Phenolic antioxidants: potent inhibitors of the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum

Patricia M. Sokolove, E.X. Albuquerque, Frederick C. Kauffman, Thomas F. Spande* and John W. Daly*

Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201 and **Laboratory of Bioorganic Chemistry, NIADDK, National Institutes of Health, Bethesda, MD 20205, USA*

Received 3 June 1986

Bis(2-hydroxy-3-*tert*-butyl-5-methylphenyl)methane (bis-phenol) is the most potent inhibitor of the $(Ca^{2+} + Mg^{2+})$-ATPase of skeletal muscle sarcoplasmic reticulum yet identified. The compound behaves as a reversible, tight-binding inhibitor with apparant $K_i = 0.3$ μM. Butylated hydroxytoluene, butylated hydroxyanisole, and 4-nonylphenol are also effective inhibitors. These observations are of particular interest in light of the widespread use of such phenolic antioxidants and stabilizers in the food industry and in the manufacture of rubbers and plastics and the ease with which the compounds are extracted into organic solvents.

$(Ca^{2+} + Mg^{2+})$-ATPase *Sarcoplasmic reticulum* *Phenolic antioxidant* *Tight-binding inhibitor*

1. INTRODUCTION

The $(Ca^{2+} + Mg^{2+})$-ATPase of skeletal muscle sarcoplasmic reticulum (SR) is a single polypeptide chain of M_r 100000, which spans the lipid bilayer. It catalyzes the central biochemical step in muscle relaxation by coupling the cleavage of ATP to the transport of two Ca^{2+} into the lumen of the SR, against a concentration gradient [1–4]. Pumiliotoxin B, an indolizidine alkaloid isolated from the neotropical frog *Dendrobates pumilio* [5,6], has been reported to be an inhibitor of the $(Ca^{2+} + Mg^{2+})$-ATPase of both rat and frog SR [7–9]. Subsequent studies revealed that the inhibition of SR $(Ca^{2+} + Mg^{2+})$-ATPase by pumiliotoxin B was due to an impurity or impurities. (Removal of the impurities by high-pressure liquid chromatography did not affect the myotonic or cardiotonic activities of pumiliotoxin B.) An exhaustive study on the source and nature of the impurities responsible for inhibition of the SR ATPase revealed them to be phenols, namely bis(2-hydroxy-3-*tert*-butyl-5-methylphenyl)methane (bis-phenol), 3,5-*tert*-butyl-4-hydroxytoluene (butylated hydroxytoluene, BHT) and 4-nonylphenols (see table 1 for structures). Because bisphenol, BHT and the nonylphenols are widely utilized by the manufacturing sector and dispersed in the environment [10,11], their effects on the SR ATPase have been examined in further detail.

2. MATERIALS AND METHODS

2.1. *Biochemical assays*

SR vesicles were prepared from the hind-limb musculature of male Sprague-Dawley rats [12] and stored frozen (−80°C) until use. The freeze-thawed vesicles were sufficiently leaky to Ca^{2+} that sonication failed to stimulate the ATPase. ATPase activity was measured spectrophotometrically by enzymatically coupling ADP production to the stoichiometric oxidation of NADH [13]. Ca^{2+} concentrations were calculated as described by Fabiato and Fabiato [14]. The program uses 5.267×10^{10} as the absolute stability constant for the Ca^{2+}-EGTA complex. This yields an apparent stability constant of 3.969×10^6 (pH 7.1, ionic

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

strength 0.16, temperature 22°C). The program considers, in addition, equilibria involving Mg^{2+}, K^+, H^+ and ATP. Protein was determined according to Lowry et al. [15]. Qualitatively similar results were obtained with a $(Ca^{2+}+Mg^{2+})$-ATPase solubilized and purified according to MacLennan [16].

