The authors would like to express their appreciation to Mr. A. CANTOR for his valuable suggestions and criticism.

Departamento de Fisiología,
Escuela de Medicina y Departamento
de Biofísica, Escuela de Farmacia,
Universidad de Concepción,
Concepción (Chile)

C. GONZÁLEZ S.
J. SÁNCHEZ O.
J. CONCHA B.

1 E. SCHOFFENIELS AND M. L. SALEE, *Comp. Biochem. Physiol.*, 14 (1965) 587.
2 C. GONZÁLEZ, J. SÁNCHEZ AND J. CONCHA, *Biochim. Biophys. Acta*, 120 (1966) 186.
3 J. SÁNCHEZ, C. GONZÁLEZ AND J. CONCHA, *Arch. Biol. Med. Exptl.*, in the press.
4 H. H. USSING AND K. ZERAHN, *Acta Physiol. Scand.*, 23 (1951) 110.
5 V. KOEFOED-JOHNSEN, H. H. USSING AND K. ZERAHN, *Acta Physiol. Scand.*, 27 (1953) 38.
6 J. MARCELIN PLAISANCE AND J. CONCHA, *J. Physiol. Paris*, 55 (1963) 425.
7 A. F. GREEN, *Advances in Pharmacology*, Vol. I, Academic Press, New York, 1962, p. 204.
8 J. H. BURN AND M. J. RAND, *J. Physiol. London*, 147 (1959) 135.
9 C. B. FERRY, *J. Physiol. London*, 164 (1963) 487.

Received October 18th, 1966
Biochim. Biophys. Acta, 135 (1967) 167–170

BBA 73000

Nuclear spin resonance evidence for complexing of sodium in frog skin

When frog skin is mounted as a flat sheet between 2 lucite chambers containing Ringer solution, a net movement of Na^+ from the outer to the inner side is observed even if the Na^+ concentration in the outside bathing solution is as dilute as 1–10 mM (refs. 1–4). Recent studies by ROTUNNO, POUCHAN AND CEREIJIDO[4] have indicated that, under these conditions, the concentration of Na^+ in the cells is about 97 mM. Under the short-circuit conditions that they have used to measure the net Na^+ flux, there exists an electrical potential difference of 15–30 mV between the outer solution and the cell (negative pole). However, this electrical potential difference is too small to explain the asymmetry of Na^+ distribution across the outer facing membrane of frog skin. Since there is no evidence that an active mechanism is operating at the outer cell border, ROTUNNO, POUCHAN AND CEREIJIDO[4] suggested the possibility that the Na^+ in the cell was contained in 2 different compartments, one of them being directly involved in Na^+ transport. This suggestion is strongly supported by recent studies carried out with Ringer solution with 1–10 mM Na^+, in which, in 90 min, only 37% of the Na^+ in the cells exchanged with the $^{22}Na^+$ in the Ringer solution ($^{22}Na^+$ flux across the frog skin equilibrated in less than 30 min*). With respect to the nature of the 2 compartments there are 2 main possibilities: (a) Physical compartments, where Na^+ is contained in 2 different spaces, one of them surrounded by a Na^+-impermeable barrier. These compartments might be represented, for instance, by Na^+ contained in different kinds of cells or cell organelles. (b) Chemical compartments, where Na^+ is contained in possibly the same physical compartments but in 2 different states. These compartments might be represented by free and bound Na^+. To study

* M. CEREIJIDO AND C. A. ROTUNNO, unpublished observations.

this last possibility a nuclear magnetic resonance (NMR) analysis of the Na^+ contained in frog skin was carried out. It was based on the demonstration of JARDETZKY AND WERTZ[5] that the NMR spectrum of Na^+ gives a peak whose height is directly proportional to Na^+ concentration and that when Na^+ is complexed with ion exchangers the signal broadens and becomes invisible.

