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# Studies on the Chemical Modification of Monensin. II. Measurement of Sodium Ion Permeability of Monensylamino Acids Using Sodium-23 Nuclear Magnetic Resonance Spectroscopy

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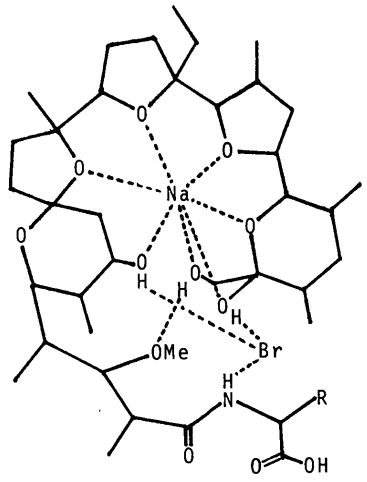
**A technique to assay $Na^+$ ions in cells is presented. Intracellular and extracellular $Na^+$ ions in a suspension of guinea pig erythrocytes were conveniently determined by using sodium-23 nuclear magnetic resonance ($^{23}$Na-NMR), in combination with two anionic shift reagents: $Dy(TTHA)^{3-}$ and $Dy(PPPi)_2^{7-}$. Monensin (1), monensylalanine (2b), monensylserine (2c), and monensylphenylalanine (2d) induced large increases of intracellular $Na^+$ ion concentration ($[Na_{in}]$), while monensylglycine (2a), monensyltyrosine (2e), monensylaspartic acid (2f), and monensylglutamic acid (2g) showed slight increases. The values of initial increasing rate ($V_i$) of 2a—g were much smaller than that of 1. This fact was probably due to the lower lipophilicity of 2a—g than 1, because a good correlation was observed between $V_i$ and $Rm_{50}$ values of 1 and 2a—g. This lower lipophilicity is a consequence of conformational differences between 1 and 2a—g.**

**Keywords** monensin; monensylamino acid; sodium ion permeability; intracellular sodium ion concentration; $^{23}$Na-NMR; guinea pig blood; shift reagent; external reference; $Rm_{50}$ value; positive inotropic effect

The polyether antibiotic monensin (**1**, Fig. 1) is one of a large class of naturally occurring ionophores, and preferentially transports $Na^+$ ions across the cell membrane by forming a pseudocyclic complex. In a preceding paper,[1] we reported chemical transformations of monensin (**1**) into monensylamino acids (**2a—g**) by condensing the carboxylic group with optically active amino acids, because amino acids have the carboxylic groups which were expected to stabilize the pseudocyclic conformations of **2a—g** by forming head-to-tail hydrogen bonds to the terminal hydroxy group. X-Ray crystallographic analysis showed that the NaBr complexes of **2a—g** were quite different from the NaBr complex of **1**. This crystallographic difference prompted us to study the $Na^+$ permeability of **2a—g** since the permeability of $Na^+$ ions into the cell is supposed to be deeply concerned with the biological activities of the polyether antibiotics.[2] In this paper, we wish to report our results on the $Na^+$ transport ability of **1** and **2a—g** across the membrane of living erythrocytes of guinea pig using sodium-23 nuclear magnetic resonance ($^{23}$Na-NMR) spectroscopy.

Recently, measurement of intracellular concentration of $Na^+$ ions in living cells has become feasible by using $^{23}$Na-NMR, in combination with anionic paramagnetic shift reagents. These reagents do not enter intact cells and remain localized in the extracellular compartments, so that the NMR resonance of extracellular $Na^+$ ions is shifted away from the resonance of intracellular $Na^+$ ions.[3,4] Gupta *et al.* reported a method to determine intracellular $Na^+$ ions by using dysprosium triphosphate ($Dy(PPPi)_2^{7-}$) as a shift reagent.[3] However, $Dy(PPPi)_2^{7-}$ is toxic to living cells,[4] and their method requires determination of the extracellular $Na^+$ concentration prior to NMR measurement. We therefore employed $Dy(PPPi)_2^{7-}$ as an external reference and nontoxic dysprosium triethylenetetramine hexacetate ($Dy(TTHA)^{3-}$)[4] as a shift reagent for discrimination of the signals due to intracellular and extracellular $Na^+$ ions in an erythrocyte suspension.[5] The integral of the intracellular $Na^+$ resonance relative to the integral of an external reference was determined at various times and compared with the calibration line prepared previously, in order to obtain pricisely the concentration of intracellular $Na^+$ ions.

