Eur. J. Biochem. 231, 40–44 (1995)

Regulation of mammalian ornithine decarboxylase
Studies on the induction of the enzyme by hypotonic stress

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(Received 28 March 1995) – EJB 95 0494/1

One of the cellular responses to hypotonic stress is a marked induction of a key regulatory enzyme in the polyamine biosynthetic pathway, i.e. ornithine decarboxylase (ODC). This increase in ODC activity appears to be a physiological response since the elevated putrescine production seen after the hypotonic shock renders the cells less sensitive to the decrease in osmolarity. In the present study, we have investigated the mechanisms by which the hypotonicity may induce ODC activity. We provide support for a translational mechanism, closely related to the polyamine-mediated feedback regulation of ODC synthesis. In addition, we have examined whether the long G+C-rich 5′ untranslated region of the ODC mRNA, which has been demonstrated to negatively affect the translatability of the message, is of any importance for the induction of ODC by hypotonic stress. Chinese hamster ovary (CHO) cells expressing ODC mRNA, with or without the 5′ untranslated region, were isolated after transfecting ODC-deficient CHO cells with the appropriate constructs. Hypotonic treatment of the stable transfectants, however, revealed no major difference in ODC induction between the cells expressing a full-length ODC mRNA and those expressing an ODC mRNA deleted of its 5′ untranslated region, demonstrating that this part of the message was not essential for the osmotic effects on ODC expression.

Keywords. Ornithine decarboxylase; polyamines; osmolarity; translational control; 5′ untranslated region.

Ornithine decarboxylase (ODC), which catalyzes a key step in the polyamine biosynthetic pathway, is subject to regulation at a variety of levels [1, 2]. The induction of ODC in connection with cellular growth appears to be caused by an increased transcription of the gene [3, 4]. In addition, ODC is regulated by translational as well as post-translational mechanisms [5–14]. The polyamine-mediated feedback control of ODC appears to be carried out at both these levels [7–15]. Exposing cells to an excess of polyamines results in an increased degradation as well as a decreased synthesis of the enzyme (without any change in the ODC mRNA level). Since the turnover of ODC is the fastest among mammalian enzymes [16], any change in the synthesis or in the degradation rate of the enzyme will rapidly affect the cellular content, and thus the activity, of ODC. The exact mechanisms by which the polyamines affect the synthesis and the degradation of ODC remain to be disclosed, even though some information has been obtained using recombinant DNA techniques [12, 14, 17–20].

The cellular ODC activity has also been shown to fluctuate, depending on the osmolarity of the growth media [21–27]. Hypotonicity gives rise to a dramatic increase in ODC activity within a very short time after the onset of the osmotic shock. Conversely, exposure to a hypertonic medium results in down-regulation of ODC. The increased putrescine production seen after the hypotonic shock appears to render the cells less sensitive to the decrease in osmolarity [28]. The mechanisms by which hypotonicity induces ODC activity is not clear. In some cells, the phenomenon has been explained mainly, if not exclusively, by post-transcriptional events [25, 26] whereas, in other cells, it appears to be fully explained by a change in ODC transcription [27]. In the present study, we provide support for a translational mechanism closely related to the polyamine-mediated feedback regulation of ODC synthesis. In addition, the importance of the G+C-rich 5′ untranslated region (UTR) of the ODC mRNA in the hypotonic induction of ODC is investigated.

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Abbreviations. ODC, ornithine decarboxylase; UTR, untranslated region; F_2MeOrn 2-difluoromethylornithine; CHO, chinese hamster ovary.

Enzyme. Ornithine decarboxylase (EC 4.1.1.17).

MATERIAL AND METHODS

Materials. L-[1-^{14}C]Ornithine (52 Ci/mol), L-[^{35}S]methionine (> 1000 Ci/mmol) and [^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham. The cDNA encoding human ODC was a generous gift from Dr Olli A. Jänne [29]. pSVL and pBluescript II were obtained from Pharmacia and Stratagene, respectively. 2-Difluoromethylornithine (F_2MeOrn) was generously provided by Dr P. P. McCann, Marion Merrell Dow.

