Experiments with 2 μM monensin yielded comparable results regarding the stimulation of the ouabain sensitive $^{86}Rb^+$-uptake and inhibition of the piretanide sensitive $^{86}Rb^+$-influx in the presence of ouabain. However, the residual $^{86}Rb^+$-influx in the presence of ouabain and piretanide was not affected by monensin.

In order to investigate whether or not the effect of the presence of amino acids on the $^{86}Rb^+$-fluxes could be brought about by the addition of only one amino acid, we studied the effects of alanine, methionine and of the model amino acid alpha-amino-isobutyric acid (AIB) on the $^{86}Rb^+$-fluxes in EBSS (table). These amino acids are taken up mostly by Na^+-cotransport[14]. Amino acids were added at a concentration of 2 mM. These results show, that the stimulation of the ouabain sensitive $^{86}Rb^+$-uptake and reduction of the piretanide inhibitable part of the ouabain insensitive $^{86}Rb^+$-uptake could be brought about by one of these amino acids, whereas reduction in the residual $^{86}Rb^+$-influx in the presence of ouabain and piretanide was not observable. This situation did not change when, in the case of alanine, a higher concentration (4 mM) was investigated.

Discussion. The results of this study present further evidence that Na^+-coupled amino acid transport is an important regulator of the Na^+, K^+-pump. The cation pumping activity may therefore respond to physiological changes in which the amino acid uptake is altered. On the other hand, the presence of the amino acids in the cell-culture medium affects also the ouabain insensitive components of the $^{86}Rb^+$-uptake. Inhibition of the $^{86}Rb^+$-influx via the Na^+, K^+, 2 Cl^--cotransport system by amino acids may be related to an increased sodium influx, since the sodium ionophore monensin had a similar effect. However, a change in cell volume due to the accumulation of amino acids may also contribute to the inhibition of the diuretic sensitive $^{86}Rb^+$-influx[17,20]. The residual $^{86}Rb^+$-uptake in the presence of ouabain and piretanide was reduced only when all the amino acids of Joklik MEM were added. This effect cannot be related to the sodium influx, since it was not observed after treatment by monensin or the investigated amino acids. The mechanism by which the presence of amino acids inhibits this $^{86}Rb^+$-influx is unclear. The reduction of the residual $^{86}Rb^+$-uptake may indicate a decreased permeability of the plasma membrane under these conditions.

Effect of amino acids on the $^{86}Rb^+$-influx in HeLa cells

Components of $^{86}Rb^+$-influx	$^{86}Rb^+$-uptake in the presence of amino acid (% of control)		
	Alanine	Methionine	AIB
Ouabain sensitive	165.4	145.0	168.2
Ouabain insensitive, piretanide inhibitable	65.8	58.7	63.4
Ouabain and piretanide insensitive	109.2	91.3	117.5

$^{86}Rb^+$-uptake was measured in EBSS with and without amino acid (2 mM). The results are expressed as percentages of the corresponding control values without amino acid. The concentration of the inhibitor used was 1 mM.

In summary, all components of the $^{86}Rb^+$-influx studied were shown to be affected by the presence of amino acids in the cell-culture medium. Thus the marked alteration in the $^{86}Rb^+$-influx due to the absence of amino acids in the incubation medium indicates that data on ion transport mechanisms obtained with cells in buffer solutions must be interpreted cautiously since these data may not reflect ionic fluxes of cells grown under normal culture conditions.

