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# The Existence of (Sodium, Potassium and Calcium) Ionophores in Different Membranes and Their Possible Clinical Importance*

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## *Introduction*

It is well known that the intracellular composition of the living cells is quite different from the extracellular fluid in respect to both organic and inorganic substances. The cell can perform its functions only when the optimal composition within the cell is maintained. This is accomplished by the selective-permeable membrane surrounding the cell.[1, 2] Two examples for this are the conductance of an impulse within a nerve cell and the contraction of a muscle cell.

Little is known about the mechanisms responsible for the selective transport of organic and inorganic substances across a cell membrane.[3] Recently much research has been done on this subject and quite important developments were achieved.[4, 5, 6, 7, 8, 9, 10, 11]

Four main mechanisms are now thought to be responsible for the transport of molecules across the membranes. These are passive, active, fascilitated transports and pore (channel) systems.[1, 2, 12] The active and fascilitated transports are mediated by 'carriers'. The mechanism of their function is similar to enzymes. They are specific for their substrates and show saturation kinetics.[13] On the other hand, small molecules called 'ionophores' form channels across the natural and artificial membranes and carry the specific ions bound on them from one side to the other side of the membrane.[14]

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As is indicated above, the dependence of the conductance of the impulse within a nerve cell and the contraction of muscle cells on the definite concentration of sodium, potassium and calcium ions within the cells, the occurrence of clinical symptoms when there is a deviation from the definite concentrations of these ions within the cells (tetany, paralysis) and the finding of an increase in sodium ions in the erythrocytes of uremic patients have led investigators to look for molecules functioning as ionophores for sodium ($Na^+$), potassium ($K^+$) and calcium ($Ca^{++}$) in some natural membranes.[15, 16]

Recently the active transport of these ions across the membranes was investigated and the results have been applied to different clinical conditions.[11,16, 17]

In case the existence of ionophores in natural membranes for these ions are proved, it will be possible to approach the cause of different clinical situations from another point of view.

## *Materials and Methods*

The chemicals were analytically pure (Sigma Chemical Co., St. Louis). The calcium ionophore, A-23187, was a gift of Wisconsin University.

Radioactivity was counted with a Nuclear Chicago gas flow counter.

**Ionophores from natural membranes:** Bovine heart mitochondria, bovine muscle endoplasmic reticulum, the white and gray matter of bovine brain, the electric organ of the eel, a membrane responsible for proton translocation from *Halobacterium halobium* were solubilized by cholic acid mainly by the method of Mac Lennan et al.[18] The fractions of the solubilized proteins were obtained by precipitating between 25-30 % ammonium sulphate and dissolving in $2.5 \times 10^{-2}$ M Tris-HCl pH=8.5. These ammonium sulphate fractions obtained from each membrane were tested for their ionophore ability.

**Liposome formation:** Liposomes were made by the method of E. Racker.[19] A solution containing 20 mg/ml phosphatidylethanolamine and phosphatidylcholine in proportions of 4:1 (w/w) was dried in a small tube under nitrogen. An equal amount of appropriate buffer was added and sonicated until a clear appearance was obtained (about 30 min). The appropriate buffers used for making the liposomes that were used for testing the sodium, potassium and calcium ionophores were $5 \times 10^{-2}$ M sodium phosphate pH=7.4, $5 \times 10^{-2}$ M potassium phosphate pH=7.4 and $4 \times 10^{-1}$ M potassium phosphate pH=7.4 containing

1 x $10^{-5}$ M $CaCl_2$, respectively. Thus three different liposomes for testing the $Na^+$, $K^+$ and $Ca^{++}$ ionophores separately were obtained. From here on these will be referred to as sodium, potassium and calcium liposomes.

Liposomes can be loaded with radioactive ions if appropriate amounts of radioactive sodium, potassium and calcium is placed in the tubes before sonication. After sonication the mixture is passed through a Dowex 50W x 8 ion exchange column prepared in 1 x $10^{-2}$ M Tris-HCl pH=7.5 (0.5 x 4 cm). Liposomes elute in the void volume of the column and the radioactive ions that have not entered the liposomes remain in the column.

**Testing for ionophore ability:** This was done mainly by the method of E. Racker et. al.[20,21] Each experiment was in duplicate.

