivatives in the absence of substrate, the

Table 1. Inhibitory Effect of Alkylberberines on the NAD-Linked Respiration of Rat Liver Mitochondria after Equilibrium Redistribution in Membrane[a]

Derivative	$I_{50}{}^{b}$ (μM)
Berberine	9.0
Methylberberine	5.5
Ethylberberine	4.0
Butylberberine	1.5

[a] Mitochondria (0.7 mg protein/ml) were preincubated with derivatives in the incubation medium 15 min. The NADH oxidase activity was measured in the presence of 4 mM glutamate with 4 mM malate and 1 mM ADP.
[b] Inhibition efficiency is expressed in terms of concentration needed to give 50% inhibition.

inhibition reached maximal value and could be measured in state 3 after the addition of glutamate with malate. During this period the activity of uninhibited NADH oxidase remained constant. The concentrations needed to give 50% inhibition by the series of the derivatives are listed in Table I. Butylberberine was the most efficient derivative tested so far. It is worth noting that the addition of 5 μM menadione to this mixture reverses the inhibition only to 60% of the original activity although this concentration of menadione was quite sufficient to restore the oxidase activity in mitochondria inhibited with rotenone. The data suggest the involvement of several inhibition sites during electron transfer from NADH to oxygen. Figure 2 presents a detailed picture of the relation between the inhibition effect and the membrane potential. It is evident from this figure that berberines inhibit the respiration of coupled mitochondria (trace b), and the uptake of oxygen stimulated with FCCP seems to be only slightly affected (trace a). If FCCP was added to mitochondria once inhibited with alkylberberines, the inhibition was released very slowly after a time lag (trace c). This result may indicate that berberines move electrophoretically and are concentrated in a site of the inner membrane or in the mitochondrial matrix. Picrate, which increases the membrane permeability for cations, stimulates markedly the inhibition effects of berberines in uncoupled mitochondria (compare traces a and d). If uncoupled mitochondria were preincubated with picrate and berberines, oxygen uptake commenced at the uninhibited rate upon the addition of substrate, followed by a progressive inhibition (Fig. 2e). On the other hand, the interaction of coupled mitochondria occurred during preincubation with berberines in the absence of exogenous substrate, as indicated by the initially oxygen uptake upon addition of substrate (Fig. 2f).

The influence of ethylberberine on the oxidation of succinate is shown in Fig. 3. The oxidation of succinate is less sensitive to the different derivatives than that of NADH. The fact that this effect can be completely

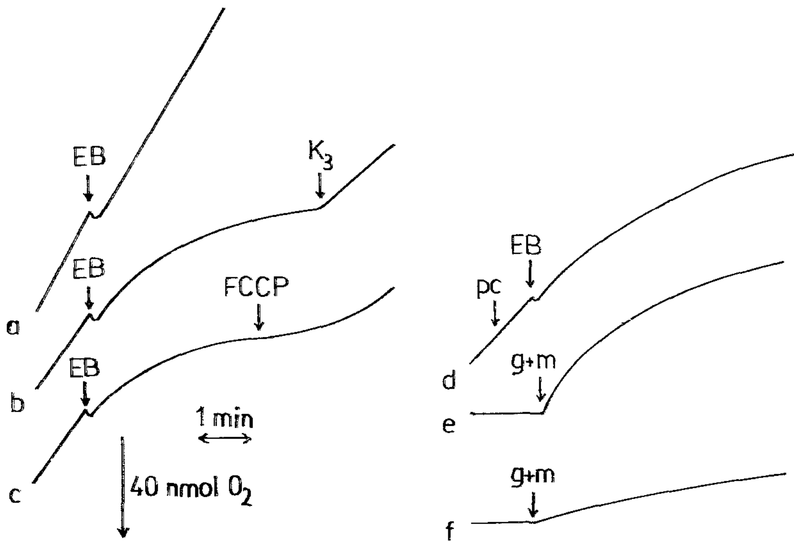


Fig. 2. Effect of ethylberberine on the oxygen consumption of rat liver mitochondria in different metabolic states. 4 mM glutamate with 4 mM malate, 1.2 mg protein of mitochondria/ml, 25 μM ethylberberine. (a) Mitochondria uncoupled with 0.1 μM FCCP; (b) 1 mM ADP, 5 μM menadione, (K_3); (c) 1 mM ADP, uncoupling with 0.1 μM FCCP; (d) uncoupled mitochondria treated with 1 μM picrate (pc); (e) mitochondria preincubated with 0.1 μM FCCP, 1 μM picrate, and 25 μM ethylberberine; concentration of glutamate (g) and malate (m) 4 mM; (f) mitochondria preincubated with 1.5 mM ADP and 25 μM ethylberberine.