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2 Post, R.L., Regulatory Functions of the Biological Membranes, p. 163. Elsevier, Amsterdam 1968.
3 Pietrzyk, C., Geck, P., and Heinz, E., Biochim. biophys. Acta *513* (1978) 89.
4 Van Dyke, R.W., and Scharschmidt, B.F., J. biol. Chem. *258* (1983) 12912.
5 Yamamoto, S., Akera, T., and Brody, T.M., Biochim. biophys. Acta *555* (1979) 270.
6 Akera, T., Yamamoto, S., Temma, K., Kim, D.-H., and Brody, T.M., Biochim. Biophys. Acta *640* (1981) 779.
7 Smith, J.B., and Rozengurt, E., Proc. natn. Acad. Sci. USA *75* (1978) 5560.
8 Mendoza, S.A., Wigglesworth, N.M., Pohjanpelto, P., and Rozengurt, E., J. Cell Physiol. *103* (1980) 17.
9 Rozengurt, E., Gelehrter, T.D., Legg, A., and Pettican, P., Cell *23* (1981) 789.
10 Moolenaar, W.H., Mummery, C.L., Van der Saag, P.T., and de Laat, S., Cell *23* (1981) 789.
11 Pouyssegur, J., Chambard, J.C., Franchi, A., Paris, S., and Van Obberghen-Schilling, E., Proc. natn. Acad. Sci. USA *79* (1982) 3935.
12 Schaefer, A., Munter, K.-H., Geck, P., and Koch, G., J. Cell Physiol. *119* (1984) 335.
13 Racker, E., Johnson, J.H., and Blackwell, M.T., J. biol. Chem. *258* (1983) 3702.
14 Shotwell, M.A., Kilberg, M.S., and Oxender, D.V., Biochim. biophys. Acta *737* (1983) 267.
15 Ikehara, T., Sakai, T., Miyamoto, H., and Kaniike, K., Jap. J. Physiol. *32* (1982) 13.
16 Geck, P., Pietrzyk, C., Burckhardt, B.-C., and Heinz, E., Biochim. biophys. Acta *600* (1980) 432.
17 Geck, P., Heinz, E., and Pfeiffer, B., Ototoxic Side Effect of Diuretics. Eds R. Klinke, W. Lahn, H. Querfurth and J. Scholtholt. Scand. Audiol., suppl. 14 (1981) 25.
18 Schaefer, A., Geck, P., Zibirre, R., Kühne, J., and Koch, G., Virology *136* (1984) 457.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
20 Ueberschar, S., and Bakker-Grunwald, T., Biochim. biophys. Acta *731* (1983) 243.

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Cytokinesis in onion roots: inhibition by vanadate and caffeine

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Summary. The effect of vanadate ions on plant cytokinesis has been studied in *Allium cepa* root meristematic cells. Vanadate induces binucleate cells by inhibiting cell plate formation. Moreover, vanadate and caffeine have additive effects in the induction of binucleate cells.

Key words. Allium cepa; cytokinesis inhibitor; root meristem; vanadate; caffeine.

Higher plant cytokinesis can be considered as a topographically organized secretion process, because the membranes of small Golgi vesicles make up the new plasma membrane during telophase and the contents of the vesicles give rise to the amorphus matrix of the new wall[1].

The induction of binucleate cells in a proliferative population by chemical and physical agents can be used to analyze cytokinesis. Caffeine, a drug which produces binucleate cells, has been shown to alter the arrangement and inhibit the fusion of Golgi vesicles[2]. Calcium and magnesium have been shown to alter the effect of caffeine on cytokinesis[3]. Cellular levels of ATP also affect the ability of caffeine to produce binucleate cells[4]. It is possible that caffeine inhibits a Ca^{++} Mg^{++}ATPase activity required for membrane fusion. More recently, the inhibitory action of vanadate has been studied on (Ca,Mg)-activated ATPase activity of sarcoplasmic reticulum[5,6], plasma membrane associated ATPase of corn and beet roots[7,8], and membrane associated ATPase in secretory vesicles of *Avena sativa* in vitro[9].

The aim of this paper is to study the possible inhibitory action of vanadate in plant cytokinesis and the role played by this in caffeine-induced binucleate cell formation.