*A. Incubation mixtures for testing the entrance of ions into the liposomes:*

1. The ionophore effect of different membrane fractions on the transport of sodium into the liposomes: 0.125 ml 5 x $10^{-2}$ M sodium phosphate pH=7.4 containing $Na^{22}$ (8 x $10^5$ - 1 x $10^6$ cpm) was mixed with 25 ul of different membrane fractions. Lastly, 0.125 ml sodium liposomes, prepared as described above, was added and the volume was made up to 0.3 ml with distilled water. These amounts could be increased or decreased in the same ratios. The mixture was kept at room temperature and at appropriate time intervals 0.1 ml of the mixture was passed through a Dowex 50W x 8 column prepared in 1 x $10^{-2}$ M Tris-HCl pH=7.5 (0.5 x 4 cm). The void volume of the column was 1 ml. The liposomes were eluted from the column with 3 ml 2.5 x $10^{-1}$ M sucrose and collected as one fraction in a small tube. The radioactive ions that did not enter the liposomes remained in the column. 1 ml of the eluate was plated and counted.

2. The ionophore effect of different membrane fractions on the transport of potassium into the liposomes: The procedure is the same as described for testing sodium ionophores in the previous paragraph except that the buffer used was 5 x $10^{-2}$ M potassium phosphate pH=7.4 containing $Rb^{86}$ (8 x $10^5$ - 1 x $10^6$ cpm) and the liposomes were potassium liposomes (cf. 'liposome formation').

3. The ionophore effect of different membrane fractions on the transport of calcium into the liposomes: The procedure was the same as described for testing sodium and potassium ionophores in the previous paragraphs except that the buffer was 4 x $10^{-1}$ M potassium phosphate pH= 7.4 containing 1 x $10^{-5}$ M $CaCl_2$ and $Ca^{45}$ (8 x $10^5$ - 1 x $10^6$cpm) and the liposomes were calcium liposomes (cf. 'liposome formation').

*B. Titration of some antibiotics known as ionophores:*

It is well known that nigericin, gramicidin and A-23187 are ionophores for potassium, sodium and calcium respectively. The procedures for the titration of these ionophores, as control experiments, was the same as described in the 1., 2. and 3rd paragraphs above. The only difference is that instead of membrane fractions, appropriate ionophores were put into the incubation mixtures.

*C. Incubation mixtures for testing the outward transport of ions from the liposomes:*

1. The effect of the mitochondrial fraction on the outward transport of sodium from the liposomes: 0.115 ml 5 x $10^{-2}$ M sodium phosphate pH=7.4 was mixed with a certain amount of mitochondrial fraction. 10 ul liposomes loaded with $Na^{22}$ (prepared as described in 'liposome formation') was added and the volume was made up to 0.15 ml with distilled water. The contents were mixed gently and, after 1 min of incubation at room temperature, 0.1 ml of the incubation mixture was passed through a Dowex 50W x 8 column as described in the previous sections. The liposomes thus obtained were counted for radioactivity.

2. The effect of the mitochondrial fraction on the outward transport of potassium from the liposomes: The procedure was the same as described in the previous paragraph except that 5 x $10^{-2}$ M potassium phosphate pH=7.4 and $Rb^{86}$ loaded liposomes were used.

3. The effect of the mitochondrial fraction on the outward transport of calcium from the liposomes: The procedure was the same as described in the previous paragraphs except that 4 x $10^{-1}$ M potassium phosphate pH=7.4 containing 1 x $10^{-5}$ M $CaCl_2$ and $Ca^{45}$ loaded liposomes were used.

*Results*

The effect of antibiotics known as ionophores on the entrance of ions into the liposomes:

1. Titration of gramicidin for the entrance of sodium into the liposomes: Incubation mixtures containing appropriate buffer and liposomes for sodium transport and five different concentrations of gramicidin (1, 2, 2.5, 3 and 4 ng per 0.1 ml incubation mixture) were prepared as described under 'Materials and Methods'. To a sixth incubation mixture no ionophore was added (control). 0.1 ml incubation mixtures were drawn at the 3rd, 6th and 12th min after the addition of the liposo-

mes and passed through Dowex 50W x 8 columns. The radioactivity in the liposomes thus isolated was counted. Figure 1 shows the entrance of sodium into the liposomes at different time intervals and at different gramicidin concentrations.

