Active extrusion of Ca^{2+} from epiphyseal chondrocytes of normal and rachitic chickens

Margherita ZANETTI, Roberta CAMEROTTO, Domenico ROMEO and Benedetto DE BERNARD
Istituto di Chimica Biologica, Università di Trieste, 34127 Trieste, Italy

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Chondrocytes isolated from the epiphyseal cartilage of chickens were exposed to either the ionophore A23187 or KCN, in the presence of 0.4 mM-extracellular Ca^{2+} . This treatment elicits a prompt release of cell Ca^{2+} , which can be measured as net cation efflux by a highly sensitive Ca^{2+} -selective electrode system. Pre-exposure of chondrocytes to the metabolic inhibitors 2-deoxy-D-glucose or oligomycin causes a parallel decrease in both cell ATP and ionophore-mediated Ca^{2+} extrusion. Conversely, substitution of extracellular Na^+ with choline produces only a very small decrease in the rate of Ca^{2+} efflux. This indicates that the ionophore A23187 and cyanide induce the mobilization of intracellular Ca^{2+} , which is then extruded from the cell mainly by an ATP-driven pump system. Chondrocytes isolated from the cartilage of rachitic chickens also exhibit the capacity for extruding Ca^{2+} by the same mechanism, with an efficiency comparable with that of their normal counterpart.

Most of the investigations on the mechanism of calcification are carried out at the level of the matrix of mineralizing tissues. This approach is prompted by the need to understand why only some matrices of connective tissues are mineralized, how precipitation of calcium phosphate occurs outside the cells, and what relationship exists between the organic and inorganic phase of the calcified areas.

Whatever the mechanism of calcification in the matrices, there is general agreement that calcification is a process controlled by the cells of connective tissue. Macromolecules involved in the mechanism of mineralization are synthesized and released by these cells. Furthermore, the concentration of phosphate and Ca^{2+} in the matrix is also likely to be controlled by histiocytes.

Many doubts still exist, however, concerning the origin of these ions and the rate and extent of their supply to the calcification area. Interesting and stimulating hypotheses have been proposed, from the oldest one by Robison (1923), concerning the origin of phosphate, to the more recent one by Lehninger (1970), on the origin and transport of calcium phosphate from cells to the matrix.

Participation of chondrocytes in the supply of Ca^{2+} to the matrix of calcifiable tissues should involve the translocation of the bivalent ion from suitable intracellular stores to the extracellular environment. In view of the steep inwardly directed Ca^{2+} gradient existing between cells and their environment, an active pump system localized at the

chondrocyte surface would thus be required for the expulsion of the cation. A system of this type, possibly active in the control of cytosolic steady-state concentrations of Ca^{2+} , has been described in various cell types (Schatzmann & Vincenzi, 1969; Babcock *et al.*, 1976; Baker, 1976a; Ferreira & Lew, 1976; Cittadini *et al.*, 1977; Dipolo, 1978; Schatzmann & Bürgin, 1978; Reeves & Sutko, 1979; Mottola *et al.*, 1980).

In the present investigation we have investigated whether chondrocytes possess such a peripheral Ca^{2+} -pump activity. In view of the altered Ca^{2+} metabolism in the cartilage of rachitic animals, the Ca^{2+} -pump system was analysed in chondrocytes of both normal and rachitic chickens. One approach we used for our study was that of increasing the membrane permeability to Ca^{2+} by the ionophore for bivalent cations, ionophore A23187 (Babcock *et al.*, 1976; Ferreira & Lew, 1976; Mottola *et al.*, 1980). This causes a release of Ca^{2+} from intracellular stores and thus may activate pump systems. If a Ca^{2+} -extrusion mechanism is operating at the level of the plasma membrane, a net efflux of Ca^{2+} is detected, whose rate and extent are dependent on the relative sizes of the pump and the ionophore-induced passive Ca^{2+} fluxes. An alternative approach was that of treating the chondrocytes with cyanide. This metabolic inhibitor causes discharge of Ca^{2+} from mitochondria (Lehninger, 1970; Rose & Loewenstein, 1975; Baker, 1976b), thereby leading to activation of the peripheral Ca^{2+} -pump ex-

trusion system without alteration of membrane permeability.