Material and methods. The material used was root tips of *Allium cepa* L. flat violet variety. The onions bulbs (15–30 g) were grown in the dark at 15±0.5°C and aerated by continuous bubbling. Sodium orthovanadate (Na_3VO_3) was prepared in a 5 mM stock solution as described by O'Neill and Spanswick[8]. When vanadate was supplied in this manner, there was no significant alteration of the pH in the reaction mixture[7,8].

Roots were incubated in vanadate and/or caffeine for 4 h in treatment solution and then returned to water for 1 h before harvesting. This short recovery was necessary to permit all affected mitotic cells to reach interphase in order to appear either as mononucleate cells, if cytokinesis has not been inhibited, or as binucleate cells when cytokinesis has been blocked.

Root samples were fixed in a 3:1 absolute ethanol:acetic acid mixture. These specimens were prepared by staining the root with acetic orcein[10] and appropriate meristem squashes were then obtained. For cytological studies, each point represents the mean value of 6000±50 meristem cells from at least six roots from two bulbs.

Results and discussion. The efficiency of vanadate as cytokinesis inhibitor was tested by submitting the roots to continuous treatment with several vanadate concentrations ranging from 0.01 to 100 mM. Vanadate concentrations higher than 10 mM proved to be lethal. 1 mM vanadate produced 1% binucleate cells at 4 h (fig. 1) and 1.5% at 6 h; the percentage of binucleate cells arising per hour is thus stable for several hours.

To determine the existence of a possible cooperative inhibitory effect of vanadate and caffeine on plant cytokinesis, the roots were treated 4 h with vanadate, at several concentrations, in the absence or presence of 2.5 mM caffeine (fig. 1). Our results show that the induction of binucleate cells by 2.5 mM caffeine is increased by vanadate concentrations ranging between 0.1 and 10 mM. The highest increase of caffeine efficiency was obtained with vanadate 1 mM. In addition, we studied the effects of vanadate 1 mM in combination with caffeine at increasing concentrations. Figure 2 shows the induction of binucleate cells by caffeine (1–5 mM) with and without 1 mM vanadate after treatment of 4 h duration, followed by 1 h recovery.

Secretory processes and higher plant cytokinesis have been considered as similar phenomena, in that they are both exocytotic mechanisms[3]. Exocytosis takes place in response to different specific or unspecific stimuli, and always requires a temporary

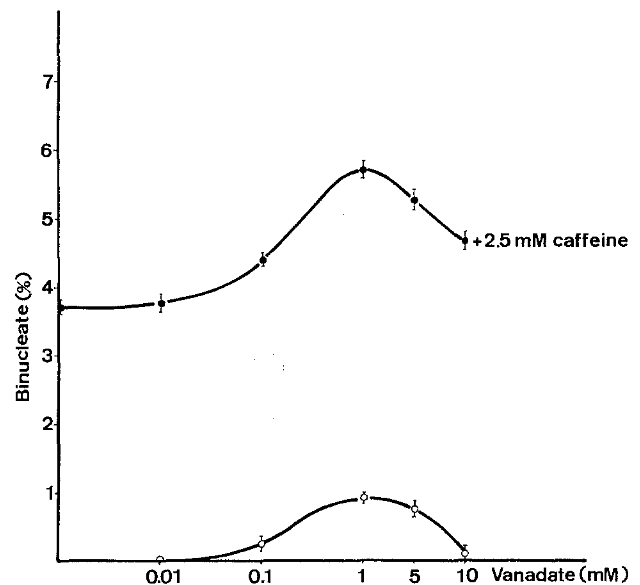

Figure 1. Binucleate cells induced by 4-h treatment with vanadate at different concentrations (○) and with +2.5 mM caffeine (●).