In addition, we attempted to evaluate the *Rm* values of **1** and **2a—g** as parameters of lipophilicity, because lipophilicity seemed to be closely related to the $Na^+$ ion-transporting capability through the cell membrane, which is constituted of lipid bilayers.




| | R |
|---|---|
| **a**: | H |
| **b**: | $CH_3$ |
| **c**: | $CH_2OH$ |
| **d**: | $CH_2Ph$ |
| **e**: | $CH_2C_6H_4OH$ |
| **f**: | $CH_2CO_2H$ |
| **g**: | $CH_2CH_2CO_2H$ |

Chart 1. Monensylamino Acid (**2a—g**) NaBr Complexes

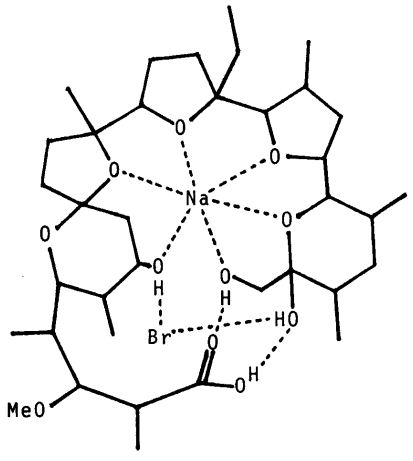



Fig. 1. Chemical Structure of Monensin (**1**) NaBr Complex

## Experimental

**Materials** Monensin (**1**) was prepared from commercially available sodium monensin by the reported method[6] while monensylamino acids (**2a—g**) were prepared by the condensation of monensin (**1**) with the corresponding amino acid benzyl esters followed by debenzylation, as described in a preceding paper.[1]

Guinea pig blood was collected by cardiocentensis of several guinea pigs and treated with heparin to avoid coagulation.

**Preparation of Shift Reagents** $Dy(TTHA)^{3-}$ was purchased from Dojin Co., Ltd. The sodium salt of $Dy(PPPi)_2^{7-}$ was prepared *in situ*[3] by treating $DyCl_3$ with $Na_5PPPi$.

**$^{23}$Na-NMR Measurement** NMR spectra were recorded using a JEOL GSX-400 spectrometer at 105.60 MHz and 37 °C. A concentric NMR tube combination (3-mm-o.d. inside a 10-mm-o.d. NMR tube) was used in the experiments (Fig. 2). The inner tube containing 20 mM $Na_7Dy(PPPi)_2 \cdot 3NaCl$ solution was used as an external reference while the annular space between the inner and outer tubes contained 1.8 ml of guinea pig blood, 0.2 ml of 100 mM $Dy(TTHA)^{3-}$ solution, and 20 $\mu$l of 10 mM test compound in dimethylsulfoxide (DMSO). A calibration line was constructed by addition of NaCl solutions (2 ml; 1, 10, 50, and 100 mM) to the annular space in the concentric NMR tube.

Concentrations of the intracellular and extracellular $Na^+$ ions ($[Na_{in}]$ and $[Na_{out}]$) were calculated from the corresponding integrals ($INT_{in}$ and $INT_{out}$) using the following Eqs. 1 and 2.

$$[Na_{in}] = (INT_{in}/A) \times (V_s/V_b \times Hem) \quad (1)$$

$$[Na_{out}] = (INT_{out}/A) \times (V_s/V_b \times (1 - Hem)) \quad (2)$$

where $V_s$ and $V_b$ represent the total volume of the sample included in the annular space and the volume of the blood used, respectively. *Hem* is the hematocrit and $A$ is the slope of the calibration line.

***Rm* Values**[7] The stationary phase was precoated thin layer chromatography (TLC) plates of Silica gel 60 F254 silanized (layer thickness 0.25 mm, Merck no. 5747), and the mobile phase was aqueous MeOH solution of various concentrations, *e.g.*, 40, 50, 60, and 70% (w/v). The test compounds were dissolved in MeOH and spotted in randomized locations in order to avoid any systematic error. The plates were developed about to 15 cm from the spotted line at room temperature, dried, sprayed with $Ce(III)_2(SO_4)_3$ solution, and heated in an oven at 85 °C. The *Rm* values were calculated from *Rf* values by means of the following equation.[7a]

$$Rm = \log(1/Rf - 1) \quad (3)$$

## Results

**$^{23}$Na-NMR** The relation between the integrals of 1, 10, 50, and 100 mM NaCl solutions and integrals of the corresponding external reference is shown in Fig. 3. The regression line was obtained by the least-squares method and is expressed by the following equation:

$$INT = 0.0471 \times [Na] + 0.00 \ (\gamma = 1.00;\ \gamma\text{: correlation coefficient})$$

where 0.0471 corresponds to the value of $A$ in Eqs. 1 and 2.