Experimental

Animals

Most of the experiments were carried out with domestic 4-week-old chickens. However, chondrocytes were also obtained from chickens that were subjected to an experimental rickets. In this case, white Leghorn chickens were caged for 4 weeks from the day of hatching in a windowless room. Illumination was provided by a fluorescent light with no u.v. spectrum. The chickens were divided into two groups, which received distilled water *ad libitum* and either a rachitogenic vitamin D-deficient diet (rachitic group) or the same diet supplemented with 100 i.u. of vitamin D/kg of diet (control group) (Hurwitz & Bar, 1972). Attainment of the rachitic conditions was demonstrated by histological examinations of the epiphyseal plates.

Cell isolation

The epiphyseal cartilage of long bones was dissected, rinsed in Gey's solution (Gey & Gey, 1936) with 0.04 g of gentamycin/litre, minced with a lancet and subjected to enzymic digestion. This was carried out at 37°C for 20 min in the presence of 2 g of collagenase/litre (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) and 1 g of trypsin/litre (Sigma Chemical Co., St. Louis, MO, U.S.A.), with continuous stirring. After decantation from the digested material, the residual tissue was further incubated at 37°C for 45 min in the presence of collagenase alone. The digested tissue was rinsed in Gey's solution with 5 g of bovine serum albumin/litre (Sigma Chemical Co., St. Louis, MO, U.S.A.) and mildly homogenized in a Potter-Elvehjem homogenizer. After filtration through six layers of cheesecloth, cells were harvested by centrifugation for 7 min at room temperature and at 200 g, and rinsed twice in a medium containing 122 mM-NaCl, 5 mM-KCl, 1.2 mM-MgCl₂ and 16 mM-Tris/HCl, pH 7.4 (Na⁺/K⁺/Tris medium). When indicated, cells were washed once in a similar medium containing 16 mM-imidazole/HCl, pH 6.1, instead of Tris buffer (Na⁺/K⁺/imidazole medium). By this procedure about 3×10^8 chondrocytes were obtained from 10 g (wet wt.) of cartilage. In the course of the experiments, chondrocytes were maintained at room temperature in Na⁺/K⁺/Tris medium for a maximum of 3 h.

Cell viability was assessed by the exclusion of the dye Trypan Blue and by the capacity of chondrocytes to adhere to plastic Petri dishes in the presence of a nutrient medium.

Ca²⁺ fluxes

Net outfluxes of Ca²⁺ from chondrocytes were followed by a very sensitive Ca²⁺-selective electrode

(Möller, Zürich, Switzerland)/amplifier/recorder system, as previously described (Flora *et al.*, 1980; Mottola *et al.*, 1980). On equilibration of the electrode with the medium (3 ml), a portion of the fluid was withdrawn and replaced by a suspension of $(2-4) \times 10^7$ cells, the final concentration of Ca²⁺ being kept at the desired value with addition of small volumes of 100 mM-CaCl₂. Ionophore A23187 (Calbiochem-Behring Corp., San Diego, CA, U.S.A.) and oligomycin (Sigma Chemical Co., St. Louis, MO, U.S.A.) were delivered in a few μ l of a stock solution in dimethyl sulphoxide and ethanol respectively. Changes in the external Ca²⁺ concentration were determined by calibration with EGTA at the end of each experiment (Flora *et al.*, 1980).

Analytical procedures

For the determination of total cell calcium, $(2-4) \times 10^8$ chondrocytes were washed once in 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.4), transferred to crucibles, dried and heated for 16 h at 550°C. Ashes were dissolved in 25 ml of triple-distilled water, containing 1 M-HCl and 10 g of lanthanum chloride/litre, and analysed for total Ca²⁺ by atomic-absorption spectrophotometry at 422 nm, utilizing a Perkin-Elmer model 306 spectrophotometer with an air/acetylene flame. Calcium standards and reagent blanks were run with each batch of samples.

ATP content of chondrocytes was assayed fluorimetrically in HClO₄ extracts with yeast glucose 6-phosphate dehydrogenase (Boehringer-Mannheim G.m.b.H., Mannheim, Germany) and hexokinase (Sigma Chemical Co.) essentially as described by Williamson & Corkey (1969).

Protein was determined by the method of Peterson (1977), with bovine serum albumin as standard.