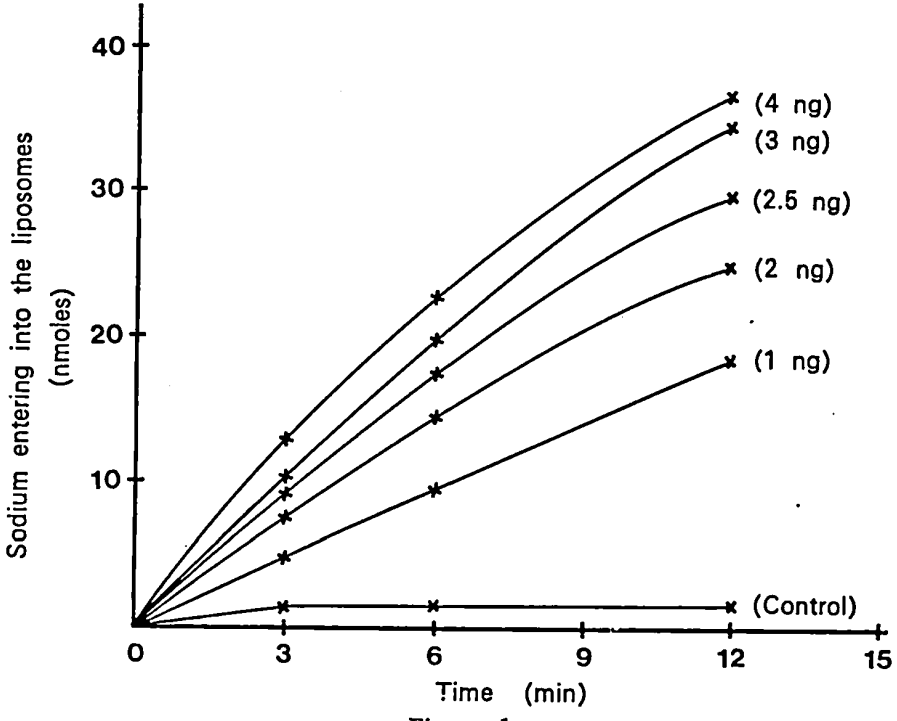


**Figure 1**

The entrance of sodium into liposomes (ordinate) as a function of time (abcissa) and at different gramicidin concentrations (shown in parenthesis at the right, per 0.1 ml incubation mixture).

The entrance of sodium into the liposomes increases as a function of time and as the concentration of gramicidin increases.

2. Titration of nigericin for the entrance of potassium into the liposomes: Incubation mixtures containing appropriate buffer and liposomes for potassium transport and four different concentrations of nigericin (2, 4, 6, 8 ng per 0.1 ml incubation mixture) were prepared as described under 'Materials and Methods'. At the 3rd, 6th and 12th min of incubation, liposomes were isolated from 0.1 ml incubation mixtures as described in the previous sections and radioactivity in the liposomes counted. Figure 2 shows the entrance of potassium into the liposomes at different time intervals and at different nigericin concentrations.

