citoplasma, dove, con molta probabilità entrano nel gioco della formazione dei globuli di vitello.

I risultati rappresentano altresì una conferma di quelli ottenuti da vari altri autori, che hanno appunto rilevato, nel materiale più diverso, l'estrusione nucleolare.

Summary. The author has found in increasing ovocytes of *Patella coerulea*, particularly in the nuclear membrane, a real passage of small nucleoli from the nucleus to the cytoplasm. Thus not all the nucleoli which appear little by little in the nucleus are amphinucleoli.

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Intracellular Phosphate Release by the Na^+-K^+-Activated Membrane ATPase

An ATPase activity associated with cell membranes has been shown to depend on the simultaneous presence of K^+ on the external and of Na^+ on the internal surface of the membrane[1,2], whereas the substrate (ATP) has to be present on the internal surface of the membrane in order to have access to the enzyme. The enzyme requires Mg^{++} in addition and is inhibited by Ca^{++} at low (intracellular) concentration and by cardiac glycosides such as ouabain[3-5]. There is good reason to assume that active transport of Na^+ and K^+ across the cell membrane and this ATPase are related. This connection has been tentatively described in terms of the mobile carrier hypothesis. It is assumed that in the presence of Na^+ the terminal phosphate group of ATP is transferred on the internal membrane surface to a compound situated in the membrane substance. The phosphorylated molecule passes the membrane in the outward direction owing to the chemical gradient and is hydrolysed in the presence of K^+ on the external surface. The phosphorylated form is thought to be the Na-carrier and the free form to be the K-carrier. This theory implies that inorganic phosphate (P_i) should be discharged directly into the external medium by this two-step enzymatic process.

This postulate has been tested in human red cells of high ATP- (and/or P_i) and Na^+-content, using WHITTAM's[6] method of reversal of haemolysis. Such cells containing P_i (which stemmed from some ATP-splitting during preparation), ATP (more than 4 μ-moles/ml cells[5]), Mg^{++} (4 μ-moles/ml cells) and Na^+ (approximately 70 μ-moles/ml cells[6]) were suspended in about a tenfold external volume and incubated for 40 min with adequate shaking in Erlenmeyer flasks at 37°C. The external medium was free of phosphate and contained 140 m*M* Na^+, 12 m*M* K^+, 10 m*M* tris buffer pH 7.3, 2 m*M* Mg^{++} but *no* glucose. P_i was estimated by the method of BERENBLUM and CHAIN[7] at the beginning and the end of the incubation period in the external medium and in the haemolysate of the cells from an aliquot of the suspension after deproteinization with an equal volume of 10% trichloroacetic acid. The initial cell to suspension volume ratio was determined by estimating the haemoglobin content of packed cells and suspension, and intracellular P_i was expressed as μ-moles/ml initial cell volume, disregarding any possible volume change taking place during the experiment. This gives an estimate of the amount of P_i per cell rather than of intracellular concentration.

Under the given conditions it was found that the cellular P_i-content decreased or remained constant while a P_i-gradient was maintained from cells to medium up to the end of the experiment thanks to the fairly large medium/cell volume ratio. The lowering of intracellular P_i content was markedly increased by adding 10^{-4} (w/v) ouabain (Merck, crystalline product DAB) to the suspension, a concentration known to inhibit the Na^+-K^+-activated ATPase completely (Figure B, Table). Omitting

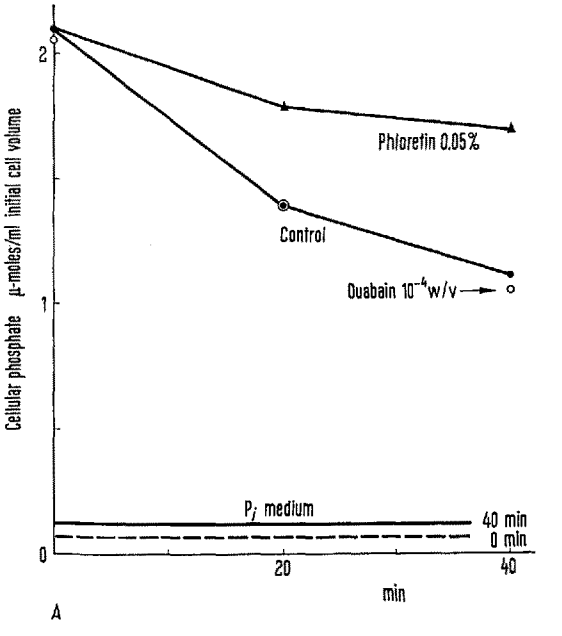

Figure A. Washed human red cells haemolysed in water in the presence of 4 m*M* KH_2PO_4-Na_2HPO_4 of pH 7.25 and 4 m*M* $MgCl_2$. Haemolysis reversed by adding NaCl. Incubated in a solution of 140 m*M* NaCl, 10 m*M* tris-buffer pH 7.3, 12 m*M* KCl, 2 m*M* $MgCl_2$ at 37°C. Cell dilution 1/7.7. Inorganic phosphate (P_i) determined in packed cells of 4 ml suspension after TAA-precipitation. Upper part of graph: cellular P_i; lower part of graph: P_i-concentration in external medium at beginning and end of experiment drawn as horizontal lines.