Results

General properties of isolated chondrocytes

Chondrocytes isolated by enzymic digestion of the epiphyseal cartilage of healthy chickens are 90–95% viable. Light and transmission-electron-microscopy examinations show that their ultrastructure is well preserved and no organized matrix at the external periphery of the cells was detected. The viability of chondrocytes of cartilage of rachitic chickens, as determined by the dye-exclusion test, is 75–85%.

Chondrocytes isolated from domestic fowls contain 131 ± 18 nmol of total Ca²⁺ (Table 1) and 0.9 ± 0.2 mg of protein (mean of seven determinations $\pm$ S.E.M.) per 1×10^7 cells. Conversely, the content of total Ca²⁺ in chondrocytes of rachitic animals is significantly lower, being 72 ± 12 nmol/ 1×10^7 cells (Table 1).

Table 1. *Effect of ionophore A23187 and of KCN on the release of Ca^{2+} from chicken epiphyseal chondrocytes*
Experiments were carried out as described in Fig. 1. Experimental chickens were fed as reported in the Experimental section. Total cell calcium was determined by atomic-absorption spectrophotometry. Data are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses; rates and extents of Ca^{2+} release were evaluated in each experiment. Values with identical symbols are significantly different ($P < 0.05$) by Student's *t* test.

		Experimental chickens		
		Domestic chickens	—Vitamin D	+ Vitamin D
Rate of Ca^{2+} release (nmol/min per 1×10^7 cells)	Ionophore A23187	$3.1^{*}\dagger \pm 0.1$ (7)	$2.4^{*} \pm 0.1$ (6)	$2.9^{\S} \pm 0.3$ (4)
	KCN	$1.8^{\dagger} \pm 0.2$ (5)	2.0 ± 0.3 (5)	$2.0^{\S} \pm 0.2$ (6)
Extent of Ca^{2+} release (nmol/ 1×10^7 cells)	Ionophore A23187	5.5 ± 0.6	7.1 ± 1.2	5.4 ± 0.1
	KCN	10.1 ± 3.3	7.2 ± 1.6	9.6 ± 1.2
Total cell calcium (nmol/ 1×10^7 cells)		131 ± 18 (8)	72 ± 12 (6)	—

Ca^{2+} fluxes

Cells maintained at room temperature in the Ca^{2+} -free $\text{Na}^{+}/\text{K}^{+}/\text{Tris}$ medium were used for these studies. When added to the electrode vessel, chondrocyte suspensions prove to contain some extracellular Ca^{2+} , probably released during storage at room temperature. Furthermore, they continue to release Ca^{2+} and this release process (i) is inversely dependent on the concentration of Ca^{2+} in the electrode medium from 0.1 to 1 mM- Ca^{2+} , (ii) is much faster at pH 6.1 than at pH 7.4 and (iii) is inhibited by the presence of phosphate or bicarbonate in the medium.

Exposure of chondrocytes to $\text{Na}^{+}/\text{K}^{+}/\text{imidazole}$ medium, pH 6.1, for 2 min, followed by cell centrifugation and resuspension in $\text{Na}^{+}/\text{K}^{+}/\text{Tris}$ medium, pH 7.4, markedly diminishes the spontaneous release of Ca^{2+} , as detected in the electrode vessel. This rinsing procedure was thus routinely adopted for the investigation of the ionophore- or cyanide-mediated activation of Ca^{2+} extrusion.

This process was followed by permitting the cells to equilibrate with the medium, containing 0.4 mM- Ca^{2+} , and by subsequently adding either ionophore A23187 at the final concentration of 2.5 μM or 2 mM-KCN. As shown in Fig. 1, addition of the ionophore to the chondrocytes results in an immediate release of Ca^{2+} , which attains the steady state by 4–5 min. Cyanide also stimulates a net efflux of Ca^{2+} , with a lag period of about 90 s. In this case the steady state is reached after about 10 min from cell exposure to the mitochondrial inhibitor.

