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Original article

Effects of oxygen, CO_2/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos

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Summary — The gas atmosphere and medium composition are critical factors in the in vitro development of one- and two-cell embryos of several species. The present study evaluated the effect of different O_2/CO_2 concentrations (2/5, 2/10, 5/2.5, 5/5, 5/10, 10/10 and 21/5) on pig one- and two-cell embryo development. The embryos were individually cultured, for 6 days at 39 °C in a medium rich in bicarbonate and glutamine and containing pyruvate and lactate but lacking glucose. When the CO_2 levels increased from 2.5% to 10%, the pH of the medium decreased from 8.2 to 7.5 and the development of the embryos was affected, but this depended mainly on the O_2 levels. Pig embryo development was inhibited by 2 and 21% O_2 levels. The optimum level for pig embryo development was 5% O_2 and 5% CO_2, whatever the criteria used to evaluate embryo development. At these optimal levels, the mean number of cells per embryo was 26 ± 1.7 (ls mean ± SE), and 50% of the one- and two-cell embryos developed to blastocysts. The substitution of 0.5% bovine serum albumin (BSA) in the medium by 0.3% polyvinyl-pyrrolidone (PVP) significantly decreased the one- and two-cell embryo development. When the calcium and chloride contents of the medium with PVP were reduced, however, the embryo development was similar to that observed in the medium containing BSA. Pig embryo development in vitro was found to be optimal under an atmosphere of 5% O_2 and 5% CO_2 and PVP could replace BSA as the high molecular weight supplement.

pig / embryo / in vitro / oxygen / medium

Résumé — Effets de différentes concentrations d'oxygène et de gaz carbonique dans l'azote sur le développement d'embryons de porcs un–deux cellules. *Dans plusieurs espèces, la composition du milieu et celle du mélange gazeux jouent un rôle essentiel pour le développement in vitro d'embryons à partir du stade un–deux cellules. L'effet de différentes concentrations d'oxygène et de gaz carbonique dans l'azote (2/5, 2/10, 5/2,5, 5/5, 5/10, 10/10 et 21/5) sur le développement d'embryons de porcs un–deux cellules a été évalué. Ces embryons ont été cultivés individuellement pendant 6 jours à 39 °C dans un milieu riche en bicarbonate et en glutamine, contenant aussi du lactate et du pyruvate mais qui était dépourvu de glucose. Lorsque la concentration de CO_2 s'élève de 2,5 à 10 %, le pH diminue de 8,2 à 7,5 et le développement de l'embryon en est affecté mais la concentration d'oxygène joue un*

rôle prépondérant. Les concentrations en oxygène de 2 et de 21 % inhibent le développement de l'embryon de porc. Le mélange gazeux optimum pour l'embryon de porc est constitué de 5 % d'oxygène et de 5 % de gaz carbonique. Dans ces conditions, le nombre moyen de cellules par embryon est de 26 ± 1,7 (moyenne ± écart) et 50 % des œufs mis en culture se développent jusqu'au stade blastocyte. Le remplacement de l'albumine bovine (fraction V) par 0,3 % de polyvinyl pyrrolidone dans le milieu de culture provoque une chute significative du développement des œufs. Cependant, si l'on réduit la concentration de calcium (1 mM) et d'ions Cl^- (86 mM), l'embryon se développe d'une manière similaire à celle d'un milieu contenant de l'albumine bovine. L'embryon de porc peut donc être cultivé individuellement dans un milieu synthétique sous une atmosphère de 5 % O_2 et de 5 % de CO_2.