abolished with TMPD which bypasses the region of cytochromes bc_1 suggests that the site of inhibition may be in this complex (Fig. 3a). Moreover, if the artificial donor of cytochrome *b*, menadione reduced with the internal menadione reductase system, was used, the inhibition was identical as it was in the case of the oxidation of succinate (trace b). The membrane potential proved to be a prerequisite for the inhibition of succinate oxidation and oxidation of NADH (Fig. 3c). However, ethylberberine acted immediately after the addition to coupled mitochondria, and the effect was not progressive. Similarly the inhibition was released immediately after uncoupling with FCCP (Fig. 3d). In state 4, 35 μM ethylberberine acted as a weak uncoupling agent: the P/O ratio decreased only by 10%. It should be noted that the effect of the uncoupler was rather complicated. FCCP (0.1 μM) uncoupled mitochondria completely, and under these conditions berberines were not inhibiting (trace c). But if the concentration of FCCP was increased to about 0.5 μM, renewal of the inhibition was observed (Fig. 3e).

The absorption difference spectra of cytochromes *b* and *c* are shown in Fig. 4. We have observed an apparent oxidation of cytochromes *c* and aa_3

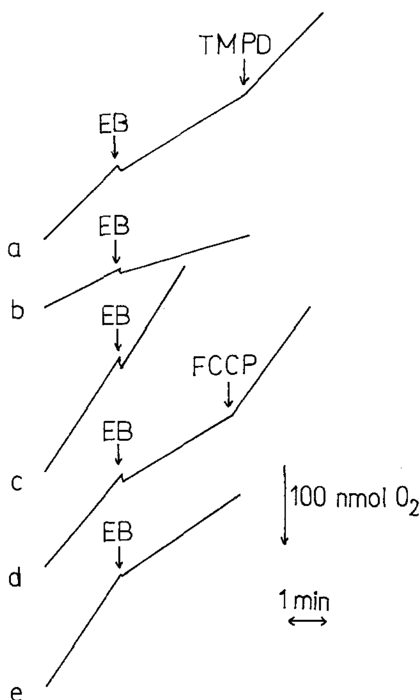


Fig. 3. Effect of ethylberberine on oxidation of succinate in rat liver mitochondria in different metabolic states. 10 mM succinate, 25 μM ethylberberine (EB), 0.6 mg protein of mitochondria/ml. (a) 1 mM ADP, 0.4 mM TMPD; (b) succinate replaced with 5 mM glutamate, 5 mM malate, and 5 μM menadione; 1 mM ADP; (c) mitochondria uncoupled with 0.1 μM FCCP; (d) 1 mM ADP, release of the inhibition with 0.1 μM FCCP; (e) mitochondria uncoupled with 0.5 μM FCCP.

upon addition of ethylberberine to mitochondria in state 4 (trace b). The redox state of cytochrome *b* remained unchanged or slightly more reduced to about 60%. This confirms the suggestion that positively charged ethylberberine can induce some specific effects in the region of cytochrome bc_1. The subsequent addition of cyanide causes complete reduction of cytochromes *c* and aa_3 while cytochrome *b* remains reduced to only 60–70% (trace c). The picture remained unchanged if a low concentration of FCCP was added just before cyanide (trace d). Although the oxidation of succinate is not more inhibited after uncoupling (Fig. 3d) the ability of cytochrome *b* to be reduced during the electron transfer seems to be restricted for a long time. On the other hand, cytochrome *b* was unaffected by high concentrations of ethylberberine if reduced previously with an artificial donor, the menadione reductase system, i.e., full reduction of cytochrome *b* was reached (Fig. 4f) although the oxidation of this artificial donor was inhibited (Fig. 3b). Thus the behavior of cytochrome *b* after interaction with ethylberberine does not correspond to the state of inhibition in complex bc_1.

Figure 5 shows the relationship between the inhibition of NADH oxidation, fluorescence of ethylberberine, and membrane potential. The experiment was carried out in parallel in three vessels where the media were