Table 1 shows the rates and amounts of Ca^{2+} released from chondrocytes isolated from either healthy or rachitic chickens and exposed to either the ionophore or cyanide. With the exception of chondrocytes from rachitic animals, the rate of Ca^{2+} extrusion is significantly higher when the stimulus is provided by the ionophore A23187 rather than by the mitochondrial inhibitor. Conversely, the extent of Ca^{2+} release is generally greater with cyanide-treated cells. When calculated as a percentage of

Fig. 1. *Release of Ca^{2+} from epiphyseal chondrocytes exposed to either the ionophore A23187 or KCN*

Ca^{2+} net fluxes were followed with a sensitive Ca^{2+} -selective electrode/amplifier/recorder system (Mottola *et al.*, 1980; Flora *et al.*, 1980). The assay medium (3 ml) contained 123 mM- NaCl , 5 mM- KCl , 1.2 mM- MgCl_2 , 0.4 mM- CaCl_2 , 10 mM-glucose and 16 mM- Tris/HCl (pH 7.4). Chondrocytes from domestic chickens were maintained at room temperature in the same medium, but in the absence of CaCl_2 and glucose. On equilibration of the electrode system at 37°C, a portion of the medium was withdrawn and replaced by a suspension of $(2-4) \times 10^7$ chondrocytes, the final concentration of Ca^{2+} in the assay medium being kept at the value of 0.4 mM. The ionophore A23187 was added in 7.5 μl of dimethyl sulphoxide. Additions of oligomycin (from a stock solution in ethanol) and of 2-deoxy-D-glucose were made 5 min before cell exposure to the ionophore. The experiment with 2-deoxy-D-glucose was carried out in the absence of glucose. Downward deflections in the traces indicate a rise in the Ca^{2+} concentration in the medium, which were quantified by calibration with portions of standard solutions of EGTA at the end of each experiment (Flora *et al.*, 1980).

total cell calcium, the amount of the bivalent cation extruded from the cells ranges from 4 to 10%.

A comparison of the efficiency of Ca^{2+} extrusion

from chondrocytes of healthy and rachitic chickens indicates that the rate of ionophore A23187-induced Ca^{2+} release from epiphyseal cells of rachitic animals is slightly, but significantly, lower with respect to chondrocytes of control domestic animals. However, no such difference is seen when the rate of Ca^{2+} extrusion from cells of diseased chickens is compared with that of chondrocytes derived from animals whose diet was supplemented with vitamin D.

Ca^{2+} is energetically extruded from the cell by two mechanisms. The first is the unidirectional export of Ca^{2+} via a high-affinity ATP-driven Ca^{2+} -extrusion pump (Schatzmann & Vincenzi, 1969; Ferreira & Lew, 1976; Dipolo, 1978; Schatzmann & Bürgin, 1978; Mottola *et al.*, 1980). The second is an antiporter-coupled exchange of extracellular Na^+ for intracellular Ca^{2+} (Baker, 1976a; Reeves & Sutko, 1979). To discriminate between these two possible mechanisms, the ionophore A23187-induced Ca^{2+} release from chondrocytes was studied either with cells that were pretreated with metabolic inhibitors known to interfere with the production of ATP or in a medium in which Na^+ was replaced by choline.

Fig. 1 shows that pre-treatment of chondrocytes with either oligomycin or 2-deoxy-D-glucose depresses the efficiency of the Ca^{2+} -extrusion system. In particular, the two metabolic inhibitors exhibit approx. 20% and 80% inhibitions of the rate of Ca^{2+} release from ionophore A23187-treated chondrocytes respectively. The extent of inhibition closely

mirrors the effect of the two inhibitors on the ATP content of chondrocytes (Table 2).

Conversely, elimination of the inwardly directed Na^+ gradient causes only a less than 20% inhibition of the rate of Ca^{2+} extrusion from chondrocytes exposed to ionophore A23187.

Discussion

Assay of Ca^{2+} movements between isolated chondrocytes and a Ca^{2+} -containing medium has shown that these cells can spontaneously release Ca^{2+} at 37°C. This release could be due to either activation of a pump system, as suggested for Ehrlich ascites-tumour cells brought from ice-cold temperature to 37°C (Cittadini *et al.*, 1977), or dissociation of the ion from extracellular-surface-bound macromolecular complexes. Although the ultrastructural analysis of the isolated chondrocytes has revealed no organized matrix in the observed specimen, the release of Ca^{2+} from microportions of contaminating matrix material cannot be ruled out. The type of dependence of this process on the concentration of H^+ , Ca^{2+} , phosphate and bicarbonate suggests that the released Ca^{2+} might derive from the inorganic phase of the matrix and/or from the calcium-acidic phospholipid-phosphate complex of the cell membrane (Boskey & Posner, 1977).