Figure 4 shows representative $^{23}$Na-NMR spectra acquired before (a; 0 time, several minutes after addition of $Dy(TTHA)^{3-}$) and 90 min after (b) addition of **2d**. The resonances of intracellular and extracellular $Na^+$ ions were clearly separated into two well-resolved signals. The resonance indicated as (B) corresponds to intracellular $Na^+$ ions while the downfield resonance (C) due to extracellular $Na^+$ ions which interacted with $Dy(TTHA)^{3-}$.

The time course of $\Delta[Na_{in}]$ is shown in Table I. DMSO, used as a solvent for the test compounds, caused a slight decrease of $[Na_{in}]$. So the corrected time course of $[Na_{in}]$, $\Delta C (= \Delta[Na_{in}]_t^i - \Delta[Na_{in}]_t^{DMSO})$, is shown in Fig. 5 and Table I. Monensin (**1**) and **2b—d** caused remarkable increases of $[Na_{in}]$ (31.3—46.7 mM) whereas the other compounds showed slight increases (5.4—7.8 mM). The characteristic features of $[Na_{in}]$ increase with monensin (**1**) were a rapid increase within a short period, followed by a moderate decrease. Compounds **2e** and **2g** showed a similar $[Na_{in}]$ tendency to monensin (**1**), though values of $[Na_{in}]$ with **2e** and **2g** were extremely small.

***Rm* Values** A linear relationship was observed between

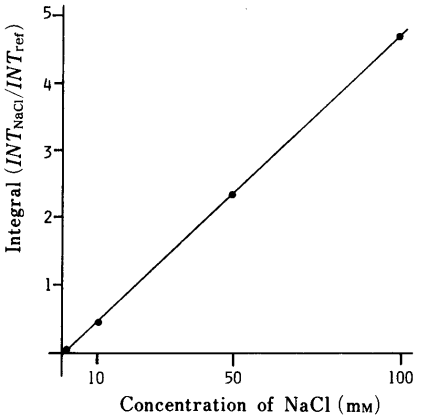



Fig. 3. Relation between the Concentration of NaCl Solutions in the Annular Space and Integral of Those Solutions Relative to That of the External Reference

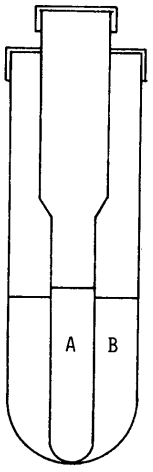



Fig. 2. The Concentric NMR Tube Combination Used for the $^{23}$Na-NMR Measurement (3-mm-o.d. Inside a 10-mm-o.d. NMR Tube)

A) The inner tube contained the external reference, 20 mM $Na_7Dy(PPPi)_2 \cdot 3NaCl$. B) The annular space contained guinea pig blood (1.8 ml), 100 mM $Dy(TTHA)^{3-}$ (0.2 ml), and 10 mM test compound DMSO solution (20 $\mu$l).

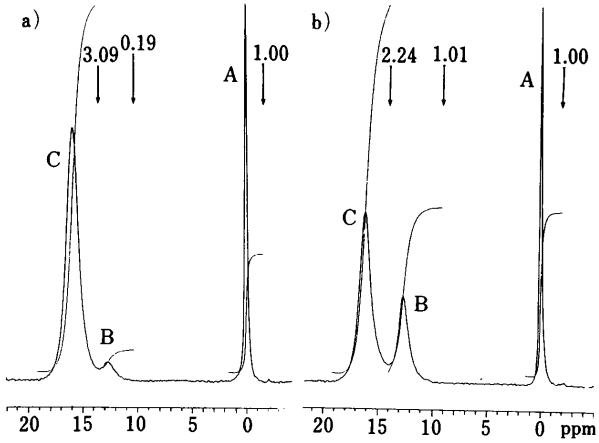



Fig. 4. $^{23}$Na-NMR Spectra Acquired a) Before and b) 90 min After Addition of **2d**

A, B, and C indicate the signals of the external reference, and intracellular and extracellular $Na^+$ ions, respectively.