DNA constructs. A *Xmn*I–*Eco*RI fragment from the human ODC cDNA was subcloned into the expression vector pSVL, which contains the simian virus 40 late promoter as well as the origin of replication. The insert contains 49 of the 338 nucleotides in the 5′ UTR, the coding sequence and the whole 3′-non-coding region. A full-length hybrid ODC cDNA was obtained by ligating the hamster ODC 5′ UTR with the coding region of human ODC (using a common *Sty*I site). This cDNA was then subcloned into a pBluescript II vector containing the hamster

ODC promoter. The construct used for stably expressing ODC mRNA deleted of the major part of the 5′ UTR was obtained by truncating the hybrid cDNA at the *XbaI* site in the 5′ UTR, then ligating it to the *NotI* site of the ODC promoter in pBluescript II using a *NotI* − *XbaI* linker.

Cell culture. The ODC-overproducing L1210 cell line, L1210-F_2MeOrn^r [11], was maintained without F_2MeOrn in RPMI 1640 medium containing 10% fetal calf serum, 50 μM 2-mercaptoethanol and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). The cells were seeded at a density of 1.0×10^5 cells/ml. COS-7 cells were grown in Dulbecco's minimum essential medium containing 10% fetal calf serum, nonessential amino acids and antibiotics. The COS cells were seeded at a density of 5000/cm^2. The Chinese hamster ovary (CHO) cells, which were grown in Dulbecco's minimum essential medium/Ham's F12 containing 10% fetal calf serum, nonessential amino acids and antibiotics, were seeded at a density of 10000/cm^2. The ODC-deficient CHO C55.7 cell line [30, 31] was maintained in the presence of 0.5 mM putrescine. A hypotonic shock was achieved by exposing the cells to a NaCl-free RPMI 1640 medium (about 100 mOsm/l) for 6 h. In some experiments, the hypotonic medium was supplemented with 1 mM ornithine.

Transfection. Transfection was performed by electroporation. Cells grown for 3 days were harvested, washed and resuspended in growth medium at a density of $7-12\times10^6$ cells/ml. The cells (0.8 ml) were mixed with DNA (15 μg), then pulsed with 0.3 kV at 250 μF. Following a 5-min recovery at room temperature, the cells were resuspended in growth medium and seeded at a density of 25000 cells/cm^2. The COS cells were used 2 days after the pulse. ODC-deficient CHO C55.7 cells transfected with the ODC constructs were selected for stable expression of the enzyme by growing the cells without putrescine.

Determination of ODC activity. Cells were sonicated in 0.1 M Tris/HCl, pH 7.5, containing 0.1 mM EDTA and 2.5 mM dithiothreitol, and were then centrifuged at $20000\times g$ for 20 min at 4 °C. ODC activity was determined in aliquots of the supernatant by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine in the presence of saturating levels of pyridoxal 5′-phosphate (0.1 mM) and ornithine (0.5 mM).

Determination of ODC synthesis. Synthesis of ODC was determined by measuring the incorporation of [^{35}S]methionine into the enzyme. Cells were collected by centrifugation ($500\times g$, 5 min) and reseeded in a methionine-free medium. After 10 min incubation at 37 °C, the cells were supplemented with [^{35}S]-methionine (10 μCi/ml) and further incubated for 25 min. The incorporation was stopped by the addition of 2 vol. ice-cold medium. Cells were harvested and sonicated in 0.1 M Tris/HCl, pH 7.5, containing 0.1 mM EDTA and 2.5 mM dithiothreitol. After centrifugation at $30\,000\times g$ for 30 min at 4 °C, the supernatants were collected and aliquots containing equal amounts of acid-insoluble radioactivity were incubated with an excess of ODC antibody [32] for 30 min at room temperature. To precipitate the antibody-ODC complex, bacterial protein A adsorbent was added and the mixture incubated for an additional 30 min. The precipitate was thoroughly washed in 10 mM Tris/HCl, pH 7.5, containing 0.1 mM EDTA, 2.5 mM dithiothreitol, 0.1% Triton X-100, 0.1% SDS and 0.1% Tween 80. Precipitated ODC was fractionated on SDS/PAGE, essentially as described by Persson et al. [33]. The radioactivity was visualized by fluorography after incubating the gels in Amplify (Amersham). Relative measurements of ODC synthesis were obtained by densitometric scanning.

Northern-blot analysis. Total cellular RNA (20 μg) was isolated by the method Chomczynski et al. [34] and size-fractionated by gel electrophoresis on a 1% agarose gel in the presence of formaldehyde. After transfer to a Hybond-N membrane (Amersham), the RNA was hybridized with a ^{32}P-labeled human ODC cDNA. Relative measurements of ODC mRNA were obtained by densitometric scanning.

