*Biochimica et Biophysica Acta*, 1024 (1990) 193–197
Elsevier



BBAMEM 70652

**BBA Report**

# The transport of $Na^+$ and $K^+$ ions through phospholipid bilayers mediated by the antibiotics salinomycin and narasin studied by $^{23}Na$- and $^{39}K$-NMR spectroscopy

Frank G. Riddell and Stephen J. Tompsett

*Department of Chemistry, The University of St Andrews, St Andrews (U.K.)*

(Received 15 January 1990)

Key words: Phospholipid bilayer; Ionophore; Antibiotic; Salinomycin; Narasin; Ion transport; NMR, $^{23}Na$-; NMR, $^{39}K$-

**Addition of the ionophoric antibiotics salinomycin or narasin to preparations of large unilamellar vesicles made from egg yolk phosphatidylcholine in sodium or potassium chloride solutions gives rise to dynamic effects in the $^{23}Na$- and $^{39}K$-NMR spectra. The dynamic spectra arise from the ionophore-mediated transport of the metal ions through the membrane. The kinetics of the transport are followed as a function of the concentrations of ionophore and the metal ion and are compatible in all cases with a model in which one ionophore molecule transports one metal ion. For both ionophores the transport of potassium ions is appreciably faster than that of sodium and in both cases the rate-limiting step for sodium transport is dissociation of the ionophore-metal complex. Assuming dissociation to be rate limiting in all four cases it is shown that the transport rate differences between the pairs of complexes of each metal arise solely from differences in the rates of formation. The stability constants for ionophore-metal complex formation in the membrane / water interface are evaluated.**

The ionophoric antibiotics of which salinomycin and narasin are amongst the most commercially important examples have widespread biological action [1]. In particular they are efficient mediators of the transport of metal ions and $H^+$ through the limiting membranes of cells. This property is presumed to be responsible for their biological activity through the dissipation of trans-membrane ion gradients. It is believed that transport occurs by the ionophore transporting the metal ion through the membrane in the form of a 1 : 1 metal-ionophore complex.

Salinomycin (Fig. 1, R = H) [2,3] has proved to be one of the most effective and widely used ionophoric antibiotics. It is extensively used as a coccidiostat in poultry and the doses of salinomycin needed to control coccidia appear to be lower than those of some other ionophores [4]. Salinomycin is effective against a wide range of other micro-organisms including Gram positive bacteria [3] and malaria [5]. Salinomycin also has potential as a cardiac drug [6].

Narasin (Fig. 1, R = $CH_3$) [7,8] is an equally effective ionophoric antibiotic. It is a potent coccidiostat in poultry with an efficacy as good if not better than monensin [9]. It is also active against many other micro-organisms including Gram positive and anaerobic bacteria [7].

The action of the ionophoric antibiotics has been ascribed to their ability to complex with and then transport alkali metal ions through biological membranes. Studies on the ability of salinomycin to migrate metal ions into organic phases showed that salinomycin exhibited a greater preference for $K^+$ than for other metal ions [10]. This conclusion was reinforced in a biological system by studies on the effects of salinomycin on the transport and membrane functions of rat liver mitochondria which showed that salinomycin ap-

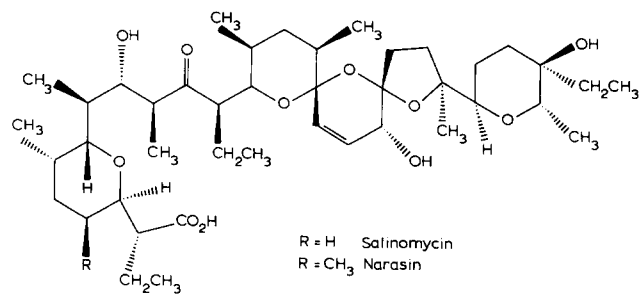


Fig. 1. Structures of the ionophoric antibiotics salinomycin and narasin.

Correspondence: F.G. Riddell, Department of Chemistry, The University of St Andrews, St Andrews, KY16 9ST, Scotland U.K.

peared to have greater effects on $K^+$ metabolism than on that of any other metal ion [11]. Painter and Pressman showed that the preference of salinomycin for $K^+$ over $Na^+$ in mixed solvents increases as the proportion of water increases [12]. For narasin the evidence of which ion is preferred is less clear cut. Kinetic transport selectivity in erythrocytes suggests preferential transport of $K^+$ over $Na^+$ [13,14] and equilibrium selectivity for uptake of $Na^+$ and $K^+$ ions into DMPC vesicles agrees with this [15]. However, studies of respiring mitochondria suggest the opposite selectivity [16].