Adbominal skins of the South American frog *Leptodactylus ocellatus* were dissected in Ringer solution containing 1 or 5 mM NaCl, 2.4 mM $KHCO_3$, 1 mM $CaCl_2$, and sucrose to adjust the osmolarity to 238 mosM. They were allowed to equilibrate for 90 min in a beaker containing 400 ml of Ringer solution gassed with compressed air. The pH was 8.2 and the temperature 22°. A group of 8–10 skins treated in exactly the same way were then removed from the beaker, blotted with filter paper, placed in a tared test tube and weighed. They were then gently packed to a volume of 7–8 ml by means of a plug and the volume occupied by the sample was carefully measured. The NMR spectra of Na^+ were obtained on a Varian DP60 NMR spectrometer with a field of 14 000 Gauss. The radiofrequency was 15.8 Mcycles/sec. Spectra were recorded on a Varian G-11 recorder. Several measurements were performed on the same sample in order to reduce errors due to instrumental noise. Each individual measurement took about 10 min. Table I shows the individual values of peak height obtained with groups of skins incubated in 1 and 5 mM Na^+ respectively. It also gives the values obtained with solutions of 10 and 30 mM NaCl. The signals for the 2 groups of skins are equivalent to a 15 mM solution.

TABLE I

NUCLEAR MAGNETIC RESONANCE ANALYSIS OF Na^+ IN THE FROG SKIN

Na^+ peak height				
NaCl standard			*Skins preincubated in Ringer solution*	
10 mM		*30 mM*	*With 1 mM Na^+*	*With 5 mM Na^+*
	35	86	43	39
	30	85	46	46
	32	80	41	45
	33	88	45	47
	35		47	43
			40	35
			44	47
			47	44
			46	48
			43	46
			43	
Mean	33	85	44	44

After the NMR analysis was carried out, the Na^+ of the samples was extracted with 1.0 M HNO_3 at 80°, and measured by flame photometry. From this information and the volume of the sample, the Na^+ content was calculated. The sample incubated in 1 mM Na^+ contained 268 μmoles, and that incubated in the 5 mM solution, 307 μmoles of Na^+ (see Table II). Since NMR analysis gives the value of 15 mM, the free Na^+ content of the samples is 117 and 123 μmoles, respectively. The skin of *Lepto-*

dactylus ocellatus weighs 26.1±0.6 mg·cm^{-2} and has 13.6±0.9 μl·cm^{-2} of extracellular space[4]. Since the samples contained 7.224 and 7.343 g of skin, the computed extracellular space would be 3.77 and 3.83 ml, respectively. Assuming that Na^+ in the extracellular space is free and has the same concentration as in the incubation Ringer solution, there would be 5 μmoles of free Na^+ in the extracellular space in the first sample and 21 μmoles in the second. The rest of the Na^+ is contained in the cells. Therefore the cells of the skin incubated in 1 mM Na^+–Ringer solution have 263

TABLE II

STATE OF Na^+ IN THE FROG SKIN

	Volume of the sample (ml)	*Na^+ content (μmoles)*					
		*Flame photometry**	*NMR***	*Extracellular space*	*Cell*		
					Total	*Free*	*Ratio*
Incubated in 1 mM	7.8	268	117	5	263	112	0.426
Incubated in 5 mM	8.2	307	123	21	286	102	0.357

* Total Na^+ content of the sample = concentration by flame photometry × volume.
** Total free Na^+ content of the sample = concentration by NMR × volume.

μmoles of Na^+ of which 112 μmoles (*i.e.* 42.6%) is free. The corresponding values for the sample incubated in 5 mM Na^+ are: 286 total, 102 free (35.7%).

The finding that 42.6 and 35.7% of the total Na^+ of the cell is in free solution in the intracellular water agrees with the observation already mentioned that only 37% of the Na^+ in the cell exchanges with $^{22}Na^+$ in the bathing solution. It also offers an explanation for the asymmetric distribution of Na^+ across the outer facing membrane where a lack of the necessary amount of (Na^+–K^+)-sensitive adenosine triphosphatase to account for an active mechanism has been demonstrated[4]. Cellular Na^+ complexing is a very well established fact in other tissues[6–8]. In the case of the frog skin it would permit the cell to have relatively high Na^+ content even when the frog is swimming in a pond with fresh water.