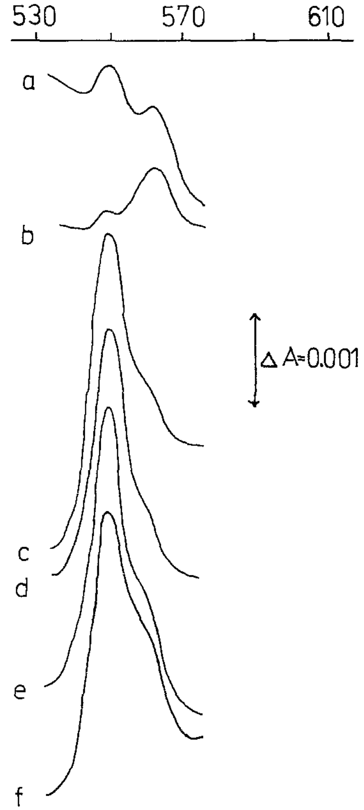


Fig. 4. Difference absorption spectra of cytochromes after the action of berberines. 0.4 mg protein of mitochondria/ml. First experiment: state 4, 10 mM succinate (a); following addition of 25 μM ethylberberine (b); following addition of 2 mM KCN (c). Second experiment: 10 mM succinate, 25 μM ethylberberine; after 30 sec, 0.1 μM FCCP and 2 mM KCN were added (d). Third experiment: full redox state, 10 mM succinate with 2 mM KCN (e). Fourth experiment: 5 mM glutamate, 5 mM malate with 5 μM menadione, 25 μM ethylberberine; after 30 sec, 2 mM KCN was added (f).

identical. To the reaction mixture containing substrate, ethylberberine and TPP^+ mitochondria were added, and estimates of the oxygen consumption, the fluorescence of ethylberberine, and the potential of the electrode sensitive to TPP^+ were made. In the equilibrium state the activity of NADH oxidase was close to zero, but it can be shown that the transmembrane potential decreases only by 40 mV. The fall of transmembrane potential after the addition of FCCP causes a quick redistribution of ethylberberine in the membrane, resulting in the decrease of the fluorescence emission followed by the very slow release of inhibition.

Discussion

The results presented in this paper show that berberines are effective inhibitors of the coupled NAD-linked respiration in rat liver mitochondria. The mechanism of inhibition is very similar to that suggested for

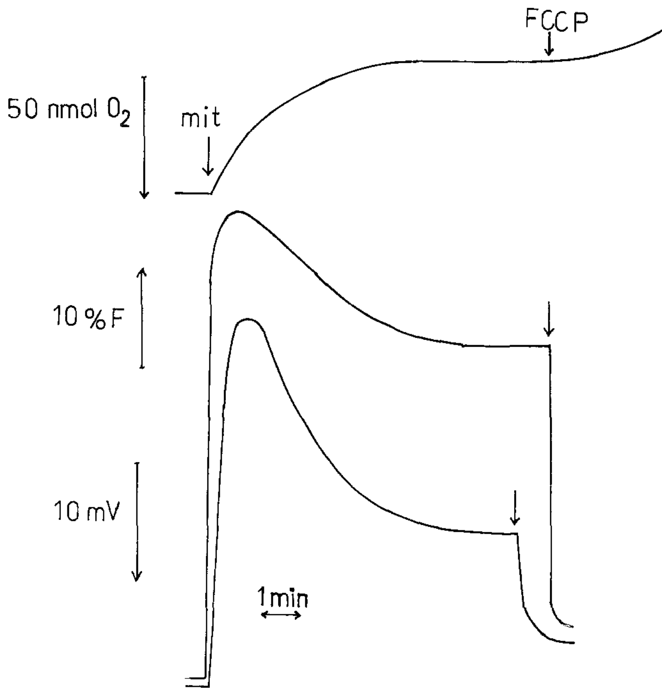


Fig. 5. Comparison of ethylberberine inhibition, ethylberberine fluorescence, and transmembrane potential in rat liver mitochondria. To the incubation medium containing 13 μM ethylberberine, 1.5 mM ADP, 4 mM glutamate, 4 mM malate, and 1 μM TPP^+, mitochondria were added (0.8 mg protein/ml); 0.1 μM FCCP. Upper trace: oxygen consumption; middle trace: fluorescence excited at 420 nm, emitted at 520 nm; lower trace: potential of electrode sensitive to TPP^+.

alkylguanidines (Papa *et al.*, 1975), cyanine dyes (Conover and Schneider, 1981), or cetylpyridinium bromide (Chavez and Bravo, 1981). The respiration of inhibited mitochondria remained suppressed for a long time after the addition of FCCP (Fig. 5). This fact suggests a possible direct inhibitory action of berberines at the NAD–ubiquinone span of the respiratory chain. The membrane potential proved to be as prerequisite for the inhibition with berberines and other cations mentioned above. We have found that berberine derivatives that are more hydrophobic are more potent inhibitors with more rapid kinetics of inhibition. It is striking that the most effective cations, i.e., cyanine dyes, alkylberberines, alkylguanidines, and cetylpyridinium bromide, possess an asymmetric structure with respect to the position of the charge. This is presumably why tetraphenylarsonium cation, safranine O, and ethidium bromide are not effective inhibitors of site I (Conover and Schneider, 1981; Higuti *et al.*, 1978, 1980a, b).