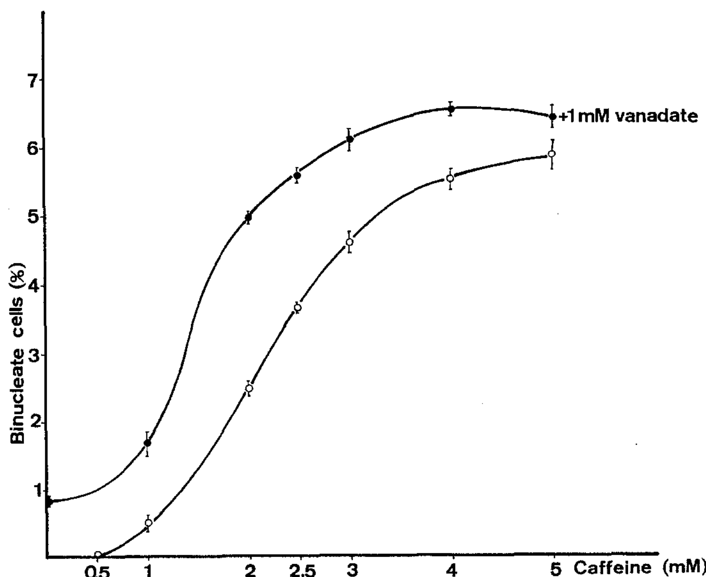

Figure 2. Production of binucleate cells by 4-h caffeine treatment at different concentrations (○), and in the presence of 1mM vanadate (●).

increase of the intracellular free calcium concentration[11]. Reed[12] suggested a requirement of calcium for plant cytokinesis and reported irregular cell plates with calcium deficiency. This ion seems to play a role in the preservation of the plasma membrane and cell wall as well as in cell wall ATPase activation[13]. Paul and Goff[14] have suggested that caffeine and calcium deficiency interfere with some aspects of membrane recognition and fusion, and it has been confirmed that caffeine interferes with cytokinesis by releasing calcium and magnesium from those sites which are required for regular building of the new cell plate[3]. Adenosine[15], and treatments which increase the cellular ATP level[4] decrease the efficiency of caffeine in inhibiting higher plant cytokinesis, and it has been postulated that caffeine can block cell plate formation by the inhibition of a certain ATPase activity[4] required for cell membrane fusion[16,17].

Hepler[18] suggests that endoplasmic reticulum might regulate the ionic conditions that allow vesicle fusion to occur. Our results show that vanadate itself interferes with plant cytokinesis, thus producing binucleate cells. The role of vanadate in the inhibition of the endoplasmic reticulum calcium-pump[6] induces us to think that vanadate may alter the control of cellular free calcium concentration through the endoplasmic reticulum.

Considering the suggested interference of caffeine with some ATPase activity required for membrane fusion[4,5,19], and the inhibitory effect of vanadate on phosphatase activities[5,9], our results showing inhibition of cell plate formation by vanadate and an additive effect of caffeine and vanadate are fully compatible with the data published. Vanadate and caffeine appear to affect plant cytokinesis at different levels, which confirms their additive effect.

1 Frey-Wyssling, A., Lopez-Sáez, J. F., and Mühlethaler, K., J. Ultrastruct. Res. *10* (1964) 422.
2 Risueño, M. C., Giménez-Martin, G., and Lopez-Sáez, J. F., Exp. Cell Res. *49* (1968) 136.
3 Becerra, J., and Lopez-Sáez, J. F., Exp. Cell Res. *111* (1978) 301.
4 Lopez-Sáez, J. F., Mingo, R., and González-Fernandez, A., Eur. J. Cell Biol. *27* (1982) 185.
5 Dux, L., and Martonosi, A., J. biol. Chem. *258* (1983) 2599.
6 Pick, U., J. biol. Chem. *257* (1982) 6111.
7 Gallagher, S. R., and Leonard, R. T., Pl. Physiol. *70* (1982) 1335.
8 O'Neill, S. D., and Spanswick, R. M., Pl. Physiol. *75* (1984) 586.
9 Binari, L. L., and Racusen, R. H., Pl. Physiol. *71* (1983) 594.
10 Tjio, J. H., and Levan, A., Ann. Est. Exp. Aula Dei *2* (1950) 21.
11 Plattner, H., Cell Biol. int. Rep. *5* (1981) 435.
12 Reed, H. S., Ann. Bot. *21* (1907) 501.
13 Jones, R. G. W., and Lunt, D. R., Bot. Rev. *33* (1967) 407.
14 Paul, D. C., and Goff, D. W., Exp. Cell Res. *78* (1973) 399.
15 González-Fernandez, A., and Lopez-Sáez, J. F., Envir. exp. Bot. *20* (1980) 455.
16 Plattner, H., Reichel, K., and Matt, H., Nature *267* (1977) 702.
17 Poste, G., and Allison, A. C., J. theor. Biol. *32* (1971) 165.
18 Hepler, P. K., Protoplasma *111* (1982) 121.
19 Becerra, J., and Lopez-Sáez, J. F., in: The Role of Calcium in Biological Systems, p. 241. Eds L. J. Anghileri and A. M. Tuffet-Anghileri. CRC Press, Boca Raton 1982.