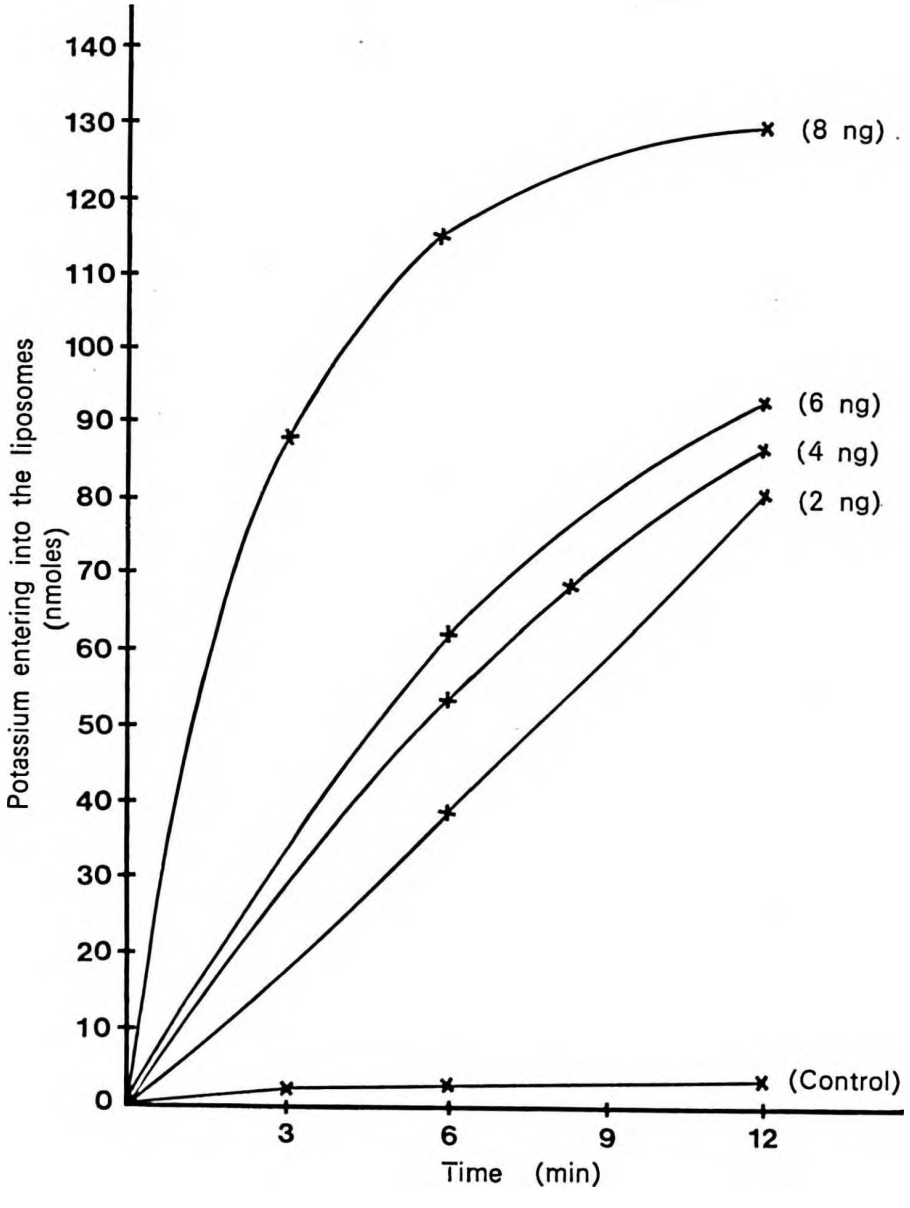


**Figure 2**

The entrance of potassium into liposomes (ordinate) as a function of time (abcissa) and at different nigericin concentrations (shown in parenthesis at the right, per 0.1 ml incubation mixture).

The entrance of potassium into the liposomes increases as a function of time and as the concentration of nigericin increases.

3. Titration of A-23187 for the entrance of calcium into the liposomes: Incubation mixtures containing appropriate buffer and liposomes for calcium transport and three different concentrations of A-23187 (15, 30, and 60 ng per 0.1 ml incubation mixture) were prepared as described under 'Materials and Methods'. Liposomes were isolated from 0.1 ml incubation mixtures drawn at the 6th and 12th min of incubation and the radioactivity counted. Figure 3 shows the entrance of calcium into the liposomes at different time intervals and at different A-23187 concentrations.