2.2. *Identification of impurities in PTX-B samples*

Both natural and synthetic [17] samples of pumiliotoxin B that had been purified by silica gel chromatography inhibited the SR ATPase, while a sample purified by high-pressure liquid chromatography was inactive. The inhibitory impurities derived both from the solvents (chloroform/methanol, 6:1; J.T. Baker, Reagent Grade) and from the apparatus (Chromatotron, Harrison Research, Palo Alto, CA) used for centrifugal thin-layer chromatography. The inhibitory principles were non-volatile and stable to heating at 150°C for 10 min. The non-volatile residue from the passage of 1 l $CHCl_3$-CH_3OH (6:1) through the Chromatotron was chromatographed on LH-20 Sepharose. Inhibition of ATPase activity was maximal in those fractions having UV absorption at 270–280 nm and exhibiting (along with minor peaks) major gas chromatographic peaks with retention times of 13.85 and 15.30 min (25 m fused silica capillary, OV-1 stationary phase, 150–300°C at 10°C/min). Combined gas chromatography-mass spectrometry (electron impact mode) identified these two peaks as corresponding respectively to bis-phenol and a methanolysis photoproduct of bis-phenol. (The methanolysis product arises subsequent to the Chromatotron operation by exposure of the extract to room daylight.) All samples of pumiliotoxin B purified by the Chromatotron revealed, superimposed on the broader peak corresponding to pumiliotoxin B (13.85 min), the sharper peak from the bis-phenol. The bis-phenol derived from the Delrin plastic of the Chromatotron and is primarily responsible for inhibition of ATPase observed with samples of pumiliotoxin B purified by the Chromatotron. The eluant also contained BHT (retention time 5.35 min) and 4-nonylphenols (retention times 7–7.8 min). These phenols could also be demonstrated in residues from commercial chloroform (J.T. Baker, Reagent Grade) and may arise in this case from the bottle's plastic cap. Such phenols can be extracted from plastic caps and/or liners even when only the vapors of organic solvents, such as methanol, are in contact with the plastic. ATPase inhibition observed for samples of pumiliotoxin B not purified by the Chromatotron but by column chromatography is accounted for by the presence of BHT and/or 4-nonylphenols.

3. RESULTS AND DISCUSSION

The inhibitory effectiveness of bis-phenol depends on the concentration of ATPase present in the assay (fig.1A). Behavior of this sort is expected when the concentration of enzyme in an assay (E_t) is substantial relative to K_i, the dissociation constant for the enzyme-inhibitor complex, e.g., $E_t/K_i > 0.1$ [18]. An Ackerman-Potter plot of ATPase

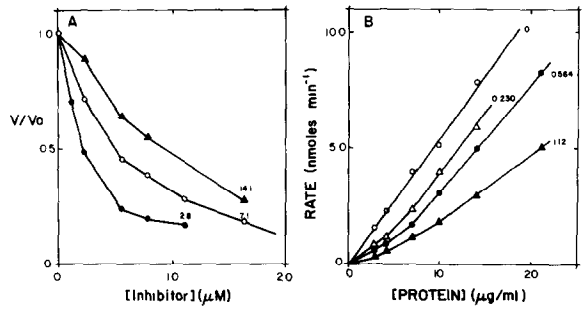


Fig.1. Inhibition of the $(Ca^{2+}+Mg^{2+})$-ATPase of rat skeletal muscle SR by bis-phenol. (A) Effect of protein concentration on inhibition by bis-phenol. Protein concentrations (in $\mu g \cdot ml^{-1}$) are indicated adjacent to the curves. (B) Ackerman-Potter plot for bis-phenol inhibition of the ATPase. Bis-phenol concentrations (μM) are indicated adjacent to the curves. Assays were conducted at 22°C in 0.3 ml containing 20 mM imidazole-HCl (pH 7.0), 100 mM KCl, 1 mM $MgCl_2$, 0.4 mM phospho*enol*pyruvate, 0.14 mM NADH, 100 μM EGTA, 50 μM ATP, sufficient $CaCl_2$ to yield Ca^{2+} concentration of 0.3 μM; and excess lactate dehydrogenase and pyruvate kinase. At these substrate concentrations >96% of the detectable ATPase activity is Ca^{2+}-dependent. Absorbance was followed continuously at 340 nm, and rates were calculated using an extinction coefficient of 6270 $cm^{-1} \cdot M^{-1}$ for NADH. Bis-phenol obtained from Pfaltz and Bauer (Flushing, NY) was added as a methanolic solution and results were corrected for minor effects of the solvent. The data obtained in the absence of inhibitor are approximated using the linear least-squares approach (R = 0.998); other lines were drawn by eye.

activity as a function of enzyme concentration for several concentrations of bis-phenol (fig.1B) is also consistent with identification of the compound as an apparently tight-binding inhibitor [19,20]. In both plots, the deviation from conventional Michaelis-Menten kinetics reflects the change in free inhibitor concentration resulting from inhibitor-enzyme interaction.