porcin / embryon / in vitro / oxygène / milieu

INTRODUCTION

Good development of porcine one- and two-cell embryos is not yet possible in completely synthetic medium. This could be due to a combination of factors such as deficiences or imbalances of some components in the medium and/or a nonoptimal atmosphere. In vivo, oxygen tension is low in the oviduct and uterus of the rabbit, hamster, rhesus monkey and rat (3–6% oxygen, Mastroianni and Jones, 1965; Mitchell and Yochim, 1968; Fischer and Bavister, 1993). In pigs, the development of embryos has been observed in 5% oxygen (Davis and Day, 1978; Lindner and Wright, 1978; Stone et al, 1984; Petters et al, 1990) or in air (Niemann et al, 1983; Hagen et al, 1991; Petters and Reed, 1991; Hajdu et al, 1994; Miyano et al, 1994; Prather et al, 1995; Rath et al, 1995). Schneider et al (1975) observed development in two different gas compositions, but according to Wright (1977), 5% oxygen was more beneficial than air. The discrepancies between these results are presumably the consequence of differences in the initial stages of development, medium composition, culture conditions (number of embryos per unit volume, presence or absence of oil, etc) and criteria for the evaluation of development (Paria and Dey, 1990; Ferry et al, 1994). In contrast to many other species, the role of CO_2/pH, in pig embryo development has not yet been investigated. Bavister (1988), however, reported an improved development of hamster embryos with 10% CO_2 in a medium containing a high concentration of $NaHCO_3$. Farrell and Foote (1995) observed the development of rabbit embryos to blastocysts in 5% oxygen and 10% CO_2; furthermore, the oviductal fluids of the rabbit and rhesus monkey are rich in HCO_3^- (Vishwakarma, 1962; Maas et al, 1979).

The main objective of this paper was to study the effect of oxygen and CO_2/pH on the development of porcine one- and two-cell embryos. The basic medium used in this study contained a high bicarbonate concentration, lactate plus pyruvate, high levels of glutamine (Rieger, 1992) and antioxidants but lacked glucose and phosphate (Petters et al, 1990; Leese, 1991; Petters and Reed, 1991). The in vitro development of the embryos of several species is dependant on the number or density of embryo cultured per unit volume (Wiley et al, 1986; Gardner, 1994; Carolan et al, 1995). To avoid this effect, the one- and two-cell embryos were individually cultured in all experiments. As in other species, progress in defining the factors involved in porcine embryo development in vitro will require a fully synthetic medium. The media used in this study was derived from NCSU 23 (Petters and Reed, 1990) and from UB medium (Petters and Wells, 1993). In a second series of experiments, bovine serum albumin (BSA) was replaced by a synthetic macromolecular substance, polyvinyl-pyrrolidone (PVP).

MATERIALS AND METHODS

Animals

Sixty-one puberal gilts were treated with Regumate (Martinat-Botté et al, 1990) to synchronize oestrus. The animals were artificially inseminated twice with six billion spermatozoa as previously described (Després et al, 1992). Large White gilts were inseminated with Meishan semen and Meishan gilts with Large White semen. Under natural conditions, these cross embryos have a similar high survival rate (Terqui et al, 1992). The females were slaughtered 48 h after the first artificial insemination.

Culture media

Culture media were prepared from cell culture-tested compounds (Sigma, France). The water was injectable grade (Laboratoire A Guettant, France). Table I presents the detailed composition of the two basic media used in this study. The BSA was Fraction V (embryo-tested, Ref A3311, Sigma, France). The antibiotic and antimycotic solution (Sigma, France) contained 10 000 IU, 10 mg and 25 µg per ml of penicillin, streptomycin and amphoterycin B, respectively. The media were sterilized by filtration through 0.22 µm filters (Millipore, France). The medium was modified from NCSU-23 (Petters and Reed, 1990). The main differences from NCSU-23 were the addition of lactate, pyruvate and vitamins, higher concentrations of glutamine and BSA and the absence of glucose.

Medium N + BSA corresponded to Medium N plus an additional 10 g/L of BSA, thus giving a total BSA concentration of 15 g/L. A similar medium was used by Whitten (1957) to culture mouse ova, and by Beckman and Day (1993) and Rath et al (1995) for porcine zygote in vitro culture. Medium N-PVP corresponded to Medium N without BSA but with 3 g/L PVP (embryo-tested Ref P093, Sigma, France). Medium U-PVP was

Table I. Composition of culture media for culture of porcine one- and two-cell embryos.