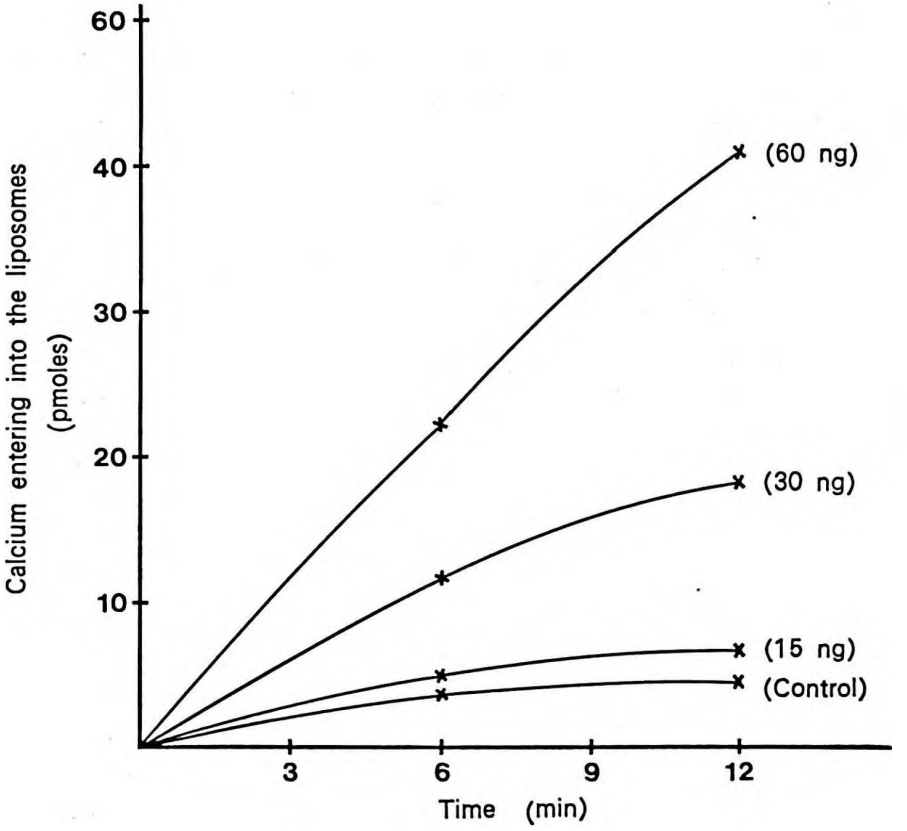


**Figure 3**

The entrance of calcium into liposomes (ordinate) as a function of time (abcissa) and at different A-23187 concentrations (shown in parenthesis at the right, per 0.1 ml incubation mixture).

The entrance of calcium into the liposomes increases as a function of time and as the concentration of A-23187 increases.

4. The effect of different natural membrane fractions on the entrance of sodium into the liposomes: Incubation mixtures were prepared as described under the lst paragraph of 'Results' except that, instead of gramicidin, different natural membrane fractions were added in different amounts as indicated in Table I.

The difference of the amounts of sodium entering the liposomes between the 6th and 15th min of incubation suggests the presence of a sodium ionophore in the white matter of the brain, in the electric organ and in the membrane fraction from *Halobacterium halobium*. Small amounts of endoplasmic reticulum fraction and the gray matter of the brain have no effect.

5. The effect of different natural membrane fractions on the entrance of potassium into the liposomes: Incubation mixtures were prepared as described under the 2nd paragraph of 'Results' except that, instead of nigericin, different membrane fractions were added in different amounts, as indicated in Table II.

The difference of the amounts of potassium entering the liposomes between the 6th and 15th min of incubation suggests the presence of a potassium ionophore in each natural membrane.

6. The effect of different natural membrane fractions on the entrance of calcium into the liposomes: Incubation mixtures are prepared as described under the 3rd paragraph of 'Results' except that, instead of A-23187, different membrane fractions were added in different amounts, as indicated in Table III.

The difference of the amounts of calcium entering the liposomes between the 6th and 12th min of incubation suggests the presence of of a calcium ionophore in the electric organ and the membrane from *Halobacterium halobium*. On the other hand, no ionophore effect could be detected in the endoplasmic reticulum membranes from muscles.

7. The effect of mitochondrial fractions on the outward transport of sodium, potassium and calcium ions from the liposomes: Appropriate liposomes and incubation mixtures were prepared as described under 'Materials and Methods'. Three incubation mixtures were prepared for each ion to be tested. One incubation mixture consisted of only the appropriate buffer and ion loaded liposomes (blank); the second incubation mixture, in addition, consisted of the mitochondrial fraction (test); and the third incubation mixture consisted of the appropriate