The sole difference between these two commercially important antibiotics is the methyl substituent at C-4. In view of the small difference we thought it important to measure the rates at which these two materials transport alkali metal ions through model biological membranes and to use our techniques to compare the effect of this difference on the recognition rates and on the stability of the metal-ionophore complex in the membrane surface.

We have previously demonstrated that dynamic NMR techniques can be used to study the ionophore-mediated transport of alkali metal ions through phospholipid bilayers [17–21]. Briefly, the experiments involve the preparation of phosphatidylcholine (PC) vesicles with equal concentrations of metal ions inside and outside, the establishment of a chemical shift difference by use of an aqueous shift reagent for the metal ions and a dynamic line broadening [17,18,21] or magnetisation transfer [19–21] experiment to obtain transport rates as the ionophore is added. The dynamic NMR results allow the rates of transport to be measured for various concentrations of ionophore and metal ion. We have also demonstrated [17–21] that the classical mobile carrier system presented by Painter and Pressman [13] is satisfactory to account for the observed kinetics. This model is presented in Fig. 2 and has been discussed by us before [17–21].

The rate equation derived from this model [17] is presented in Eqn. 1.

$$\frac{1}{\tau_{M^+,in}} = \frac{A \cdot k_{diff} \cdot k_d \cdot [L]_T}{V_{in}(k_d + 2k_{diff})([M^+] + k_d/k_f)} \quad (1)$$

(This equation describes the lifetime ($\tau$) of a metal ion $M^+$ inside a vesicle of volume $V_{in}$ and surface area $A$ with a total ionophore concentration (complexed + uncomplexed) $[L]_T$.)
Eqn. 1 can be rewritten as:

$$\frac{1}{\tau_{M^+,in}} = \frac{A \cdot V_m \cdot [L]_T}{V_{in}(K_m + [M^+])} \quad (2)$$

where $K_m = k_d/k_f = K_s^{-1}$ ($K_s$ is the stability constant of the membrane-bound ligand-metal complex). The value of $V_m$ depends upon the relative values of $k_{diff}$ and $k_d$.
Eqns. 1 and 2 predict the following:

$$\frac{1}{\tau_{M^+,in}} = k_2 \cdot [L]_T \quad (3)$$

i.e., first-order kinetics in $[L]_T$ where

$$k_2 = \frac{A \cdot V_m}{V_{in}(K_m + [M^+])}$$

and

$$\tau_{M^+,in} = \frac{K_m}{V_m \cdot [L]_T} + \frac{[M^+]}{V_m \cdot [L]_T} \quad (4)$$

Eqn. 4 indicates that a plot of $\tau_{M^+,in}$ vs. $[M^+]$ at constant $[L]_T$ (i.e., 1/first-order rate constant for efflux) should be linear with a slope $1/V_m[L]_T$ and intercept $K_m/V_m[L]_T$. Thus, for this graph the ratio of slope to intercept is the stability constant $K_s$ for the membrane-bound complex. If diffusion is rapid the slope and intercept will be proportional to $1/k_d$ and $1/k_f$, respectively. For slow diffusion the graph will have a slope proportional to $1/k_{diff}$ and intercept proportional to $k_d \cdot k_{diff}/k_f$. For intermediate situations the functions describing slope and intercept will be somewhat more complex.

We now present our results for salinomycin and narasin transporting sodium and potassium ions through PC bilayer membranes obtained by similar methods to those described in previous papers [17–21]. The results are in accord with the model in Fig. 2 and Eqns. 1–4. The results show that both ionophores transport sodium ions through PC bilayers at rates comparable with the

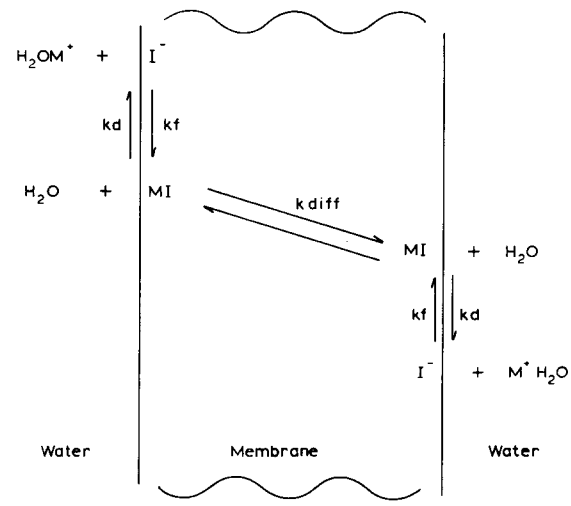


Fig. 2. Model used to describe ionophore-mediated metal ion transport through phospholipid bilayers. The rate constants are: $k_f$, formation rate for metal-ionophore complex; $k_d$, dissociation rate for the metal-ionophore complex; $k_{diff}$, the diffusion coefficient for diffusion through the membrane.