Ingredients in mmol.L^{-1}	*Medium N*	*Medium U*
NaCl	94	80.42
KCl	5.36	5.36
$MgSO_4$, $7H_2O$	0.81	0.81
$NaHCO_3$	44	44
(DL) lactate, sodium salt	–	12.08
(L) $^{1/2}$Ca(lactate) (mw = 109)	3.25	1
$CaCl_2$, $2H_2O$	1.8	–
Sodium pyruvate	0.2	0.2
Glutamine	3.42	3.42
Taurine	7	7
Hypotaurine	5	5
Vitamin C	10	10
Vitamin E	0.2	0.2
Androstenedione (mg/L)	0.250	0.25
Phenol Red (mg/L)	15	15
BSA (g/L)	5	–
PVP (g/L)	–	3
Antibiotic and antimycotic solution (ml/L)	10	10
K^+	5.36	5.36
Na^+	138.2	136.7
Cl^-	102.96	85.78
Ca^{2+}	5.05	1
Osmotic pressure (mOsm)	291	266

derived from UB medium (Petters and Wells, 1993). The U medium differs mainly from UB by its higher concentration of glutamine and the absence of other amino acids, sorbitol and BSA.

Eggs collection

Within 30 min of slaughter, the eggs were collected by flushing each oviduct with 25 ml of saline solution containing 5% (v/v) newborn calf serum (BioWhittaker, France). The eggs were washed three times with 0.5 ml of the culture medium.

Eggs culture

One- and two-cell embryos were individually cultured in wells with 200 µl of medium without oil (96 well plates, NUNC, France). Embryos from the same gilt were allotted between different treatments, with at least two embryos (two wells) of the same female in a given treatment. Each treatment was repeated at least three times. The total number of gilts and the number of replicates are indicated in tables II and III for each treatment. The embryos were cultured for 6 days at 39 °C without any medium renewal. In experiment 1, different atmospheres were tested (table II); in experiment 2, the gas mixture was 5% CO_2, 5% O_2 and 90% N_2.

pH determination

A volume of 25 ml of medium was equilibrated for 16 h under the conditions described earlier. The pH was determined immediately. For 5% oxygen and 5% CO_2, the measurements were repeated three times. The recorded values did not differ by more than 0.1 pH unit.

Assessment of embryo development

Qualitative evaluation of development

After culture, the embryos were examined under a phase contrast microscope at 200x magnification and were classified according to their development stage as either not developed (< four cells), abnormal or morula/blastocyst.

Quantitative measurements

The nuclei were stained and counted by the addition of Hoescht dye (No 33342) to the culture medium at a final concentration of 10 µg/ml (Papaioannou and Ebert, 1988). The embryos were classified into one of the three categories defined by Beckmann and Day (1993) according to their number of cells: < 6 cells; > 6 to 19 cells; > 19 cells.

Number of dead cells

Cells stained red by propidium iodide (Ockleford et al, 1981) were considered as dead cells.

Statistical analysis

The number of cells were analysed with the GLM procedure of the SAS system (1989) and Splus (Statistical Sciences, 1993). The distribution of the number of cells was adjusted to a quasi-Poisson distribution. The hypotheses were tested with females nested in replicates as error term and also with female and replicate*female and replicate as random factors. The model included the initial stage of development (one or two cells). The number of embryos in each class were analysed with the CATMOD procedure of the SAS system (1989). A P value of less than 0.05 was considered as significant.

RESULTS

The number of one- and two-cell embryos used in the experiments (tables II and III) were very similar (48.5% of one cell) and there was no significant difference between treatment groups, at the beginning of the culture, in the number of one- and two-cell embryos. There was a significant effect, however, of the initial stage of development on the number of cells at the end of the culture. This effect represented a mean

Table II. Effect of atmosphere composition on porcine embryo development after 6 days of culture in medium N at 39 °C.

Atmosphere composition in p 100							
O_2	*2*	*2*	*5*	*5*	*5*	*10*	*21*
CO_2	*5*	*10*	*2.5*	*5*	*10*	*10*	*5*
No of replicates	5	5	3	11	5	2	3
No of gilts	11	10	10	26	10	7	8
No of eggs	27	27	50	99	25	30	35
pH	nd	nd	8.2	7.8	nd	7.5	7.8
No of dead cells/zygote ls mean ± SE	0.04 ± 0.43	0.08 ± 0.42	nd	0.09 ± 0.31	0.0 ± 0.45	1.5* ± 0.38	0.06 ± 0.35
No of cells/zygote ls mean ± SE	6 ± 3.2	8 ± 3.2	15 ± 2.8	26* ± 1.6	11 ± 3.4	12 ± 3.8	5 ± 3.6
Distribution in percentage between classes of cell number							
< 6 cells	70	66	40	22**	60	33	46
> 6 and ≤ 19 cells	30	30	24	24	24	47	48
> 19 cells	0	4	36	53	16	20	6

* The least square mean (ls mean) is different from the other ls means of the same row ($P < 0.01$); ** distribution of this group is different from the other groups ($P < 0.05$). nd: not determined.

increase of seven cells for the two-cell embryos by the end of the culture. The effects of treatments were similar for both the one and two cells. There was no significant interaction between treatments and initial stage ($P > 0.10$). Thus, in the table and figure, no distinction was made between one- and two-cell embryos.