## TABLE I

THE EFFECT OF DIFFERENT NATURAL MEMBRANES ON THE ENTRANCE OF SODIUM INTO THE LIPOSOMES

| | | Sodium entering into the liposomes (nmoles) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endoplasmic reticulum | | White matter of the brain | | Gray matter of the brain | | Electric organ | | Membrane o *Halobacterium halobium* | | Control |
| Amount of membrane fraction put in the incubation mixture (ul) | | 5 | 20 | 5 | 20 | 5 | 20 | 10 | 30 | 10 | 30 | No membrane |
| Time | 6 th min | 0.77 | 1.41 | 1.30 | 1.20 | 1.68 | 1.44 | 4.66 | 5.42 | 5.90 | 4.80 | 0.77 |
| | 15 th min | 1.00 | 1.81 | 3.35 | 3.34 | 1.59 | 2.08 | 5.56 | 6.67 | 8.02 | 8.26 | 1.06 |
| Difference of 6-15 min | | 0.23 | 0.40 | 2.05 | 2.14 | (–) | 0.64 | 0.90 | 1.25 | 2.12 | 3.46 | 0.29 |

The amount of sodium entering into the liposomes (nmoles) at the 6th and 15th min of incubation, when indicated amounts of membrane fractions are used, are shown. Each value is the average of two experiments. (cf. 'Materials and Methods' for the preparation of incubation mixtures).

## TABLE II

THE EFFECT OF DIFFERENT NATURAL MEMBRANES ON THE ENTRANCE OF POTASSIUM INTO THE LIPOSOMES

| | | Potassium entering into the liposomes (nmoles) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endoplasmic reticulum | | White matter of the brain | | Gray matter of the brain | | Electric organ | | Membrane of *Halobacterium halobium* | | Control |
| Amount of membrane fraction put in the incubation mixture (ul) | | 10 | 25 | 10 | 25 | 10 | 30 | 10 | 30 | 10 | 30 | No membrane |
| Time | 6 th min | 5.35 | 10.12 | 5.60 | 13.51 | 7.28 | 5.46 | 4.60 | 4.15 | 6.25 | 11.10 | 5.50 |
| | 15 th min | 10.45 | 19.55 | 9.16 | 17.08 | 9.09 | 7.77 | 6.85 | 7.75 | 11.00 | 17.60 | 6.60 |
| Difference of 6-15 min | | 5.10 | 9.43 | 3.56 | 3.57 | 1.81 | 2.31 | 2.25 | 3.60 | 4.75 | 6.50 | 1.10 |

The amount of potassium entering into the liposomes (nmoles) at the 6th and 15th min of incubation, when indicated amounts of membrane fractions are used, are shown. Each value is the average of two experiments (cf. 'Materials and Methods' for the preparation of incubation mixtures).

## TABLE III

THE EFFECT OF DIFFERENT NATURAL MEMBRANES ON THE ENTRANCE OF CALCIUM INTO THE LIPOSOMES

| | | Calcium entering into the liposomes (pmoles) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Endoplasmic reticulum | | Electric organ | | Membrane of *Halobacterium halobium* | | Control |
| Amount of membrane fraction put in the incubation mixture (ul) | | 2 | 4 | 10 | 30 | 10 | 30 | No membrane |
| Time | 6 the min | 3.27 | 4.46 | 2.08 | 2.32 | 4.49 | 4.49 | 3.37 |
| | 12 th min | 2.95 | 3.56 | 2.92 | 3.39 | 6.18 | 6.48 | 4.05 |
| Difference of 6-12 min | | (–) | (–) | 0.84 | 1.07 | 1.69 | 1.99 | 0.68 |

The amounts of calcium entering into the liposomes (pmoles) at the 6 th and 12 th min of incubation, when indicated amounts of membrane fractions are used, are shown. Each value is the average of two experiments (cf. 'Materials and Methods' for the preparation of incubation mixtures).

## TABLE IV

THE EFFECT OF DIFFERENT NATURAL MEMBRANES ON THE OUTWARD TRANSPORT OF Na+, K+ AND Ca++ IONS FROM THE LIPOSOMES[1]

| Radioactivity remaining in the liposomes (cpm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sodium[2] | | | Potassium[4] | | | Calcium[6] | | |
| Blank | Mitochondrial fraction | Gramicidin[3] | Blank | Mitochondrial fraction | Nigericin[5] | Blank | Mitochondrial fraction | A–23187[7] |
| 559 | 201 | 48 | 346 | 30 | 23 | 259 | 36 | 30 |

1. Cf. 'Materials and Methods' for experimental conditions. 8 ul of mitochondrial fraction is used for each experiment. The values are the average of two experiments.
2. The radioactivity in 10 ul of liposomes at the beginning of the incubation was 1321 cpm.
3. Gramicidin concentration was 5 ug per 0.125 ml incubation mixture.
4. The radioactivity in 10 ul of liposomes at the beginning of the incubation was 756 cpm.
5. Nigericin concentration was 2 ug per 0.125 ml incubation mixture.
6. The radioactivity in 10 ul of liposomes at the beginning of the incubation was 687 cpm.
7. A–23187 concentration was 3 ug per 0.125 ml incubation mixture.

buffer, liposomes loaded with ions and the ionophore specific for the ion to be tested (control). The results from each incubation mixture prepared for three ions are compared in Table IV.