The release of Ca^{2+} can be markedly reduced by briefly exposing the chondrocytes to a slightly acidic medium (pH 6.1). The steady-state concentration of Ca^{2+} in the cytosol of these 'washed' cells can then be induced to increase, thereby causing an activation of a Ca^{2+} -extrusion system localized at the cell surface. Such transient elevation in the cytoplasmic concentration of free Ca^{2+} can be brought about by exposing the cells to ionophore A23187 (Ferreira & Lew, 1976; Babcock *et al.*, 1976; Mottola *et al.*, 1980) or to the inhibitor of mitochondrial energy production, cyanide (Lehninger, 1970; Rose & Loewenstein, 1975; Baker, 1976b). In the former case, the permeability to Ca^{2+} of cell membranes is severalfold increased and this results in mobilization of the cation from intracellular stores as well as passive diffusion from the extracellular environment. In the latter case, Ca^{2+} is released from mitochondria. When either treatment is applied to chondrocytes, Ca^{2+} is extruded at a considerable rate. With cyanide, the amount of Ca^{2+} expelled is higher than with the ionophore, probably because with the latter agent the passive influx of the cation from the extracellular medium partially counteracts its active extrusion.

It is interesting that intracellular cell Ca^{2+} is extruded against what likely amounts to a significant electrochemical ion gradient and, at least for the ionophore, in the presence of large inward leaks. Thus a suitable amount of cell energy must be

Table 2. *Effect of metabolic inhibitors on the ATP content of epiphyseal chondrocytes*

Isolated chondrocytes, suspended in $\text{Na}^+/\text{K}^+/\text{Tris}$ medium (2×10^7 cells/ml), containing 5 mM-glucose, were incubated at 37°C for 5 min in the presence or in the absence of the indicated metabolic inhibitors. When the effects of 2-deoxy-D-glucose were tested, glucose was not added. At the end of the incubation, cells were centrifuged for 20 s at 10000g. The sediments were extracted for 5 min with 0.3 ml of ice-cold HClO_4 (30 g/litre) and the extracts were neutralized with K_2CO_3 (40 g/litre). ATP was measured by a fluorimetric method with glucose 6-phosphate dehydrogenase and hexokinase (Williamson & Corkey, 1969). Data are means of at least three experiments $\pm$ S.E.M. and are expressed either as nmol/ 1×10^6 chondrocytes or as a percentage with respect to untreated cells.

	ATP content	
	Healthy chickens	Rachitic chickens
Untreated cells	0.54 ± 0.04 (100%)	0.56 ± 0.06 (100%)
2-Deoxy-D-glucose (10 mM)	$20 \pm 1\%$	$22 \pm 2\%$
Oligomycin (0.5 $\mu\text{g}/\text{ml}$)	$75 \pm 3\%$	$78 \pm 2\%$
KCN (2 mM)	$66 \pm 6\%$	$68 \pm 8\%$

concomitantly consumed. The experiments here described indicate that this energy mainly derives from the hydrolysis of ATP and not from a coupled $\text{Na}^+/\text{Ca}^{2+}$ exchange. In fact, ATP depletion, but not removal of extracellular Na^+ , markedly affects the efficiency of the Ca^{2+} -extrusion system of chondrocytes.

Chondrocytes isolated from rachitic chickens also exhibit the capacity for extruding Ca^{2+} by the same mechanism, with an efficiency comparable with that of their normal counterpart. However, when the stimulus for Ca^{2+} mobilization and extrusion is provided by the ionophore, chondrocytes of rachitic cartilage expel a higher percentage of total cell calcium, but at a slightly lower rate, than cells of normal tissue. Since the ionophore effects are controlled by its diffusion across the membrane lipid barrier and rachitism brings about a profound alteration of cell membrane lipids (Fontaine *et al.*, 1981), the possibility exists that the apparent change in extent and rate of Ca^{2+} extrusion observed with chondrocytes of rachitic chickens are due to a reduced passive inward diffusion of the ionophore A23187- Ca^{2+} complex.