TABLE I. Time Courses of Intracellular $Na^+$ Ion Concentration

| Time (min) | DMSO, $n=4$ $\Delta[Na_{in}]_t$ (±S.E.) | **1**, $n=5$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2a**, $n=5$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2b**, $n=6$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2c**, $n=3$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ |
|---|---|---|---|---|---|---|---|---|---|
| 5 | −0.3 (±0.7) | 46.4 (±2.1) | 46.7 | — — | — | — — | — | — — | — |
| 10 | −1.2 (±0.7) | 44.3 (±3.4) | 45.5 | — — | — | — — | — | — — | — |
| 20 | −2.7 (±0.7) | 37.6 (±1.9) | 40.3 | −0.1 (±0.5) | 2.6 | 5.9 (±1.0) | 8.6 | 10.3 (±1.6) | 13.0 |
| 40 | −3.1 (±1.0) | 35.0 (±1.7) | 38.1 | 0.4 (±0.6) | 3.5 | 10.8 (±1.8) | 13.9 | 18.6 (±2.6) | 21.7 |
| 60 | −3.1 (±0.8) | 35.8 (±0.9) | 38.9 | 0.5 (±1.2) | 3.6 | 14.9 (±1.0) | 18.0 | 23.0 (±2.8) | 26.1 |
| 90 | −3.4 (±0.9) | 37.5 (±2.5) | 40.9 | — — | — | 19.0 (±1.3) | 22.4 | 31.2 (±3.6) | 34.6 |
| 120 | −3.2 (±1.0) | 38.6 (±3.2) | 41.8 | 1.9 (±1.8) | 5.1 | 21.9 (±1.1) | 25.1 | 32.3 (±2.6) | 35.5 |
| 180 | −4.7 (±0.5) | — — | — | 3.5 (±2.4) | 8.2 | 24.9 (±1.8) | 29.6 | 38.9 (±2.8) | 43.6 |
| 240 | −3.3 (±0.8) | — — | — | 4.5 (±2.9) | 7.8 | 28.0 (±3.2) | 31.3 | 42.9 (±1.9) | 46.2 |

| Time (min) | **2d**, $n=7$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2e**, $n=3$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2f**, $n=4$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ | **2g**, $n=5$ $\Delta[Na_{in}]_t$ (±S.E.) | $\Delta C$ |
|---|---|---|---|---|---|---|---|---|
| 5 | 7.5 (±2.7) | 7.8 | — — | — | — — | — | — — | — |
| 10 | 17.0 (±3.5) | 18.4 | — — | — | — — | — | — — | — |
| 20 | 27.3 (±1.3) | 30.0 | 1.2 (±0.5) | 3.9 | 0.2 (±0.9) | 2.9 | 0.6 (±1.6) | 3.3 |
| 40 | 37.2 (±1.0) | 40.1 | −0.5 (±0.7) | 2.6 | −0.4 (±1.0) | 2.7 | −1.3 (±1.0) | 1.8 |
| 60 | 39.8 (±2.0) | 42.9 | 1.3 (±0.4) | 4.4 | −0.8 (±0.5) | 2.3 | 0.7 (±1.0) | 3.8 |
| 90 | 39.9 (±2.7) | 43.3 | 1.2 (±0.9) | 4.6 | — — | — | — — | — |
| 120 | 39.7 (±2.6) | 42.9 | 1.6 (±0.8) | 4.8 | 1.0 (±1.1) | 4.5 | 0.8 (±1.7) | 4.0 |
| 180 | — — | — | 3.7 (±0.5) | 8.4 | 1.9 (±1.7) | 6.6 | 0.1 (±2.6) | 4.8 |
| 240 | — — | — | 3.7 (±0.6) | 7.0 | 3.1 (±2.7) | 6.4 | 2.1 (±1.2) | 5.4 |

$\Delta[Na_{in}]_t (=[Na_{in}]_t-[Na_{in}]_0)$, S.E., and $\Delta C (=\Delta[Na_{in}]_t^{i}-\Delta[Na_{in}]_t^{DMSO})$.