The values suggest that the mitochondrial fraction is effective in carrying the sodium ions about 36 % and the potassium and calcium ions almost 100 % out of the liposomes.

## *Discussion*

The dependence of ion transport across the liposome membranes by known ionophores (gramicidin, nigericin, and A-23187) on time and the concentration of the ionophore is shown in Figures 1, 2, and 3. Various results were obtained when different natural membrane fractions used instead of known ionophores. These findings are described under 'Results'. The values suggest that the white matter of the brain, the electric organ and the membrane fraction of *Halobacterium halobium* can transport sodium; in addition to these natural membrane fractions, the gray matter of the brain and the endoplasmic reticulum fraction of the muscle can transport potassium; and, only the electric organ and the membrane fraction of *Halobacterium halobium* can transport calcium. On the other hand, it was found that the endoplasmic reticulum fraction has no effect on the transport of calcium. Calcium is a very important ion for muscle contraction so this last finding suggests the necessity of a second factor (e.g. a phospholipid) for the transport of calcium by endoplasmic reticulum membranes.

*In vivo* experiments indicate the existence of sodium and potassium channels in nerves.[22,23] This findings, at least, support one of the results of this work and that is that the white matter of the brain can transport sodium and potassium with a mechanism other than active transport. The molecules which make these channels have not yet been identified.

The mitochondrial fraction was found to be effective in carrying out of the liposomes almost 100 % of the potassium and calcium ions and about 36 % of the sodium ions. These findings can well be applied to some mechanisms or processes thought to occur in mitochondria . For example, there is evidence that calcium is accumulated and there is a proton exchange in the mitochondria during electron transport.[12, 24, 25] It is possible that ionophores, besides active transport, may have a role in these ion translocations.

The results are not stoichiometric neither with known ionophores nor with natural membrane fractions although they are supported by physiological findings. Fractions obtained from the same sources and by

the same procedures, do not always reveal the same values. The reasons for these may include inevitable differences in the preparation procedures, the hydrophobic characteristics of the ionophores and the way of reaction of the ionophores with the lipids of the liposomes. Therefore we tried not to give exact amounts for natural membrane fractions. The amount necessary for maximum transport should be determined for each new preparation.

Little is known about cell membranes because their structure and function(s) are very complicated. Recently, to elucidate the membrane structure and function(s), it was attempted to isolate separately and then reconstitute the structural units of the membranes.[20, 21, 26, 27]

There are some clinical situations which have not been explained in the molecular level yet. Since cell function mostly depends on the intactness of the cell membrane, disturbances of the cell membrane may be the cause of some disorders encountered in clinics. For example, it has been suggested that the $Na^+ - K^+$ adenosine-triphosphatase activity of the erythrocytes and leukocytes of uremic patients is not normal.[16]

Some membrane proteins responsible for anion permeability are described in human erythrocyte membranes.[28] In this study we tried to look for the existence of organic molecules which may act as ionophores for the three ions, sodium, potassium and calcium. It is now necessary to isolate and identify these ionophores and to find out if there is a change in their characteristics under different clinical situations.

*Summary*

As an approach to elucidate the complicated structure of cell membranes, we looked for the existence of molecules with ionophore characteristics in different natural membrane fractions. It was found that a fraction of the white matter of bovine brain, the electric organ of the eel and a membrane of *Halobacterium halobium* act as an ionophore for sodium; in addition to this, a fraction of the gray matter of bovine brain and muscle endoplasmic reticulum act as an ionophore for potassium. There was also evidence that bovine heart mitochondria membrane fractions are very effective in the transport of potassium and calcium.

Now it is necessary to identify these structures, like the proteins responsible for the transport of anions in the human erythrocyte membranes, and to look for their role in different clinical situations.

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