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Acetylcholine receptors in the gastrulating chick embryo

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Summary. Muscarinic acetylcholine receptors are present in the gastrulating chick embryo.
Key words. Chick embryo; gastrulation; muscarinic acetylcholine receptors; cGMP.

Studies on the embryos of various organisms have indicated the possible regulatory role of acetylcholine (ACh) in the early stages of development[1-4]. However, the mechanism of ACh action in the early embryogenesis has not been investigated yet. The observation that atropine, a muscarinic antagonist, prevents the normal process of chick gastrulation[3], implies the existence of muscarinic ACh-receptors in gastrulating chick embryos. Using the [^{3}H]QNB binding assay and biochemical study of the ACh responses in vitro, we demonstrate here the presence of muscarinic ACh-receptors in the gastrulating chick embryo.

Materials and methods. Binding assay. White Leghorn embryos (area pellucida only), at the stage of development 4 HH (Hamburger and Hamilton)[5], were dissociated in Ca-Mg free Ringer solution with 0.2 mM EDTA, and the number of cells counted. Cells were homogenized in 0.05 M sodium-potassium phosphate buffer, pH 7.4, using a conical glass-to-glass ice-cooled microhomogenizer. Protein was determined by the method of Lowry et al.[6]. The binding assay was performed according to Yamamura and Snyder with slight variations[7]. The incubation volume of each sample was 1 ml, containing 200 µl of the homogenate. The specific binding of [^{3}H]QNB (Amersham, sp. act. 32 Ci/mmol) was determined from the difference of binding in the absence and presence of 10 µM atropine. The [^{3}H]QNB concentration used was 2 nM; this was assumed to be a saturating concentration, as shown by a previous study of muscarinic receptors in chick limb buds[8]. The incubation was terminated by rapid filtering of the suspension through a glass fiber filter (GF/B). The filters were washed four times with 5 ml of the ice-cold sodium-potassium buffer, and immersed in 10 ml dioxane-naphthalene scintillation mixture. Radioactivity was counted after 24 h in a liquid scintillation counter ('RackBeta', LKB).

cGMP assay. In each experiment, about 30–40 chick embryos (area pellucida only) at stage 4 were dissociated in 1 ml Ca-Mg-free Ringer solution with 0.2 mM EDTA. The dissociated embryo cells were preincubated at 37°C for 1 h in 180 µl Ringer bicarbonate buffer (pH 7.4). The cells were further incubated for 5 min in the presence of 0.1 mM IBMX and in the presence or absence of 1 mM atropine. A control sample (52.5 µl) was taken

Effects of various agents on levels of cGMP in dissociated chick embryo cells at stage 4 (HH)

Conditions	Percent of control ± SE
Acetylcholine (500 µM)	224 ± 43
Acetylcholine (500 µM) + atropine (1 mM)	120 ± 10
Acetylcholine (500 µM) in Ca^{2+}-free medium	30 ± 15
A23187 (10 µg/ml)	170 ± 30