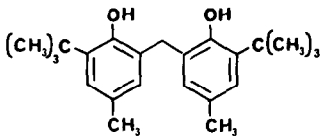
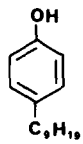
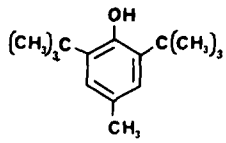
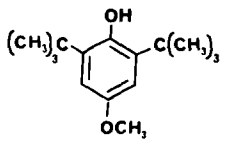
Although C_{50} (the concentration of inhibitor required to produce 50% inhibition) depends on the concentration of enzyme in the assay, the apparent K_i does not [20]. An apparent K_i value for the inhibitor of 0.29 ± 0.09 μM was estimated, using data from three experiments of the sort shown in fig.1A. K_i is given by the y-intercept of a plot of C_{50} vs relative enzyme concentration [20–22]. An apparent K_i of 0.32 ± 0.08 μM (five experiments) was calculated using the equation

$$K_i = 2(C_{75})/3 - C_{50},$$

where C_{50} and C_{75}, the inhibitor concentration producing 75% inhibition, are determined for a single enzyme concentration [22]. SR protein concentrations employed in these assays fell between 2 and 24 $\mu g \cdot ml^{-1}$. Assuming that the SR ATPase accounts for 60% of the protein in the preparation and assuming an M_r of 100000 [1–4], an enzyme

Table 1

Effect of several phenolic antioxidants on the ($Ca^{2+} + Mg^{2+}$)-ATPase of rat skeletal muscle SR

Antioxidant	Structure	C_{50} (μM)
Bis-phenol[a]		0.70 ± 0.05 (n = 6)
4-Nonylphenols[b]		1.68 (n = 2)
3,5-Dibutyl-4-hydroxytoluene (BHT)[c]		4.6 ± 0.7 (n = 3)
3,5-Dibutyl-4-hydroxyanisole[c]		12.2 (n = 2)

[a] Pfaltz and Bauer (Flushing, NY)
[b] A complex mixture of branched chain isomers. Fluka (Hauppauge, NY)
[c] Aldrich (Milwaukee, WI)

Assay conditions were as described for fig.1, except that protein concentration was fixed at 7.05 $\mu g \cdot ml^{-1}$. As demonstrated in fig.1, C_{50} values for these compounds are not constant but vary with enzyme concentration. Values shown are the mean ± SD of the number of determinations indicated in parentheses

concentration of between 0.012 and 0.125 μM, corresponding to E_t/K_i ratios of 0.04–0.40, can be calculated. Greco and Hakala [22] suggest that the most precise estimations of K_i can be made in this range, in which appreciable dissociation of the enzyme-inhibitor complex occurs.

For true tight-binding inhibitors (K_i < 1 nM), slowness of association-dissociation reactions often precludes steady-state analysis of data. This is not the case for inhibition of the SR ($Ca^{2+} + Mg^{2+}$)-ATPase by bis-phenol. No curvature in reaction progress curves was observed. Furthermore, no difference in reaction course could be detected when the reaction was begun by (i) addition of enzyme or (ii) addition of substrate(s) (Ca^{2+}, ATP, or Ca^{2+} + ATP) to enzyme preincubated with inhibitor. In this enzyme system, behavior characteristic of a tight-binding inhibitor is a reflection of the E_t/K_i ratio rather than a K_i value in the nanomolar range. An apparent $K_i > 10^{-7}$ M is, in fact, compatible with steady-state kinetics [21].

C_{50} values for several phenolic antioxidants, assayed under standard conditions, are summarized in table 1. Inhibitory potency varies over an order of magnitude with bis-phenol > 4-nonylphenols > dibutylhydroxytoluene > dibutylhydroxyanisole. Quercetin, a flavanoid isolated from plant sources, has also been characterized as a potent, reversible inhibitor of the SR ($Ca^{2+} + Mg^{2+}$)-ATPase [23–24]. The C_{50} for quercetin is approx. 10 μM. However, quercetin is a general inhibitor of energy conversion reactions, active at similar concentrations against the F_1/F_0 ATPases of mitochondria [25] and chloroplasts [26]. Bis-phenol is, by contrast, without inhibitory effect on the ATPase of rat heart mitochondria (not shown). The phenolic antioxidant is, thus, both more potent and more selective than quercetin.