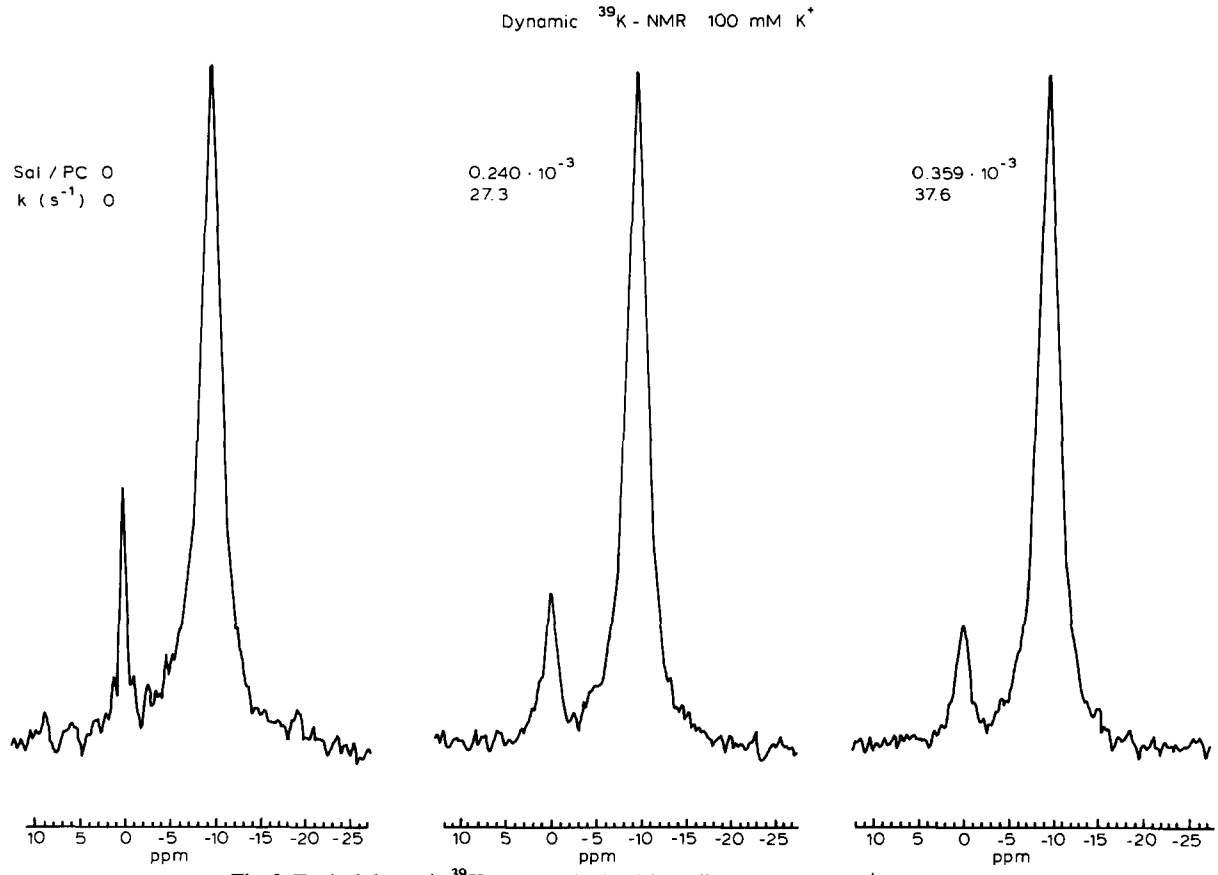


Fig. 3. Typical dynamic $^{39}$K spectra obtained for salinomycin with $[K^+] = 0.100$ M.

monensin-mediated transport of potassium and both ionophores transport potassium at rates faster than those of any other ionophore/metal combination that we have so far reported. Assuming that diffusion is rapid, for both ionophores interacting with the same metal the dissociation rates are indistinguishable within experimental error. Transport rate differences and stability constant differences arise solely from differences in the formation rates of the complexes at the membrane surface. For sodium the narasin complex forms at about twice the rate of the salinomycin complex but for potassium the narasin complex forms at about half the rate of the salinomycin complex.