Effect of gas atmosphere

The results shown in table II demonstrate that CO_2 concentration modified the pH of the medium; the pH decreased by 0.7 pH units as CO_2 percentage increased from 2.5 to 10%. The pH of the medium N under 5% O_2 and 5% CO_2 was identical to that of 21% O_2 and 5% CO_2.

All the cultured eggs were recovered and analysed. The composition of the culture atmosphere had a large effect on development ($P < 0.05$). According to the O_2 and CO_2 percentages, the mean total number of cells after 6 days of individual culture varied from less than eight cells to more than 25 cells. The concentrations of the two gases affected the embryo development in a nonlinear manner ($P < 0.05$). A mixture of 5% O_2 and 5% CO_2 led to maximum development. The number of dead cells per zygote was extremely low and was not significantly different from zero except when the eggs were exposed to 10% O_2 and CO_2 (table II). Under these conditions, one to two cells of the embryos were dead. The distribution of the cultured embryos between the three classes of cell counts, as defined by Beckman and Day (1993), are presented in table II. This clearly shows that both low (2%) and high (air) oxygen concentration reduced embryo development in comparison to that observed under a concentration of 5% of both gases and also that porcine egg development is very sensitive to CO_2. The mean number of cells per embryo of the category > 19 cells was also higher after culture under 5% O_2 and CO_2 than those cultured under other gas mixtures (43 vs 22 to 37 cells, $P < 0.05$).

The results of the morphological examination after culture are presented in figure 1. The general pattern was similar to the distribution in cell number class; 5% O_2 and 5% CO_2 appeared again to be the best atmosphere for porcine embryos with close to 50% reaching the blastocyst stage ($P < 0.05$). The morphological examination and cell counts, however, were not always in agreement. For example, some embryos were considered as blastocysts in the group 2% O_2 and 5% CO_2, although no embryo had more than 19 cells.

Whatever the criteria considered, gas mixture of 5% O_2 and 5% CO_2 was the optimal one for porcine embryos cultured singly in medium N.

Effect of medium composition

The high BSA concentration in medium N slightly decreased the number of cells per zygote (table III). The mean number of cells per embryo in class > 19 cells, however, was higher in this media as compared to that obtained in the others (47 vs 33 to 37 cells, $P < 0.05$). The replacement of BSA by PVP without any other media change (medium N-PVP) significantly reduced, by more than half ($P < 0.05$), the number of cells per zygote. Indeed, as shown in table III, the percentage of embryos in the category > 19 cells was reduced to 14%, which was much lower than percentages recorded for the other media (range 43 to 57%). The medium U-PVP, which was entirely synthetic, yielded similar number of cells (table III), percentage of embryos > 19 cells as indicated in table III and mean number of cells of the category > 19 cells ($P > 0.19$) than medium N. The number of dead cells remained low and did not differ between media (table III).