The ($Ca^{2+} + Mg^{2+}$)-ATPase of SR couples the vectorial movement of Ca^{2+} to the hydrolysis of ATP by means of a reaction mechanism that comprises at least 8 steps [1–4], namely: (1) Two Ca^{2+} interact with the enzyme at high-affinity sites oriented towards the cytoplasm. (2) ATP binds to the catalytic site of the enzyme and (3) a covalent enzyme-phosphate intermediate is formed. (4) Ca^{2+} is translocated through the SR membrane and the affinity of the enzyme for Ca^{2+} decreases such that (5) Ca^{2+} is released into the lumen. Finally, in a two-step process (6,7), the enzyme-phosphate bond is hydrolyzed, and (8) the enzyme returns to a conformation with high-affinity Ca^{2+}-binding sites facing the cytoplasm. Two of these steps, 4 and 8, can be considered isomeric rearrangement reactions; orientations rather than binding interactions are altered. These steps are of particular interest because they require interplay between the enzyme and its membrane environment and because step 4 appears to be rate-limiting under most circumstances. Several manipulations (an increase in temperature, lowered pH, and the presence of organic solvents such as dimethyl sulfoxide) shift the equilibria of these two reactions in favor of the low-affinity form of the enzyme [27].

Preliminary characterization of the inhibitory interaction of bis-phenol with the ($Ca^{2+} + Mg^{2+}$)-ATPase suggests that bis-phenol also acts on these two conformational rearrangement steps. Inhibitory effectiveness increases with increasing assay temperature, i.e. bis-phenol decreases the absolute value of the slope of an Arrhenius plot for the ATPase reaction (fig.2A). Inhibition is antagonized by concentrations of the non-ionic detergent Triton X-100 well below the critical micelle concentration [28] particularly at higher temperatures (fig.2A). Inhibition is enhanced as the assay pH is lowered. For example, a decrease in assay pH from 8.1 to 6.6 increased the inhibition obtained with 0.56 μM bis-phenol from 17.8 to 56.6% (assay conditions as in fig.1; protein concentration 7.05 $\mu g \cdot ml^{-1}$). Inhibition of the ($Ca^{2+} + Mg^{2+}$)-ATPase by bis-phenol is non-competitive with respect to Ca^{2+} (fig.2B,C), but "the complexity of tight-binding inhibition equations mitigate against their use for determination of values of kinetic parameters" [20]. The interactions of Ca^{2+}, bis-phenol, and the ATPase are strongly influenced by temperature (cf. fig.2B and C). At lower temperature an increase in Ca^{2+} concentration decreases ATPase activity. Presumably Ca^{2+} interacts with the low-affinity form of the Ca^{2+}-binding site and inhibits step 4. Under these conditions, bis-phenol enhances enzyme activity. The dependence of inhibition on ATP concentration is influenced by Ca^{2+} concentration: at 0.3 μM Ca^{2+} bis-phenol inhibition is non-competitive with respect to ATP (fig.2D). At a

