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THE USE OF ^{60}Co-EDTA AS AN EXTRACELLULAR MARKER IN FROG SKIN

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Summary

^{60}Co-EDTA was tested as an extracellular marker in isolated epithelia of frog skin.

It was found to be non-toxic for frog skin and 0.1 mM EDTA proved to be enough to saturate any adsorption sites.

Comparing with [^{14}C]inulin, ^{60}Co-EDTA marks a slightly greater extracellular space and this volume is constant between 15 min and 2 h.

Furthermore it is reproducible and its use simplifies the methodology of the determination of water and electrolyte contents in the cells.

Introduction

The use of extracellular markers to distinguish between extracellular and intracellular water is important for the determination of ion content in tissues or cells, uptake and washout experiments, to study osmotic and other phenomena. The ideal choice is the use of an impermeant molecule, but the criteria of impermeability is very difficult to assess in absolute terms. A molecule that diffuses rapidly in the extracellular water with even distribution, and slowly in the cells can be an acceptable marker. Small molecules, in general, diffuse quickly in the extracellular space, but also penetrate cells. Larger molecules may not diffuse homogeneously in the extracellular space due to the existence of blind channels with small diameter. It is known that the apparent volume of distribution increases in small steps with decreasing molecular size [1].

Another problem is the binding of these molecules to membrane structures, giving volumes of distribution greater than the volume of tissue water [2].

In tissues such as frog skin, different results have been obtained with different extracellular markers [3—7]. Mannitol gives a larger extracellular space in

short time incubation and levels off with inulin at 2 h [5]. Both substances may, however, bind to the stratum corneum giving larger extracellular space than the Na space estimated with ^{22}Na or the volume of tissue water in this layer [2].

Brading and Jones [8] studied the complex, ^{60}Co-EDTA, as an extracellular marker in smooth muscle. This complex seems to be stable, non-toxic, easy to measure and a small piece of tissue can be used for determinations of the levels of Na^+, K^+, Cl^- and H_2O. Therefore, we decided to study the possibility of using ^{60}Co-EDTA in isolated frog skins.

Methods

Isolated frog epithelia were obtained from *Rana ridibunda pallas*, kept in the cold room in running tap water, at 4—6° C.

The isolated epithelia were obtained according to the methods of Aceves and Erlij [9] and Rajerison et al. [10]. The skins were mounted on funnel-shaped, Perspex holders with the chorion side upwards. The outside was immersed in usual Ringer at 25° C and well-aerated. The chorion side was bathed with a Ringer solution containing collagenase (Sigma Type I). On this side of the skin, a hydrostatic pressure of 15—20 cm^2 was exerted and incubation lasted for 2 h. The skin was then placed on a Petri dish and desiccation performed.

Experiments were carried out in beakers or in Ussing-type chambers using an automatic voltage-clamp devide for short-circuit current and conductance measurements. The usual NaCl Ringer was 112 mM Na^+, 119 mM Cl^-, 2.4 mM K^+, 1 mM Ca^{2+}, 1 mM Mg^{2+} and 5 mM glucose. Collagenase-Ringer contained 40 U/ml collagenase and 5 mM Ca^{2+} (according to Seglen [11], in isolated liver cells the collagenase efficiency is increased in Ringer solution at this Ca^{2+} concentration). $CoCl_2$ was tested at 0.1, 0.5 and $1 \cdot 10^{-4}$ M and EDTA at 0.05, 0.1, 0.5 and $1 \cdot 10^{-3}$ M. The final ^{60}Co-EDTA Ringer had $0.5 \cdot 10^{-4}$ M $CoCl_2$, $1 \cdot 10^{-4}$ M EDTA and no Ca^{2+} or Mg^{2+}.

[*carboxy*-^{14}C]Inulin was also used to compare the results with those obtained with ^{60}Co-EDTA.