As we have observed previously for monensin, nigericin and M139603, adding small aliquots of salinomycin and narasin to vesicle preparations broadened the $Na^+$ and $K^+$ lines consistent with a dynamic exchange process between the $M^+_{in}$ and the $M^+_{out}$ populations. Typical $^{39}$K spectra for salinomycin mediated transport are shown in Fig. 3. The rates of transport are given in Tables I–IV where the derived $k'$ values incorporate terms in area and volume of the vesicles and in ionophore to lipid ratio. For both metals at every

TABLE I

*Rate constants for salinomycin-mediated $Na^+$ transport in egg PC vesicles*

From these results $k'_f = (2.611 \pm 0.277) \cdot 10^4\ s^{-1}$, $k'_d = (0.425 \pm 0.062) \cdot 10^4\ M \cdot s^{-1}$, $K_s = 6.1 \pm 1.8\ M^{-1}$. $k'_f$ and $k'_d$ are calculated on the basis that diffusion is not the rate-limiting step.

| $[Na^+]$ (M) | $k$(mol lipid·(mol salinomycin)$^{-1}$·s$^{-1}$) |
|---|---|
| 0.025 | $(2.147 \pm 0.250) \cdot 10^4$ |
| 0.050 | $(2.161 \pm 0.038) \cdot 10^4$ |
| 0.100 | $(1.201 \pm 0.018) \cdot 10^4$ |
| 0.125 | $(1.657 \pm 0.044) \cdot 10^4$ |
| 0.150 | $(1.237 \pm 0.035) \cdot 10^4$ |
| 0.200 | $(1.131 \pm 0.019) \cdot 10^4$ |

TABLE II

*Rate constants for salinomycin-mediated $K^+$ transport in egg PC vesicles*

From these results $k'_f = (28.32 \pm 1.62) \cdot 10^4\ s^{-1}$, $k'_d = (1.39 \pm 0.30) \cdot 10^4\ M \cdot s^{-1}$, $K_s = 20.4 \pm 9.5\ M^{-1}$. $k'_f$ and $k'_d$ are calculated on the basis that diffusion is not the rate-limiting step.

| $[K^+]$ (M) | $k$(mol lipid·(mol salinomycin)$^{-1}$·s$^{-1}$) |
|---|---|
| 0.050 | $(1.179 \pm 0.034) \cdot 10^5$ |
| 0.100 | $(1.068 \pm 0.050) \cdot 10^5$ |
| 0.150 | $(0.860 \pm 0.080) \cdot 10^5$ |
| 0.175 | $(0.524 \pm 0.032) \cdot 10^5$ |
| 0.200 | $(0.561 \pm 0.037) \cdot 10^5$ |

TABLE III

*Rate constants for narasin-mediated $Na^+$ transport in egg PC vesicles*

From these results $k_f' = (5.859 \pm 2.229) \cdot 10^4\ s^{-1}$, $k_d' = (0.535 \pm 0.121) \cdot 10^4\ M \cdot s^{-1}$, $K_s = 11.0 \pm 8.56\ M^{-1}$. $k_f'$ and $k_d'$ are calculated on the basis that diffusion is not the rate-limiting step.

| $[K^+]$ (M) | $k$(mol lipid·(mol narasin)$^{-1}$·s$^{-1}$) |
|---|---|
| 0.050 | $(0.432 \pm 0.056) \cdot 10^5$ |
| 0.100 | $(0.269 \pm 0.010) \cdot 10^5$ |
| 0.150 | $(0.192 \pm 0.005) \cdot 10^5$ |
| 0.200 | $(0.203 \pm 0.007) \cdot 10^5$ |

TABLE IV

*Rate constants for narasin-mediated $K^+$ transport in egg PC vesicles*

From these results $k_f' = (14.7 \pm 3.8) \cdot 10^4\ s^{-1}$, $k_d' = (1.67 \pm 0.34) \cdot 10^4\ M \cdot s^{-1}$, $K_s = 8.8 \pm 2.7\ M^{-1}$. $k_f'$ and $k_d'$ are calculated on the basis that diffusion is not the rate-limiting step.

| $[K^+]$ (M) | $k$(mol lipid·(mol narasin)$^{-1}$·s$^{-1}$) |
|---|---|
| 0.050 | $(0.880 \pm 0.093) \cdot 10^5$ |
| 0.100 | $(0.926 \pm 0.055) \cdot 10^5$ |
| 0.150 | $(0.658 \pm 0.097) \cdot 10^5$ |
| 0.200 | $(0.505 \pm 0.027) \cdot 10^5$ |

concentration studied, the transport rates in the direction in → out vary linearly with the both salinomycin and narasin concentrations, indicating a first-order relationship between ionophore concentration and the transport rate (Eqn. 3). The linear relationship between $k^{-1}$ and metal ion concentration shown in Fig. 4 is also in accord with our model (Eqn. 4). Taken together these results present strong evidence that the transporting species is a 1 : 1 complex between ionophore and $M^+$.