Miscellaneous methods. The cellular polyamine content was determined as previously described using an amino acid analyzer (Biotronik LC 5001). Protein concentrations were measured by the method of Bradford [35].

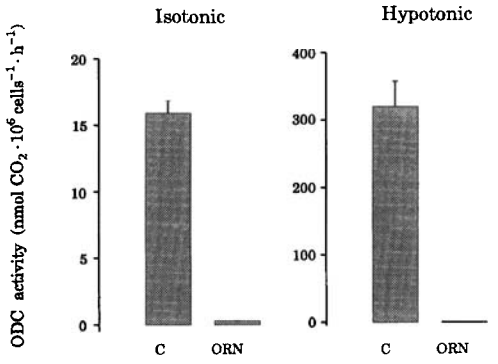

Fig. 1. Effects of hypotonic stress and ornithine on ODC activity in L1210-F_2MeOrn^r cells. The cells were grown in a medium without F_2MeOrn. One day after seeding in fresh medium, the cells were exposed for 6 h to an isotonic or hypotonic medium in the presence (ORN) or absence (C) of 1 mM ornithine. Mean ± SEM, n = 6.

Table 1. Effects of ornithine and hypotonicity on polyamine content in L1210-F_2MeOrn^r cells. The cells were grown in a medium without F_2MeOrn. One day after seeding in fresh medium, the cells were exposed for 6 h to an isotonic or hypotonic growth medium in the presence or absence of 1 mM ornithine. Mean ± SEM, n = 6.

Osmolarity	Polyamines			
	ornithine	putrescine	spermidine	spermine
		nmol/10^6 cells		
Isotonic	−	3.2 ± 0.3	4.9 ± 0.2	1.5 ± 0.1
Isotonic	+	4.3 ± 0.5	4.7 ± 0.4	1.4 ± 0.1
Hypotonic	−	3.2 ± 0.5	3.8 ± 0.9	1.1 ± 0.1
Hypotonic	+	21.2 ± 0.9	3.3 ± 0.1	1.3 ± 0.1

RESULTS

Hypotonic induction of ODC in L1210-F_2MeOrn^r cells. Studies on ODC synthesis are usually hampered by the fact that the expression of ODC is very low, even when fully induced. The L1210-F_2MeOrn^r cells used in this study were isolated by selection for resistance to the ODC inhibitor F_2MeOrn and express ODC to a very high level [11]. When grown in the absence of F_2MeOrn, ODC expression in the L1210-F_2MeOrn^r cells is down-regulated, due to the polyamine-mediated feedback control of the enzyme. Exposure of the L1210-F_2MeOrn^r cells (grown in the absence of F_2MeOrn) to hypotonic stress, by lowering the osmolarity of the growth medium from about 300 mOsm/l to 100 mOsm/l, resulted in a 20-fold increase in the cellular ODC activity within 6 h (Fig. 1); this hypotonic induction of ODC activity was abolished if the hypotonic medium was supplemented with 1 mM ornithine. In fact, when ornithine was supplied to the medium, whether it was hypotonic or not, the ODC activity attained levels (1.1 nmol $CO_2 \cdot 10^6$ cells$^{-1} \cdot h^{-1}$ and 0.3 nmol $CO_2 \cdot 10^6$ cells$^{-1} \cdot h^{-1}$, respectively)