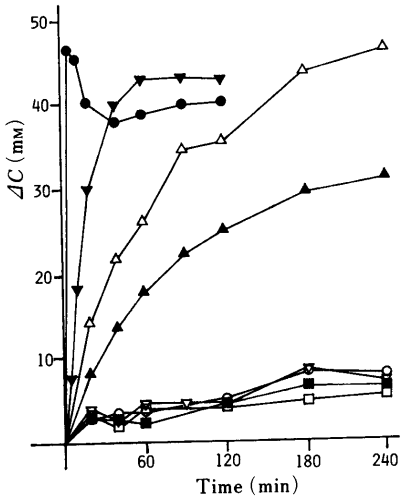



Fig. 5. Time Courses of $\Delta C$ Induced by **1** and **2a—g**
●, **1**; ○, **2a**; ▲, **2b**; △, **2c**; ▼, **2d**; ▽, **2e**; ■, **2f**; □, **2g**.

TABLE II. Parameter of Lipophilicity ($Rm_{50}$), Logarithmic Increasing Rates at 0—20 min (log $V_i$), and Ratio of Surface Area of Lipophilic and Hydrophilic Site at Amino Acid Moieties ($A_l/A_h$)

| Compd. | $Rm_{50}$ | log $V_i$ | $A_l/A_h$ |
|---|---|---|---|
| **1** | 1.85 | 0.97[a] | — |
| **2a** | 0.65 | −0.78 | 0.65 |
| **2b** | 0.73 | −0.36 | 1.18 |
| **2c** | 0.63 | −0.18 | 0.63 |
| **2d** | 1.17 | 0.18 | 2.72 |
| **2e** | 0.83 | −0.65 | 1.75 |
| **2f** | −0.04 | −0.83 | 0.43 |
| **2g** | −0.08 | −0.78 | 0.65 |

a) log $V_i$ at 0—5 min.

*Rm* values of **1**, **2a—g** and concentration of MeOH in the mobile phase in the range from 40 to 60%. By means of the least-squares method, linear regressions were calculated from the *Rm* values, and the equation of each straight line was used in order to calculate an $Rm_{50}$ value corresponding to 50% MeOH in the mobile phase. The $Rm_{50}$ values are listed in Table II. There was no compound more lipophilic than monensin (**1**).

## Discussion

We have developed a method to determine the concentrations of intracellular and extracellular $Na^+$ ions using $^{23}$Na-NMR spectroscopy. The hematocrit value of the guinea pig blood used in this study remained unchanged during the NMR measurement, which routinely took about 4 h. The initial extracellular $Na^+$ concentration of blood cells in the absence of the test compound was determined by measuring the $^{23}$Na-NMR spectrum several minutes after addition of $Dy(TTHA)^{3-}$. The resulting concentration was 123.2 mM (S.E. ± 1.1 mM) which was in good agreement with the standard value (125.0 mM).[8] The blood of guinea pig therefore appears to be a convenient experimental preparation to determine precisely the influx of $Na^+$ ions across the erythrocyte membrane. Our NMR technique has the advantage of separating directly the resonances from intracellular and extracellular $Na^+$ ions, and allowing us to determine quantitatively the influx and efflux of $Na^+$ ions. The increase of integrals for intracellular $Na^+$ ions was always parallel to the decrease of the corresponding integrals for extracellular $Na^+$ ions. Since $Dy(TTHA)^{3-}$ introduced in this study was nontoxic to the blood cells and caused effective resonance separation, this reagent should be widely useful in $^{23}$Na-NMR studies of intact cells and tissues.

Monensin (**1**) and **2b—d** showed remarkable maximal $\Delta C$. The positive inotropic effect (PIE) was then examined on isolated guinea pig papillary muscle since monensin (**1**)

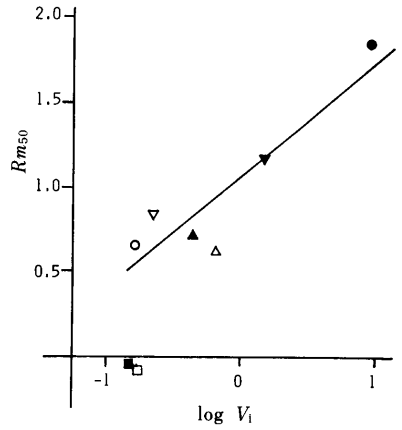



Fig. 6. Relation between $Rm_{50}$ and log $V_i$ of **1** and **2a**—**g**
●, **1**; ○, **2a**; ▲, **2b**; △, **2c**; ▼, **2d**; ▽, **2e**; ■, **2f**; □, **2g**.