In conclusion, our results indicate that chondrocytes have an ATP-dependent machinery for the expulsion of Ca^{2+} , which is localized at the cell surface and is very likely similar to the Ca^{2+} pump present in the plasma membrane of other cell types (Schatzmann & Vincenzi, 1969; Ferreira & Lew, 1976; Dipolo, 1978; Schatzmann & Brigin, 1978; Mottola *et al.*, 1980). A decreased activity of this pump, which under normal conditions is very likely involved in the maintenance of low steady-state Ca^{2+} concentrations, might be a crucial event in the release of calcium-loaded matrix vesicles (Bonucci, 1967) from chondrocytes present at the calcification front. In fact, Brighton & Hunt (1978) have observed that in costochondral growth plates there is a shift of Ca^{2+} from an intracellular, mainly mitochondrial location, in the top part of the hypertrophic cells, to an extracellular, mainly matrix vesicle location, in the bottom part of the zone of the hypertrophic cells. Release of calcium from the mitochondria is likely to result from an impairment of the cell Ca^{2+} high-affinity buffering systems, also affecting the peripheral Ca^{2+} -extrusion mechanism. Ca^{2+} would therefore accumulate in the cortical cytoplasm, causing evagination and vesiculation of the plasma membrane (Allan & Michell, 1975). This might promote exfoliation of Ca^{2+} -loaded right-side-out vesicles (Wuthier *et al.*, 1978; Cecil & Anderson, 1978), which would thus carry the cation to the extracellular matrix. Hydrolysis of residual ATP in the vesicles, catalysed by the ATPase present in these organelles (de Bernard *et al.*, 1981),

would further damp the activity of the pump, thereby enhancing Ca^{2+} accumulation by the vesicles and facilitating the formation of calcium phosphate precipitates.

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References

- Allan, D. & Michell, R. H. (1975) *Nature (London)* **258**, 348–349
- Babcock, D. F., First, N. L. & Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 3881–3886
- Baker, P. F. (1976a) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2589–2595
- Baker, P. F. (1976b) *Symp. Soc. Exp. Biol.* **30**, 67–88
- Bonucci, E. (1967) *J. Ultrastruct. Res.* **20**, 33–50
- Boskey, A. L. & Posner, A. S. (1977) *Calcif. Tissue Res.* **23**, 251–258
- Brighton, C. T. & Hunt, R. M. (1978) *J. Bone Joint Surg.* **60A**, 630–639
- Cecil, R. N. A. & Anderson, H. C. (1978) *Metab. Bone Dis. Relat. Res.* **1**, 89–95
- Cittadini, A., Bossi, D., Rosi, G., Wolf, F. & Terranova, T. (1977) *Biochim. Biophys. Acta* **469**, 345–349
- de Bernard, B., Camerotto, R., Stagni, N., Vittur, F. & Zanetti, M. (1981) in *International Cell Biology 1980–81* (Schweiger, H. G., ed.), pp. 983–992, Springer Verlag, Berlin
- Dipolo, R. (1978) *Nature (London)* **276**, 390–392
- Ferreira, H. G. & Lew, D. L. (1976) *Nature (London)* **259**, 47–49
- Flora, U., Gennaro, R. & Romeo, D. (1980) *Anal. Biochem.* **102**, 77–79
- Fontaine, O., Matsumoto, T., Goodman, D. B. P. & Rasmussen, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1751–1754
- Gey, G. O. & Gey, M. K. (1936) *Am. J. Cancer* **27**, 55
- Hurwitz, S. & Bar, A. (1972) *Am. J. Physiol.* **222**, 761–767
- Lehninger, A. L. (1970) *Biochem. J.* **119**, 129–138
- Mottola, C., Dolzani, L. & Romeo, D. (1980) *Cell Calcium* **1**, 371–379
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Reeves, J. P. & Sutko, J. L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 590–594
- Robison, R. (1923) *Biochem. J.* **17**, 286–295
- Rose, B. & Loewenstein, W. R. (1975) *Nature (London)* **254**, 250–252
- Schatzmann, H. J. & Brigin, H. (1978) *Ann. N.Y. Acad. Sci.* **307**, 125–147
- Schatzmann, H. J. & Vincenzi, F. F. (1969) *J. Physiol. (London)* **201**, 369–395
- Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513
- Wuthier, R. E., Linder, R. E., Warner, G. P., Gore, S. T. & Borg, T. K. (1978) *Metab. Bone Dis. Relat. Res.* **1**, 125–136