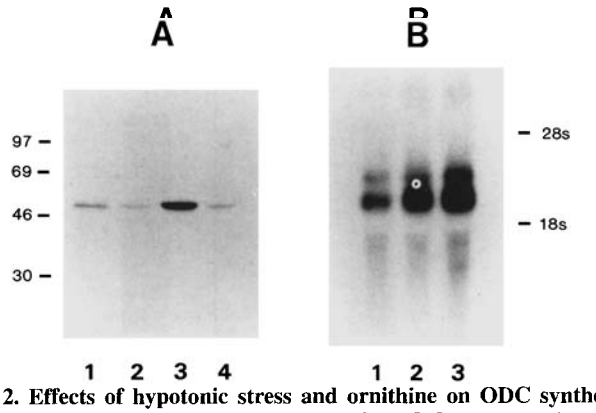

Fig. 2. Effects of hypotonic stress and ornithine on ODC synthesis (A) and ODC mRNA levels (B) in L1210-F_2MeOrn^r cells. The cells were treated as described in Fig. 1. (A) Synthesis of ODC was determined by measuring the incorporation of [^{35}S]methionine into the enzyme as described in Materials and Methods. Lane 1, pure mouse kidney ODC labeled with [^{3}H]F_2MeOrn (M_r = 53000); lane 2, cells exposed to an isotonic medium; lane 3, cells exposed to a hypotonic medium; lane 4, cells exposed to a hypotonic medium containing 1 mM ornithine. ^{14}C methylated proteins were used as molecular-mass markers (M_r = 97400; 69000; 46000; 30000). (B) Total cellular RNA was isolated, fractionated on a 1% agarose gel containing formaldehyde, transferred to a Hybond-N membrane and hybridized with a ^{32}P-labeled human ODC cDNA. Lane 1, cells exposed to an isotonic medium; lane 2, cells exposed to a hypotonic medium; lane 3, cells exposed to a hypotonic medium containing 1 mM ornithine. The size heterogeneity of the two ODC mRNA species is due to the alternative utilization of two polyadenylation signals [36, 45].

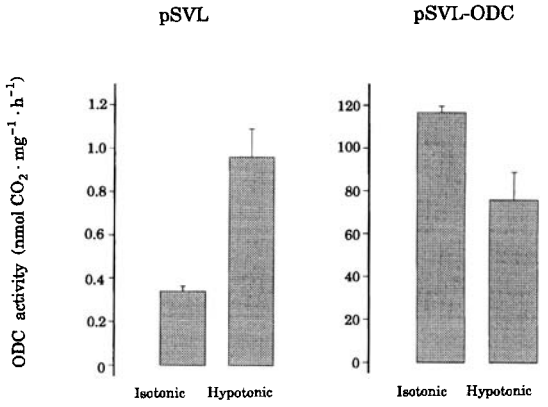

Fig. 3. Effects of hypotonic stress on ODC activity in transfected COS cells. COS cells were transfected with pSVL or pSVL-ODC as described in Materials and Methods. Two days after transfection, the cells were incubated for 6 h in an isotonic or hypotonic medium. It should be noted that the scales differ. Mean ± SEM, n = 4.

which were much lower than those normally found in the L1210-F_2MeOrn^r cells grown in isotonic medium (Fig. 1).

Interestingly, the marked increase in ODC activity caused by hypotonic stress did not give rise to a significant change in the cellular polyamine content (Table 1); neither were the cellular spermidine and spermine contents affected by the supplementation of the isotonic medium with ornithine, in spite of the fact that ODC activity decreased 40-fold when ornithine was added to the medium (Table 1). Nevertheless, when ornithine was supplied to the hypotonic medium, the putrescine content increased sevenfold (Table 1).

Pulse labeling of ODC with [^{35}S]methionine revealed that the ODC synthesis was markedly increased in the L1210-F_2MeOrn^r cells 6 h after the onset of the hypotonic shock (Fig. 2). This increase in ODC synthesis appeared to be mainly due to an increased translational efficiency, since the amount of ODC mRNA only increased 2.2-fold, whereas the ODC synthesis increased 14-fold in response to hypotonic stress (Fig. 2). If the hypotonic medium was supplemented with 1 mM ornithine, no induction of ODC synthesis was observed, in spite of the fact that ODC mRNA was increased 2.3-fold (Fig. 2).

Effects of hypotonicity on the transient expression of ODC in COS cells. Mammalian ODC mRNAs have a G+C-rich 5′ UTR of about 300 nucleotides [36–39], which may be of importance for the translational control of the enzyme. Since the hypotonic induction of ODC appears to be, at least partly, caused by a more effective translation of the ODC mRNA, the question arises as to whether or not the 5′ UTR is of any importance for this induction. In order to test whether the 5′ UTR of ODC mRNA is essential for the hypotonic induction of ODC, we subcloned a human ODC cDNA, deleted of most of its 5′ UTR, into the mammalian expression vector pSVL. This vector contains the simian virus 40 late promoter as well as the simian virus 40 origin of replication and, thus, gives rise to a very high transient expression in COS cells. The ODC activity in the COS cells increased more than 300-fold when the cells were transfected with the vector containing the ODC cDNA (pSVL-ODC) as compared to transfection with the control vector (pSVL) alone (Fig. 3). Although not as marked as in the L1210-F_2MeOrn^r cells, the ODC activity increased (threefold) in the mock-transfected COS cells when these were exposed to a hypotonic medium for 6 h. However, in the pSVL-ODC transfected cells, the hypotonicity did not induce a rise in the ODC activity. Instead, a 35% decrease in the enzyme activity was seen after hypotonic shock (Fig. 3).