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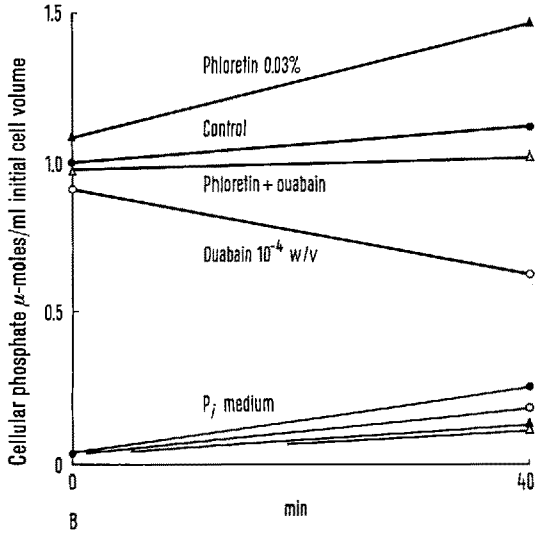
Change in intracellular inorganic phosphate during 40 min at 37°C in ATP-loaded cells

Experiment No.	No phloretin			Phloretin 0.03%		
	Change in intracellular P_i in 40 min (μmoles/ml cells[a])		Δ a–b	Change in intracellular P_i in 40 min (μmoles/ml cells[a])		Δ a–b
	No ouabain (a)	Ouabain 10^{-4} (b)		No ouabain (a)	Ouabain 10^{-4} (b)	
1	−0.51	−1.41	0.9	–	–	–
2	−0.71	−1.24	0.53	+1.19	+0.56	0.63
3 (starved cells[c])	+0.11	−0.28	0.39	+0.38	+0.03	0.35
	$[K]_e$[b] 12 mM (a)	$[K]_e$[b] 0 mM (b)		$[K]_e$[b] 12 mM (a)	$[K]_e$[b] 0 mM (b)	
4	−0.18	−0.42	0.24	+0.25	+0.03	0.22
5	−0.20	−0.98	0.78	+1.69	+0.82	0.87
6	−0.06	−0.57	0.51	+0.65	+0.19	0.46

[a] Calculated for initial cell volume. [b] K added to external medium. [c] Cells incubated in glucose-free medium for 5 h previous to experiment

K^+ from the external medium had a similar effect to ouabain (Table). Since ouabain has no effect on the passive transfer of P_i through the membrane (Figure A), these observations suggest that the P_i liberated by the Na^+-K^+-activated, ouabain-sensitive ATPase remains associated with the membrane or appears inside the cells. Carrying out the same experiment in the presence of phloretin led to a result which is in favour of the latter possibility.

Phloretin is known to inhibit the passive phosphate transfer across the cell membrane[8] (see Figure A). Phloretin (laboratory reagent, British Drug Houses), which is very sparingly soluble, was suspended in a small volume of hot water and added to the cell suspension as 0.03%. This treatment resulted in a reduction of rate of P_i-loss from the cells, as shown in Figure A. In such experiments cells were loaded with a 4 mM solution of Na_2HPO_4 and KH_2PO_4 of pH 7.25 during haemolysis, no ATP being added.



B. Starved human red cells haemolysed with water in the presence of 4 mM ATP (di-sodium salt) and 4 mM $MgCl_2$. Haemolysis reversed by adding NaCl. Cell dilution 1/7.2. Incubated at 37°C in the same solution as in A. Phloretin and ouabain added shortly before incubation. Upper part: cellular P_i; lower part: P_i-concentration in external medium (same symbols as for cellular phosphate).

ATP-loaded cells under phloretin poisoning showed an *increase* in cellular P_i during 40 min incubation at 37°C, which is obviously due to the leakage of P_i from the cells being smaller than the formation of P_i from the ATP-splitting. This increase of intracellular P_i-content could be reduced by adding 10^{-4} (w/v) ouabain or by removing K^+ from the external solution (Table). The outcome of such experiments was not altered by rigorously depleting the cells of glucose by thorough washing and subsequent incubation in glucose-free medium for 5 h at 37°C previous to the experiment (Figure B).

Phosphate transfer through the red cell membrane is looked upon as an equilibrating carrier transport, no experimental evidence being available as to a possible uphill transport for P_i[9,10]. The appearance of P_i in ATP-loaded cells which can be partly abolished by ouabain and K^+-deprival is therefore not likely to be due to an external release of P_i from ATP with subsequent reaccumulation. The most probable explanation seems to be that P_i is primarily released inside the cell by the Na^+-K^+-activated 'transport-ATPase' and leaves the cell by way of the passive transfer mechanism which can be inhibited by phloretin. Under physiological conditions, ATP-splitting is balanced by ATP-resynthesis so that no P_i-gradient arises and P_i-loss is prevented.

In conclusion, the present results do not support the idea of phosphate being shifted through the membrane by the Na^+-K^+-ATPase system as visualized in the carrier theory in its form given above.

Zusammenfassung. An menschlichen Erythrocyten hemmt Phloretin den passiven Austritt von anorganischem Phosphat. Mit Hilfe dieser Wirkung konnte gezeigt werden, dass die Na^+-K^+-aktivierte ATPase anorganisches Phosphat nicht direkt nach aussen befördert, sondern im Innern der Zelle freisetzt.

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