Amiloride was used at 10^{-4} M and ouabain at 10^{-2} M. Syntocinom (Sandoz) was used at 2 U/100 ml Ringer. The aerated Ringer solutions were all titrated to pH 8 with Tris base.

At the end of each experiment, the skins were blotted with filter paper, placed on tared aluminium boxes, weighed, dried at 100° C to a constant weight and placed in vials for counting of the ^{60}Co. Suitable amounts of 0.1 M HNO_3 were added to the vials with slow agitation at room temperature for 24 h for extraction of inulin, Na^+ and K^+. For [^{14}C]inulin determination, 1 ml tissue extract was used with 5 ml scintillation solution (10% naphthalene, 0.7% 2,5-diphenyloxazole (PPO), 30% 1,4-bis-(5-phenyloxazolyl-2-benzene (POPOP) in dioxane) and counted in a β-counter. Na^+ and K^+ of the Ringer solution and tissue extracts were measured by flame photometry after proper dilutions.

Total water content was calculated from the difference between wet and dried weight, extracellular space was calculated assuming that ^{60}Co-EDTA or [^{14}C]inulin equilibrate in the extracellular space reaching the same concentration of the medium and were expressed as kg per kg dry weight and percent of tis-

sue water, respectively. Intracellular Na^+ and K^+ were calculated from the total amount measured and corrected for the extracellular fluid, assuming they are at the same concentrations in this space as in the Ringer solution. These results were then expressed in mequiv./kg cell water.

Results are expressed as means and standard errors of the means.

Results and Discussion

The toxicity of $CoCl_2$ was tested on isolated frog skins at 0.1, 0.5 and $1 \cdot 10^{-4}$ M. No effect was observed on I_{sc} or on conductances when $CoCl_2$ was applied either to the outer or to the inner side of the preparation.

Tolerance to EDTA. Isolated frog skins were subjected to EDTA either from the outside or from the inside at 0.05, 0.1, 0.5 and $1.0 \cdot 10^{-3}$ M. It was observed that skin with small conductance did not change this parameter with any of these concentrations on each side. However, skin with high conductance may start to increase its conductance even with low EDTA concentrations. When, in these latter type of skin, a control period was made, the conductance recovered its initial value. Therefore, we think that the change in conductance is reversible and does not mean any permanent damage to the skin.

To exclude any adsorption of ^{60}Co-EDTA to the tissue structure, two concentrations of carrier were tested. The results of experiments with 11 pairs of half skins show that the extracellular space is the same with the $0.1 \cdot 10^{-3}$ and $0.5 \cdot 10^{-3}$ M EDTA (33.3% ± 2.3 versus 32.5% ± 2.3 of total water) suggesting that any adsorption sites were already saturated at the low carrier concentration.

^{60}Co-EDTA and [^{14}C]inulin extracellular spaces were compared at different times (Table I). It can be seen that [^{14}C]inulin gave lower values initially, but steadily increasing and reaching similar values to ^{60}Co-EDTA at 2 h. The ^{60}Co-EDTA extracellular space had steady values from 15 min to 2 h. The linear regression (least mean squares) gave a slope of $4.9 \cdot 10^{-3}$ that represents an increase of 1.8% in 2 h.

^{60}Co-EDTA seems therefore to fulfill some of the requirements needed for a new extracellular marker. It has values not very different from a known extracellular marker, has a plateau for almost 2 h, suggesting very little, if any, penetration into the cells and a homogeneous distribution in the extracellular space.

TABLE I

THE EXTRACELLULAR SPACE IN ISOLATED FROG SKIN MEASURED WITH ^{60}Co-EDTA AND [^{14}C]INULIN AT DIFFERENT TIMES

The extracellular water is expressed as percentage of total tissue water. Number of experiments, 10.