Substantially higher berberine concentrations are needed for inhibition of succinate oxidation. The effect is induced immediately after the addition of alkyl derivatives and disappears after the addition of the uncoupler (Fig. 3). Thus we assume that berberines inhibiting the oxidation of succinate are located on the water–membrane interface unlike the part of the dye interacting with NADH dehydrogenase. Complex bc_1 seems to be influenced mostly by the binding of the excess of dye because the addition of TMPD restored fully the oxidation activity of inhibited mitochondria. In addition, we observed a slight stimulation of cytochrome oxidase activity in the presence of ethylberberine (not shown). The results we have obtained are in agreement with those obtained with ethidium bromide (Higuti *et al.*, 1978), tetraphenylarsonium (Higuti *et al.*, 1980a), rhodamine 6G (Higuti *et al.*, 1980b), and alkylguanidines (Pressman, 1963). We suppose that the respiration can be inhibited through the nonspecific interaction of berberines with negative charges on the outer surface of the inner mitochondrial membrane. It is not clear why further increase in FCCP concentration leads to new inhibitory effects of berberines (Fig. 3e). It is possible that our derivatives possess other unrecognized inhibition properties when forming an ionic pair with negative charged FCCP, and caution should be taken in the use of negative uncouplers together with organic cations.

The results we obtained with berberines suggest the involvement of a secondary effect in the region of cytochromes bc_1 in addition to inhibition in the NADH–ubiquinone span and their nonspecific interaction with surface dipoles. It seems that these dyes change energy transfer through the complex III. Some experimental evidence may be cited in favor of this hypothesis: (1) Cytochrome *b* is changed upon the addition of berberines (Fig. 4). (2) The behavior of cytochrome *b* correlates with the effect of berberines on the oxidation of NADH and not succinate. The addition of FCCP to coupled mitochondria if succinate is the substrate is followed by the immediate release of inhibition (Fig. 3d). In spite of this, berberines remain in the membrane, as can be seen in the absorption spectra (Fig. 4d). The oxidation of the artificial donor, menadiol, is inhibited with excess berberines without the secondary effect on cytochrome *b* (Fig. 4e). (3) Inhibition of NADH oxidation by low concentrations of berberines cannot be fully eliminated with menadione although considerably higher concentrations are needed for the inhibition of complex III. This effect may be due to the influence of berberines which had penetrated into the membrane following the initiation of the menadione reductase bypass. If menadione were added before berberines, the bypass is completed and no special effect on cytochrome *b* is observed.

As we showed in the previous study, berberines fluoresce intensively when bound to mitochondria, and the effect is linearly dependent on the magnitude of the transmembrane potential (Mikeš and Dadák, 1983). The

interaction of berberines with the polarized mitochondrial membrane, reflected in the fluorescence emission, is relatively fast when compared with the kinetics of the inhibition of NADH oxidation. Similarly, the addition of uncoupler causes a very rapid fall of berberine fluorescence and release of the inhibition of succinate oxidation, while the oxidation of NADH remains suppressed for several minutes (Fig. 5). Thus we assume that dye bound on the outer surface of mitochondria is highly fluorescent and inhibits complex III if added in excess. On the other hand, dye bound in site I does not fluoresce.

References

Chance, B., and Holunger, G. (1963). *J. Biol. Chem.* **238**, 432–437.
Chappel, J. B. (1963). *J. Biol. Chem.* **238**, 410–417.
Chavez, E., and Bravo, C. (1981). *Arch. Biochem. Biophys.* **213**, 81–86.
Conover, T. E., and Schneider, R. F. (1981). *J. Biol. Chem.* **256**, 402–408.
Higuti, T., Yokota, M., Arakaki, N., Hattori, A., and Tani, I. (1978). *Biochim. Biophys. Acta* **503**, 211–222.
Higuti, T., Arakaki, N., Niimi, S., Nakasima, S., Saito, R., Tani, I., and Ota, F. (1980a). *J. Biol. Chem.* **225**, 7631–7636.
Higuti, T., Niimi, S., Saito, R., Nakasima, S., Ohe, T., Tani, I., and Yoshimura, T. (1980b). *Biochim. Biophys. Acta* **593**, 463–467.
Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, Y. (1979). *J. Membr. Biol.* **49**, 105–121.
Mikeš, V., and Kovář, J. (1981). *Biochim. Biophys. Acta* **640**, 341–351.
Mikeš, V., and Dadák, V. (1983). *Biochim. Biophys. Acta* **723**, 231–239.
Papa, S., Tuena de Gomez-Puyou, M., and Gomez-Puyou, A. (1975). *Eur. J. Biochem.* **55**, 1–8.
Pavelka, S., and Kovář, J. (1976). *Collect. Czech. Chem. Commun.* **41**, 3654–3669.
Pressman, B. C. (1963). *J. Biol. Chem.* **238**, 401–408.
Schneider, W. C., and Hogeboom, G. H. (1950). *J. Biol. Chem.* **183**, 123–133.