Importance of the mRNA 5′ UTR in the hypotonic induction of ODC. The results with the transient expression of ODC in the COS cells may suggest that the 5′ UTR of the ODC mRNA is essential for the hypotonic induction of ODC activity. However, the induction of ODC in the mock-transfected COS cells was much less than that found in the L1210-F_2MeOrn^r cells, indicating that this transient expression system may not be optimal for such studies. Thus, we next used a stable expression system in an attempt to further clarify whether or not the 5′ UTR of the ODC mRNA was of any importance for the induction of ODC by hypotonic shock. Mammalian ODC cDNA, with or without the 5′ UTR, was subcloned into a pBluescript II plasmid containing the hamster ODC promoter, as described in Materials and Methods. The DNA constructs were then transfected into the ODC-deficient CHO cell line C55.7 [30, 31] and stable transfectants were isolated by selection for cells expressing an enzymically active ODC (translated from an ODC mRNA with and without a 5′ UTR) and thus capable of growing in a medium lacking polyamines.

ODC activity in the CHO wild-type cells was usually increased 6–10-fold after 6 h hypotonic exposure (Fig. 4). The ODC activity in the transfected cells grown in an isotonic medium was found to be less than that of the wild-type cells (Fig. 4). Nevertheless, hypotonic treatment of the stable transfectants revealed no major difference between the cells expressing a full-length ODC mRNA and those expressing an ODC mRNA deleted of its 5′ UTR. The ODC activity was increased about 13–17-fold, which actually is somewhat larger than in the wild-type cells.

DISCUSSION

It is well established that hypotonic shock results in a dramatic increase in cellular ODC activity [21–27]. However, the

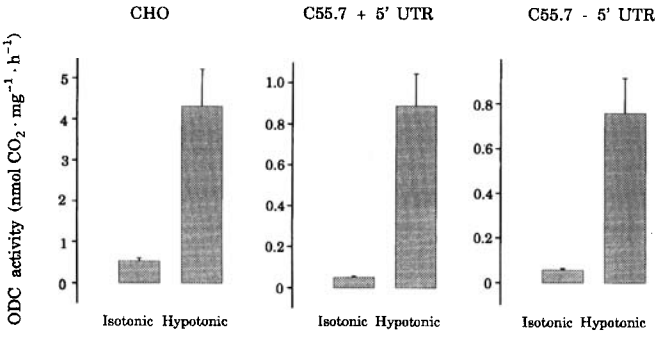

Fig. 4. Hypotonic induction of ODC in CHO cells expressing full-length and 5′ truncated ODC mRNA. Stable transfectants of the mutant ODC-deficient CHO cell line C55.7 expressing ODC full-length mRNAs or mRNAs devoid of the 5′ UTR were isolated as described in Materials and Methods. One day after seeding, the stable transfectants and control wild-type CHO cells were exposed to either an isotonic or a hypotonic medium for 6 h before harvested and analyzing for ODC activity. It should be noted that the scales differ. Mean ± SEM, $n = 8$.

mechanism by which the hypotonic stress induces ODC activity is still obscure. It appears that no single mechanism is responsible for the hypotonic induction of ODC. Poulin and Pegg [25] have demonstrated that the rise in ODC activity in a F_2MeOrn-resistant L1210 cell line after hypotonic stress is mainly due to a combination of an increased ODC mRNA translation and decreased ODC degradation. Nevertheless, in other investigated cell lines, such as LLK-PK_1 (an epithelial cell line derived from pig kidney), 3T3 (derived from Swiss mouse embryos), A549 (derived from human lung carcinomatous tissue), Wish cells (derived from human amnion) and human skin fibroblasts, the induction of ODC activity by hypotonic treatment is correlated with an increase in the cellular ODC mRNA level [27], indicating an effect on the level of transcription (or mRNA turnover). In the F_2MeOrn-resistant L1210 cell line used in the present study, hypotonicity gave rise to only a very small elevation of the ODC mRNA level, whereas the synthesis of ODC was increased several-fold, indicating mainly a post-transcriptional mechanism. The results are similar to those of Tohyama et al. [26], who used primary cultures of rat hepatocytes.