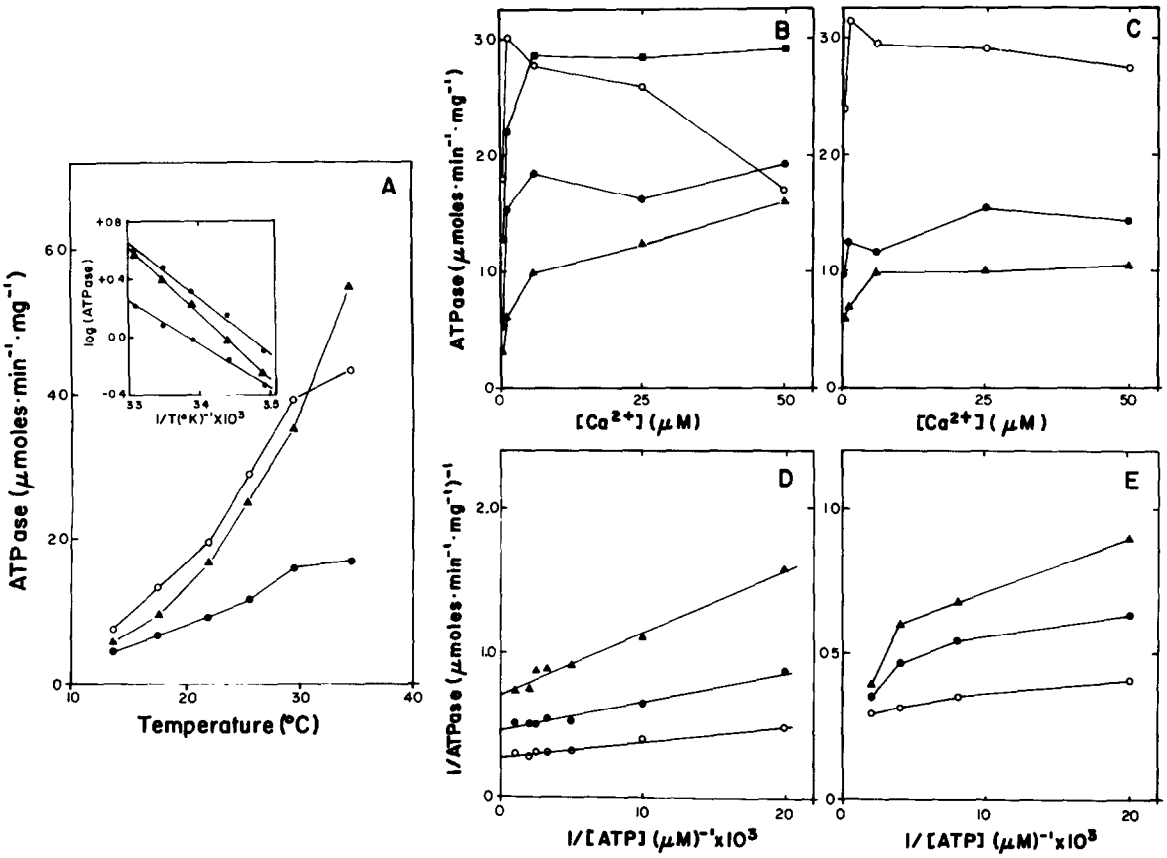


Fig.2. Characterization of bis-phenol inhibition of the SR ($Ca^{2+}+Mg^{2+}$)-ATPase. Assay conditions were as outlined in fig.1, except that protein concentration was held constant at 7.05 $\mu g \cdot ml^{-1}$. (A) Effect of temperature and of Triton X-100. The data shown were obtained in the absence of further additions (○), in the presence of 0.56 μM bis-phenol (●) and in the presence of both 0.56 μM bis-phenol and 0.005% Triton X-100 (▲). When the assay mixture was supplemented with 0.005% Triton only, a curve similar to that shown for Triton plus bis-phenol, but slightly steeper, resulted. Measured rates ran from 0.407 $\mu mol \cdot min^{-1} \cdot ng^{-1}$ at 13.5°C to 5.69 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at 34.5°C. Arrhenius plots, all of which had $|R| > 0.996$ for the temperature range 13.5–29.5°C, had slopes of −3.89 (control), −2.96 (bis-phenol), −4.56 (Triton plus bis-phenol) and −5.58 (Triton). (B,C) Dependence on Ca^{2+} concentration (B, 22°C; C, 34.5°C). The data shown are for controls (○), and for assays in the presence of 0.23 μM (■), 0.56 μM (●) and 1.12 μM (▲) bis-phenol. (D,E) Lineweaver-Burk plots for the effect of ATP (D, 0.3 μM Ca^{2+}; E, 1.2 μM Ca^{2+}). Symbols as in panels B and C. The concave downward curves in E are characteristic both of the ATP dependence of the enzyme and of Lineweaver-Burk plots for tight-binding inhibitors.

higher Ca^{2+} concentration (1.2 μM), Lineweaver-Burk plots suggest competitive interactions between ATP and bis-phenol (fig.2E).

In summary, factors that are believed to shift the equilibria of steps 4 and 8 toward the low-affinity forms of the enzyme (increased temperature, low pH) enhance inhibition by bis-phenol; elevated Ca^{2+} concentrations, which decrease the flux through step 4, create a situation in which low concentrations of bis-phenol are stimulatory. Finally, the detergent Triton X-100, which might be expected to influence primarily enzyme conformation alterations occurring within the SR membrane, overcomes bis-phenol inhibition. These findings suggest that bis-phenol and related compounds act on the crucial steps in the ATPase reaction cycle during which reorientation of the Ca^{2+}-binding sites and ion translocation occur. Bis-phenol has also been shown to inhibit Ca^{2+} uptake into SR vesicles, measured in the presence of

inorganic phosphate to keep the ionized Ca^{2+} concentration low. Inhibition is dependent on protein concentration, and inhibitor potency is similar to that observed for the uncoupled ATPase reaction (Beeler, T.H., personal communication). The phenolic antioxidants will therefore be valuable tools for probing and manipulating the complex reaction sequence of the ATPase and the ion translocation steps in particular.