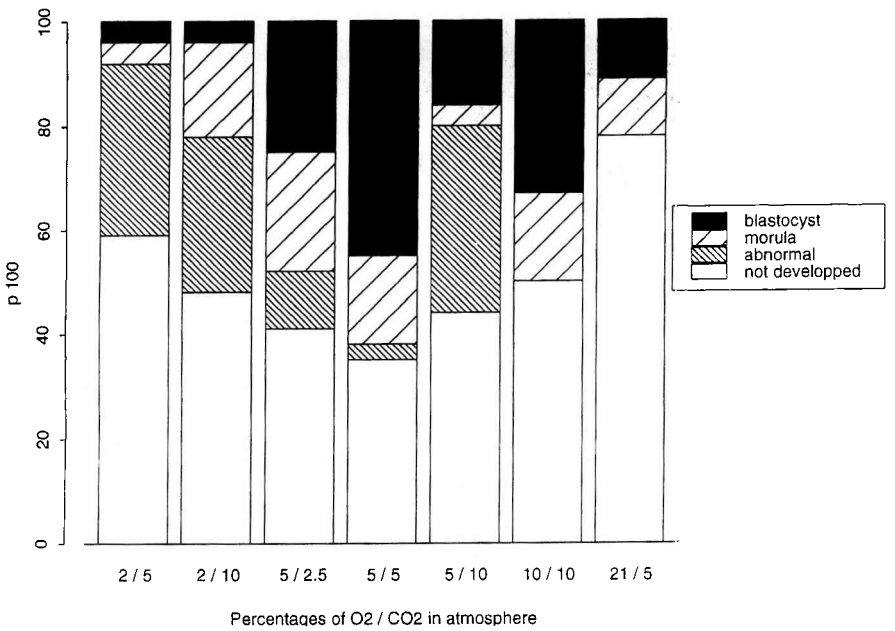

Fig 1. Morphological evaluation of the development of porcine one- and two-cell embryos after 6 days of culture in different oxygen and CO_2 atmospheres. Maximum development was obtained under 5% O_2 and 5% CO_2 atmosphere ($P < 0.05$).

Table III. Effects of bovine serum albumin (BSA) and medium composition on porcine embryo development after 6 days of culture at 39 °C and under 5% O_2 and 5% CO_2.

	Medium N + BSA	*Medium N*	*Medium N–PVP*	*Medium U/PVP*
No of replicates	5	7	9	7
No of gilts	14	18	20	18
No of eggs	33	63	69	70
No of dead cells/embryo ls mean ± SE	nd	0.2 ± 0.08	0.28 ± 0.12	0.11 ± 0.04
No of cells/embryo ls mean ± SE	16 ± 3.13	27 ± 2.3	10* ± 1.8	25 ± 2.15
Distribution in percentage between classes of cell number				
< 6 cells	30	19	40**	14
> 6 and ≤ 19 cells	27	24	45	43
> 19 cells	43	57	15	43

* The least square mean (ls mean) is different from the other ls means of the same row ($P < 0.01$); ** distribution of this group is different from the other groups ($P < 0.05$). nd: not determined; PVP: polyvinyl-pyrrolidone.

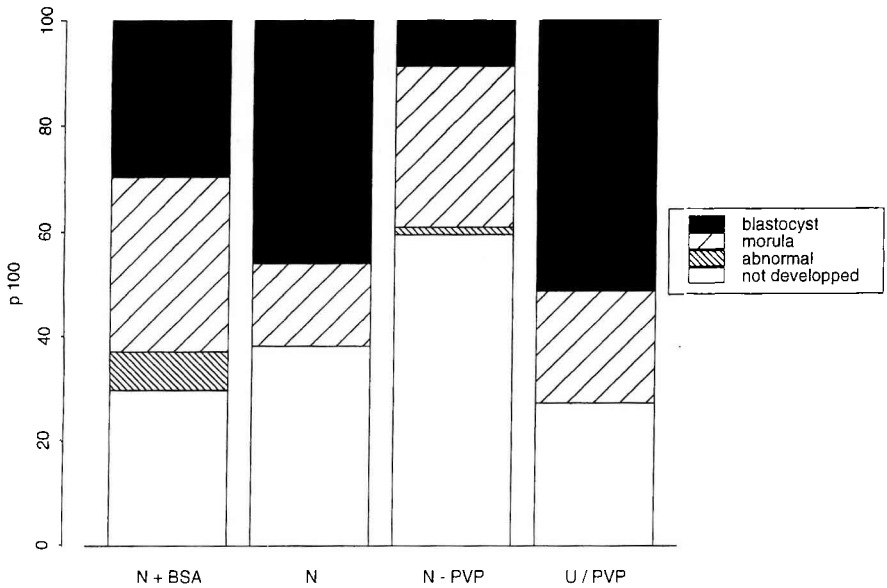

Fig 2. Morphological evaluation of the development of porcine one- and two-cell embryos after 6 days of culture in different media. In medium N-PVP, the development is significantly reduced ($P < 0,05$). BSA: bovine serum albumin; PVP: polyvinyl-pyrrolidone.