Our model predicts that if diffusion is the rate-limiting step the slope in Fig. 4 would be proportional to $k_{diff}^{-1}$. Since it would be expected that the $Na^+$ and $K^+$ complexes would have almost identical molecular volumes and shapes, the diffusion coefficients should be almost identical for both metals and therefore the slopes should be identical. Fig. 4 shows that the slopes of the graphs for $Na^+$ are about three times those of the slopes of the graphs for $K^+$. The metal with the steeper slope, sodium, cannot therefore have diffusion as its

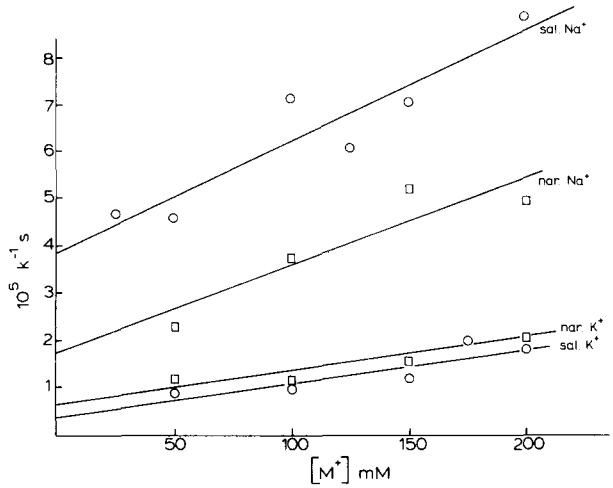


Fig. 4. Graph showing the linear relationships between $k^{-1}$ and $[M^+]$ from which the values of $k_f'$ (1/intercept) and $k_d'$ (1/slope) are obtained on the assumption that diffusion is not the rate-limiting step.

rate-limiting step. It is possible that diffusion is rate limiting for potassium. We note that the much smaller value of slope observed for $K^+$/salinomycin than for any previous observation gives further weight to our conclusions that diffusion is not rate limiting for monensin and nigericin [17,18] and probably not for M139603 [21].

The observations of earlier workers that salinomycin is an ionophore that prefers to transport $K^+$ are borne out by the current work. For narasin we conclude that $K^+$ is transported more rapidly than $Na^+$ in agreement with most of the earlier observations but that the difference in transport rates for narasin is less than for salinomycin.

It is of interest that the slopes of the graphs in Fig. 4 are similar for the same metal. As indicated above this means that the dissociation rates of the two sodium complexes are similar. The same conclusion may well hold for the two potassium complexes. The differences in the transporting ability of each ionophore for the same metal arise from differences in the formation rates of the complexes.

Salinomycin not only transports $K^+$ more rapidly than $Na^+$, it also forms a more stable complex with $K^+$.

TABLE V

*Comparison of rate and stability constants for M139603, monensin, nigericin, salinomycin and narasin in egg PC vesicles*

| | Li | | | Na | | | K | | |
|---|---|---|---|---|---|---|---|---|---|
| | $k_f'$ ($10^4\ s^{-1}$) | $k_d'$ ($10^4\ M \cdot s^{-1}$) | $K_s$ ($M^{-1}$) | $k_f'$ ($10^4\ s^{-1}$) | $k_d'$ ($10^4\ M \cdot s^{-1}$) | $K_s$ ($M^{-1}$) | $k_f'$ ($10^{-4}\ s^{-1}$) | $k_d'$ ($10^4\ M \cdot s^{-1}$) | $K_s$ ($M^{-1}$) |
| M139603 | 0.549 | 0.0479 | 11.5 | 20.52 | 0.855 | 24.0 | 5.65 | 0.804 | 7.03 |
| Monensin | 0.103 | 0.00558 | 18.5 | 4.878 | 0.150 | 32.6 | 2.30 | 0.433 | 5.3 |
| Nigericin | too slow to measure | | | 7.838 | 0.352 | 22 | 9.61 | 0.0997 | 96 |
| Salinomycin | no measurements yet attempted | | | 2.611 | 0.425 | 6.1 | 28.32 | 1.39 | 20.4 |
| Narasin | no measurements yet attempted | | | 5.859 | 0.535 | 11.0 | 14.7 | 1.67 | 8.8 |