	Minutes				
	15	30	60	90	120
^{60}Co-EDTA	32.7 ± 1.7	33.9 ± 1.3	31.2 ± 1.6	33.2 ± 1.7	33.7 ± 2.7
[^{14}C]Inulin		24.1 ± 1.2	23.5 ± 1.7	27.1 ± 1.3	33.3 ± 1.2

TABLE II

DETERMINATION OF WATER AND ION CONTENT IN PAIRED SKINS INCUBATED IN BEAKERS OR USSING-TYPE CHAMBERS

Number of experiments, 8.

	Total water (kg/kg dry wt.)	Extracellular water (percent of total water)	Total Na^+ content (mequiv./kg dry wt.)	Intracellular ion concentration (mequiv./kg cell water)	
				Na^+	K^+
Chamber	3.41 ± 0.12	29.7 ± 1.6	175.9 ± 9.2	27.6 ± 5.1	132.6 ± 2.0
Beaker	3.26 ± 0.14	37.3 ± 1.4	206.4 ± 13.5	28.8 ± 2.8	129.4 ± 3.2

The use of ^{60}Co-EDTA in the determination of fluid and ion content

(1) A comparison of results from half skins immersed in beakers or mounted in Ussing-type chambers was made (Table II).

It can be seen that the extracellular space and total Na^+/unit dry weight were larger in those immersed in the beaker. But Na^+ and K^+ concentrations in the cells were similar. This can be explained by the fact that the pieces of skin in the beaker were smaller and the contribution of the cut edges for the extracellular spaces was appreciable, whereas, for the epithelia mounted in the chambers, they are only cut at the end of the incubation period.

(2) In Table III, we compare the results of pairs of half skins mounted in 2-cm^2 chambers. It can be seen that the pairs of half skins are comparable and can be used as controls for each other.

(3) The determination of water and ion contents were made under the influence of different compounds, namely amiloride, pitresin, ouabain and controls. This is considered to be under short-circuit conditions as the skins are shorted by the fluid in the beaker [12]. Amiloride is known to act only at the external side, while pitresin and ouabain only act when applied at the internal side (Table IV).

The most striking results are those obtained with ouabain, where an increase of Na^+ and a decrease of K^+ are observed. This agrees with the results of Macknight et al. [13,14]. It is also known that amiloride and pitresin, affecting the Na^+ permeability of the external border, do not necessarily change the K^+ concentrations in the skin [15,16]. The effect of these drugs on Na^+ content,

TABLE III

DETERMINATION OF WATER AND ION CONTENT IN PAIRED HALF SKINS MOUNTED IN USSING-TYPE CHAMBERS AND USED AS CONTROL OF EACH OTHER

Number of experiments, 12.

	Total water (kg/kg dry wt.)	Extracellular water (percent of total water)	Intracellular ion concentrations (mequiv./kg cell water)	
			Na^+	K^+
Odds	3.84 ± 0.12	26.6 ± 1.3	25.4 ± 4.8	147.7 ± 6.7
Evens	3.92 ± 0.14	27.5 ± 1.6	22.5 ± 3.0	140.3 ± 5.9

TABLE IV

DETERMINATION OF WATER AND ION CONTENT IN ISOLATED FROG SKIN EPITHELIA UNDER DIFFERENT EXPERIMENTAL CONDITIONS: CONTROL, AMILORIDE, PITRESIN AND OUABAIN

In brackets number of determinations.

	Total water (kg/kg dry wt.)	Extracellular water (percent of total water)	Intracellular ion concentrations (mequiv./kg cell water	
			Na^+	K^+
Control (9)	4.19 ± 0.11	30.5 ± 2.5	31.7 ± 3.2	117.7 ± 3.1
Amiloride (9)	4.04 ± 0.28	30.9 ± 2.0	28.4 ± 1.6	118.5 ± 1.9
Pitresin (8)	3.98 ± 0.08	30.2 ± 2.6	38.4 ± 3.6	116.6 ± 3.0
Ouabain (8)	3.68 ± 0.10	34.3 ± 2.8	89.2 ± 6.8	75.0 ± 5.5

although qualitatively in agreement with the literature, are not statistically significant.

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