Because phenolic antioxidants or stabilizers are common, unregulated additives employed in a broad spectrum of industries, their severe effects on the activity of a key enzyme in muscle physiology are of clear, general concern. The agents utilized in this study were identified initially as contaminants of an alkaloid purified by standard chromatographic procedures [5,6]. They were found to originate from the Delrin plastic of the chromatographic apparatus and to be present in commercial chloroform. Similarly, incubation of disposable pipet tips in methanol at 4°C for 1 h elutes compounds capable of inhibiting the ATPase, as does extended contact of caps of vials, tubes and bottles with vapors from methanol. The ease with which such inhibitory compounds are extracted by organic solvents from some plastic laboratory ware and caps of glassware should be recognized by research scientists.

ACKNOWLEDGEMENTS

Research supported in part by a grant from the Muscular Dystrophy Association to P.M.S., by grants NS 14728 (F.C.K.) and NS 12063 (E.X.A.) from USPHGS, and Army Research Office Grant DAAG 29-81-K-0161 (E.X.A.). $(Ca^{2+} + Mg^{2+})$-ATPase (R_3) purified according to MacLennan [16] was graciously supplied by Dr Giuseppi Inesi. The technical assistance of Diana Kunes is gratefully acknowledged.

REFERENCES

[1] Inesi, G. (1978) Aging 6, 159–177.
[2] DeMeis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292.
[3] Ikemoto, N. (1982) Annu. Rev. Physiol. 44, 297–317.
[4] Inesi, G. (1985) Annu. Rev. Physiol. 47, 573–601.
[5] Daly, J.W. and Myers, C.W. (1967) Science 156, 970–973.
[6] Daly, J.W., Tokuyama, T., Fujiwara, T., Highet, R.J. and Karle, I.L. (1980) J. Am. Chem. Soc. 102, 830–836.
[7] Albuquerque, E.X., Warnick, J.E., Maleque, M.A., Kauffman, F.C., Tamburini, R., Nimit, Y. and Daly, J.W. (1981) Mol. Pharmacol. 19, 411–424.
[8] Tamburini, R., Albuquerque, E.X., Daly, J.W. and Kauffman, F.C. (1981) J. Neurochem. 37, 775–780.
[9] Sokolove, P.M., Albuquerque, E.X., Daly, J.W. and Kauffman, F.C. (1982) Fed. Proc. 43, 1228.
[10] Koch, J. (1972) Deut. Lebensm.-Rudsch. 68, 216–221, from Chem. Abstr. 77, 163105.
[11] Hoffman, W. and Ostromow, N. (1972) Kaut. Gummi. Kunstst. 25, 260–264, from Chem. Abstr. 77, 102991.
[12] Froelich, J.P. and Taylor, E.W. (1975) J. Biol. Chem. 250, 2013–2021.
[13] Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Proc. Natl. Acad. Sci. USA 71, 622–626.
[14] Fabiato, A. and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] MacLennan, D.H. (1970) J. Biol. Chem. 245, 4508–4518.
[17] Overman, L.E., Bell, K.L. and Ito, F. (1984) J. Am. Chem. Soc. 106, 4192–4201.
[18] Straus, O.H. and Goldstein, A. (1943) J. Gen. Physiol. 26, 559–585.
[19] Ackerman, W.W. and Potter, V.R. (1949) Proc. Soc. Exp. Biol. Med. 72, 1–9.
[20] Williams, J.W. and Morrison, J.F. (1979) Methods Enzymol. 63, 437–467.
[21] Cha, S. (1975) Biochem. Pharmacol. 24, 2177–2185.
[22] Greco, W.R. and Hakala, M.T. (1979) J. Biol. Chem. 254, 12104–12109.
[23] Shoshan, V., Campbell, K.P., MacLennan, D.H., Frodis, W. and Britt, B.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4435–4438.
[24] Shoshan, V. and MacLennan, D.H. (1981) J. Biol. Chem. 256, 887–892.
[25] Lange, D.R. and Racker, E. (1974) Biochim. Biophys. Acta 333, 180–186.
[26] Deters, D.W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041–1047.
[27] DeMeis, L. and Inesi, G. (1982) J. Biol. Chem. 257, 1289–1294.
[28] Furth, A.J. (1980) Anal. Biochem. 109, 207–215.