Polyamines have been demonstrated to exert a strong feedback control on cellular ODC [7, 8, 10–15]. This control appears to be mainly post-transcriptional. In a situation when the cellular polyamines are depleted, the amount of ODC rises due to increased synthesis (in spite of no change in the ODC mRNA level) and decreased degradation. An excess of polyamines, in contrast, causes a decreased synthesis and an increased degradation of ODC. As shown in the present study, addition of ornithine counteracted the hypotonic induction of ODC in the F_2MeOrn-resistant L1210 cells. A similar finding has been observed by Poulin et al. [25] in a different F_2MeOrn-resistant L1210 cell line. The reason for this effect is not known. It may be partly due to a putrescine-mediated repression of ODC. The very high ODC activity found in these cells appeared to deplete the cells of ornithine and thus make the substrate for polyamine production a limiting factor. This explains the finding that, although ODC activity was increased almost 20-fold by the hypotonic shock, the cellular putrescine content did not rise. However, when ornithine was provided in the hypotonic medium, the cellular putrescine level increased markedly. That ornithine was a limiting factor for putrescine production in the F_2MeOrn-resistant L1210 cells was confirmed by the finding that the putrescine content was not significantly changed, in spite of a marked repression of ODC activity, when ornithine was added to cells grown in an isotonic medium. This finding also emphasizes the efficiency by which the feedback control of ODC may occur. Interestingly, the addition of ornithine to the medium did not affect the increase in the ODC mRNA level caused by the hypotonic shock, indicating a mechanism unrelated to polyamines.

The physiological function of the long G+C-rich 5′ UTR of the ODC mRNA is still obscure. Since it is extremely G+C rich, it can form strong secondary structures with a very high free energy of formation ($-DG > 460$ kJ/mol). These secondary structures may negatively affect the translatability of the message and may thus be of regulatory importance [40]. That the ODC mRNA is indeed poorly translated *in vivo* is indicated by the finding that most of the ODC mRNA is found associated with ribosomal subunits or monosomes [15, 41, 42]. Only a very small fraction of the ODC mRNA is found in polysomes. Furthermore, the inhibition of translation by 5′ UTR of ODC mRNA has been demonstrated in reticulocyte lysates as well as in cells transfected with various DNA constructs containing the leader sequence [39, 43, 44]; it is conceivable that this is of importance for the polyamine-mediated translational control of ODC. However, a study using various DNA constructs has failed to demonstrate such a function [15]. Since at least part of the osmotic control of ODC occurs by a translational mechanism, it appeared of interest to determine whether the 5′ UTR may be essential for the hypotonic induction of ODC. Using stable transfectants expressing 5′ truncated and full-length ODC mRNAs, we were unable to find a difference in response to hypotonic stress, demonstrating that the hypotonic induction of ODC was not dependent on the presence of a full-length 5′ UTR. Since the truncation was not complete, leaving 27 nucleotides of the 5′ UTR, we cannot rule out the possibility that the part closest to the initiation codon may be important for the increase in translation of the ODC mRNA seen after hypotonic stress. Nevertheless, it is clear from the present results that the major part of the 5′ UTR of the ODC mRNA is unessential for the osmotic effects on ODC expression.

Interestingly, when ODC was transiently expressed in COS cells, there was no increase in ODC activity when the cells were exposed to a hypotonic medium, whereas in the mock-transfected COS cells, endogenous ODC was induced threefold by hypotonic stress. In fact, the transiently expressed ODC activity decreased during the hypotonic treatment. This is most likely due to an inhibitory effect on general protein synthesis. Poulin and Pegg [25] have demonstrated that a 4-h exposure of F_2MeOrn-resistant L1210 cells to hypotonic medium results in a 50% reduction in L-[^{35}S]methionine incorporation into total soluble protein. However, the decrease in general protein synthesis cannot explain the lack of a hypotonic induction of ODC in the COS cells transiently expressing the enzyme, since ODC is induced in the mock-transfected cells. The reason for this unresponsiveness is not known. Nevertheless, further studies of this phenomenom in COS cells may provide important information for the elucidation of the exact mechanism by which hypotonicity can induce ODC activity.

This study was supported by grants from the Swedish Cancer Society, the Medical Faculty (University of Lund, Sweden) and the John and Augusta Persson, the Crafoord, the Wiberg, the *Gyllenstiernska Krapperup* and the Gunnar, Arvid and Elisabeth Nilsson foundations.

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