exhibits the anticoccidial activity and PIE, which is known to be induced by influx of $Na^+$ ions into the cells. The PIE of **2a**—**g** was then examined. However, none of these compounds exhibited PIE although the maximal $\Delta C$ values for **2c** and **2d** were comparable to that of **1**. From these observations, it could be suggested that appearance of PIE might depend not on the maximal increase but on the initial increasing rate of $[Na_{in}]$ ($V_i$). Actually the $V_i$ of **1** within 0—5 min is significantly faster than those of **2a**—**g** within 0—20 min. Therefore, those differences in $V_i$ values are considered to be due to the differences in lipophilicity, which plays an important role in penetration of the compounds into the cell membrane consisting of lipid bilayers. Consequently, we have investigated the correlation between the logarithmic $V_i$ and $Rm_{50}$. As shown in Fig. 6, a good correlation was observed except for **2f** and **2g**, thus suggesting that high lipophilicity could result in a more rapid $Na^+$ influx into the cells. The smaller $Rm_{50}$ values of **2f** and **2g** are considered to be due to hydration of the two carboxyl groups in aqueous MeOH solution, used as the developing solvent for *Rm* value calculation, while the $V_i$ values for **2f** and **2g** were comparable to that of **2a** because **2f** and **2g** may penetrate the lipid bilayers of erythrocyte membrane with the formation of intramolecular or intermolecular association between the two carboxylic groups in the molecule.

It is noteworthy that low lipophilicity of **2a**—**g** relative to that of **1** can be explained in terms of the molecular conformations coordinating to the $Na^+$ ion, as illustrated in Chart 1. The NaBr complex of monensin (**1**) possesses head-to-tail intramolecular hydrogen bonds leaving no hydrophilic substituent on the molecular surface, whereas the NaBr complexes of **2a**—**g** have hydrophilic amino acid substituents such as carboxyl and hydroxyl groups occupying positions toward the outside of the pseudocyclic molecule. A further study of the lipophilicity of **2a**—**g** was carried out to clarify the relationship between $Rm_{50}$ and relative ratio of Van der Waals surface areas of the amino acid substituents, which could be calculated from the parameters of lipophilic groups (alkyl and aryl groups; $A_l$) and hydrophilic ones (hydroxy and carboxyl groups; $A_h$) according to the method reported by Yamakawa and coworkers.[9] As is clear from Fig. 7, a good correlation was observed between $Rm_{50}$ and $A_l/A_h$ values, except for **2f** and

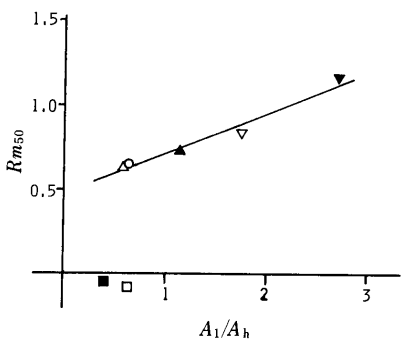



Fig. 7. Relation between $Rm_{50}$ and $A_l/A_h$
○, **2a**; ▲, **2b**; △, **2c**; ▼, **2d**; ▽, **2e**; ■, **2f**; □, **2g**.

**2g**, which seemed to be more hydrophilic than other compounds on account of the presence of the two carboxylic groups. This correlation reveals that lower lipophilicity of **2a**—**g** is attributable to the hydrophilicity of the amino acid residues.

In conclusion, we have established a convenient method to determine precisely the concentrations of intracellular and extracellular $Na^+$ ions in a suspension of guinea pig erythrocytes using $^{23}$Na-NMR spectroscopy, in combination with two anionic shift reagents; $Dy(PPPi)_2^{7-}$ and $Dy(TTHA)^{3-}$. This method could also be applicable for determination of $K^+$ and $Ca^{2+}$ concentrations if suitable nontoxic shift reagents are available.

It is most likely that the initial increasing rate ($V_i$) of the compound is an important factor to predict the biological activity, and this may be closely related to the lipophilicity of the compounds. Similarly, lack of PIE and anticoccidial activity is also probably due to the less lipophilic character of the test compounds. Further investigation of chemical modifications of the monensin molecule with much more lipophilic functional groups is in progress.

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