The qualitative observations are summarized in figure 2. The results were similar to those recorded in table III. Medium N and Medium U/PVP yielded approximately 50% blastocysts. Medium N-PVP (medium N with PVP instead of BSA) produced a limited percentage of blastocysts ($P < 0,05$). A fully synthetic medium, therefore, allowed the development of one- to two-cell embryos up to the blastocyst stage; the number of cells per blastocyst was only slightly lower than that obtained with very high BSA levels in the medium.

DISCUSSION

Oxygen concentration appears to be a key factor controlling the in vitro development of the porcine embryo. Under our conditions, the optimal concentration was close to 5%. This is similar to the situation existing in other species, except perhaps in the cat (Johnston et al, 1991). The in vitro development of the one- or two-cell embryos in the mouse (Whitten, 1957; Auerbach and Brinster, 1968; Quinn and Harlow, 1978), rabbit (Li and Foote, 1993; Lindenau and Fischer, 1994), sheep and cow (Thompson et al, 1990a; Carolan et al, 1995), goat (Batt et al, 1991) and human (Noda et al, 1994) is optimal under low oxygen levels (3–7%). Lower and higher oxygen concentrations were detrimental, suggesting that an optimal amount of active oxygen intermediates are required to stimulate development (McCord, 1995). An excess of free oxygen radicals, however, can negatively affect lipids, proteins, carbohydrates and DNA, cause major cell damage (see review, Jaeschke, 1995) and retard cell growth during culture (Joenje, 1989). The propidium iodide method used for dead cell determination presumably underestimates the proportion

of damaged cells. In the present study, however, there was no increase in dead cell number. This was perhaps due to the presence of vitamins E and C, taurine and hypotaurine in the medium, which act as free radical scavengers (Olson and Seidel, 1995; Thompson et al, 1990b).

This study also demonstrated that embryo development in medium N is also dependent on CO_2/pH. Low and high CO_2 concentrations inhibited porcine embryo development. This differs from the report of Bavister (1988) for the hamster, but the species and media are not the same, and intracellular pH was not measured in our experiment. Our results, however, supported Bavister's idea (1988) of a weak acid role in the control of the development of embryos during in vitro culture.

It is always difficult to compare cell numbers and morphology from one study to another, mainly because duration of cultures are not the same. However, development obtained in medium N and U/PVP were in the same range as that observed by Youngs et al (1993) and by Prather et al (1995), who also performed individual culture, and by other groups (Petters and Reeds, 1991; Beckman and Day, 1993; Petters and Wells, 1993; Hadju et al, 1994; Rath et al, 1995). Thus, the one- and two-cell porcine embryos can develop in the absence of glucose, but with lactate, pyruvate and glutamine even without BSA. These results support the work of Leese (1995) and Rieger (1992) and Petters and Wells (1993), who have postulated that the embryo is able to use these substrates instead of glucose. A high HCO_3^- concentration is important since it is involved with pyruvate in the synthesis of oxaloacetate (Attwood, 1995). Furthermore, bicarbonate seems essential to pig fertilization in vitro (Suzuki et al, 1994). As in other reports, the in vitro development is 1 to 2 days behind the in vivo development (Papaioannou and Ebert, 1988). The addition of foetal calf serum to culture medium did not improve the development rate (Pollard et al, 1995).

In Medium N without BSA (N-BSA), there was a significant decrease in embryo development. This was perhaps due to the high calcium concentration, which exists in a free state in the absence of BSA and of the contaminants of BSA such as citrate (Rorie et al, 1994). This could also be an effect of the high levels of Cl^-. In medium U/PVP with lower levels of Ca^{2+} and Cl^- (table I), the development was improved. A putative inhibitory effect of high Cl^- concentrations on embryo development (Lawitts and Biggers, 1991) is also supported by the results of Beckman and Day (1993) who improved embryo development by reducing the NaCl concentration in their medium. Whatever the reason, these results underline the ambiguous role of BSA, and the interest in developing a synthetic medium.

In conclusion, porcine embryo development in vitro is optimal in an atmosphere of 5% oxygen and 5% CO_2. It does not require glucose with a reduced calcium and chlorine concentration in the medium and PVP could replace BSA as a high molecular weight supplement.

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