For narasin, as we observed earlier for monensin and nigericin, the opposite is the case and the more stable complex in the membrane is with the more slowly transported ion. These results emphasise that for these materials the thermodynamic stability of the metal/ionophore complex bears little relationship to the transporting ability and the transport rate. Transport rates are determined by the kinetic parameters $k_f$, $k_d$ and $k_{diff}$. Stabilities are determined by the ratio of the kinetic parameters $k_f$ and $k_d$. Thus, the more stable complex can be either the faster or slower transporting species. The stability of the complex only becomes important in determining transport rates if it is exceptionally high in which case the metal ion is never released by the ionophore (low value of $k_d$) or it is exceptionally low in which case the complex never forms (low value of $k_f$). The most important role of the stability constant in those ionophores we have examined so far (Table V) is that it is such that between approx. 20% and 80% of the ionophore molecules are bound to metal ions at any one time. When this is the case, provided that $k_f$, $k_d$ and $k_{diff}$ are sufficiently large, rapid transport will occur.

We thank the SERC for a studentship (to S.J.T.) to carry out this work. We thank Hoechst AG and Eli Lilly for generous gifts of the sodium salt of salinomycin and narasin as the acid, respectively.

## References

1 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530.
2 Japanese Patent 72 25, 392 (20 Oct 1972) (Chem. Abstr. 78, 41561).
3 Miyazaki, Y., Shibuya, M., Sugawara, H., Kawaguchi, O., Hirose, C., Nagatsu, J. and Esumi, S., (1974) J. Antibiot. 27, 814–821.
4 Mehlhorn, H., Pooch, H. and Raether, W. (1983) Z. Parasitenkd. 69, 457–471.
5 Mehlhorn, H., Ganster, H.J. and Raether, W. (1984) Zentralbl. Bakteriol., Mikrobiol. Hyg., Ser. A 256, 305–313.
6 Fahim, M., Del Valle, G. and Pressman, B.C. (1986) Cardiovasc. Res. 20, 145–152.
7 US Patent Application 477954 (10th June 1974) (Chem. Abstr. 84, 103844).
8 Berg, D.H. and Hamill, R.L. (1978) J. Antibiot. 31, 1–6.
9 Ruff, M.D., Reid, W.M., Rahn, A.P. and McDougald, L.R. (1980) Poult. Sci. 59, 2008–2013.
10 Mitani, M., Yamanishi, T. and Miyazaki, Y. (1975) Biochem. Biophys. Res. Commun. 66, 1231–1236.
11 Mitani, M., Yamanishi, T., Miyazaki, Y. and Otake, N. (1976) Antimicrob. Agents Chemother. 9, 655–660.
12 Painter, G. and Pressman, B.C. (1979) Biochem. Biophys. Res. Commun. 91, 1117–1122.
13 Painter, G.R. and Pressman, B.C. (1982) Top. Curr. Chem. 101, 83–110.
14 Pressman, B.C. and Painter, G.R. (1983) in The Biochemistry of Metabolic Processes (Lennan, D.L.F., Stratman, F.W. and Zahlten, R.N., eds.), p. 41, Elsevier, Amsterdam.
15 Caughey, B., Painter, G.R. and Gibbons, W.A. (1986) Biochem. Pharmacol. 35, 4103–4105.
16 Wong, D.T., Berg, D.H., Hamill, R.H. and Wilkinson, J.R. (1977) 26, 1373–1376.
17 Riddell, F.G., Arumugam, S., Brophy, P.J., Cox, B.G., Payne, M.C.H. and Southon, T.E. (1988) J. Am. Chem. Soc. 110, 734–738.
18 Riddell, F.G., Arumugam, S. and Cox, B.G. (1988) Biochim. Biophys. Acta 944, 279–284.
19 Riddell, F.G. and Arumugam, S. (1988) Biochim. Biophys. Acta 945, 65–72.
20 Riddell, F.G., Arumugam, S. and Cox, B.G. (1987) J. Chem. Soc. Chem. Commun., 1890–1891.
21 Riddell, F.G. and Arumugam, S. (1989) Biochim. Biophys. Acta 984, 6–10.