This work was partially supported by research grants from the Consejo Nacional de Investigaciones Científicas of Argentina and the U.S. Public Health Service. We also wish to thank Miss M. BATTELLI for technical assistance.

Department of Physical Chemistry
and Department of Physics, Junín 956,
Universidad de Buenos Aires,
Buenos Aires (Argentina)

C. A. ROTUNNO
V. KOWALEWSKI
M. CEREIJIDO

1 L. B. KIRSCHNER, *J. Cellular Comp. Physiol.*, 45 (1955) 61.
2 F. M. SNELL AND C. P. LEEMAN, *Biochim. Biophys. Acta*, 25 (1957) 311.
3 M. CEREIJIDO, F. C. HERRERA, W. J. FLANIGAN AND P. F. CURRAN, *J. Gen. Physiol.*, 47 (1964) 879.
4 C. A. ROTUNNO, M. I. POUCHAN AND M. CEREIJIDO, *Nature*, 210 (1966) 597.
5 O. JARDETZKY AND J. E. WERTZ, *J. Am. Chem. Soc.*, 82 (1960) 318.

6 A. S. TROSHIN, in A. KLEINZELLER AND A. KOTYK, *Membrane Transport and Metabolism*, Academic Press, London, 1961, p. 45.
7 G. N. LING, *Federation Proc.*, 24 (1965) 103S.
8 F. W. COPE, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 225.

Received August 23rd, 1966

Biochim. Biophys. Acta, 135 (1967) 170–173

BBA 73002

Inability of anti-insulin serum to neutralize insulin after the hormone has become bound to muscle

Incubation of isolated frog sartorius muscles with insulin causes a gradual increase in permeability to sugar. After about 3 h of incubation at 19°, permeability reaches a plateau that is proportional to the concentration of insulin in the range of approximately 10 to 500 or 1000 microunits (μU) per ml (ref. 1). Subsequent washing of the muscles with Ringer's solution at 19° causes a slow reversal of the insulin effect, and insulin that has been bound by the tissue is concomitantly degraded under these conditions. On the other hand, neither degradation of bound insulin nor a reversal of the effect of insulin on permeability occurs to an appreciable extent when washing is performed for several hours at 0°. Reversal of the hormonal effect may depend upon removal of insulin from its site of action. Since anti-insulin serum rapidly combines with insulin in solution and effectively neutralizes it[2–4], it was of interest to see whether or not anti-insulin serum would also accelerate reversal of the hormonal effect on permeability.

A question arises as to the ability of antibodies to penetrate into the interstitial space of skeletal muscle. There is histological evidence that γ-globulin can penetrate into the interstitial space of cardiac muscle, at least in patients with rheumatic heart disease[5,6]. Furthermore, HUXLEY[7] has demonstrated that ferritin, a protein with a molecular weight of approximately 750 000, can penetrate into the transverse tubules of muscle cells within a few minutes when frog sartorius muscles are incubated *in vitro*. Therefore, it seems likely that γ-globulin molecules should be able to diffuse through the extracellular space of isolated sartorius muscles and reach the surface of the fibers.

Changes in permeability were measured by observing the initial rate of penetration of sugar into sartorius muscles at 19°. Tritium-labeled 3-*O*-methyl-D-glucose has been shown to be suitable for such measurements because it is not metabolized by frog muscles, and entry into the cells appears to be mediated by the same transport system that facilitates the entry of glucose[8]. For the present experiments, tritium-labeled 3-methylglucose was obtained, purified and counted in the manner described previously[8]. ^{14}C-Labeled mannitol was used for the simultaneous measurement of extracellular space, as in the earlier studies.

Antisera to insulin were obtained (*cf.* ref. 3) by injecting 0.5 mg of beef insulin in Freund's adjuvant into the foot pads of each of six guinea pigs on two occasions, one month apart, and then collecting heart blood. The relative potency of the antisera was tested by their ability to protect [^{131}I]insulin (Abbott Laboratories) from degrada-