Biochimica et Biophysica Acta, 1107 (1992) 299–304

BBAMEM 75674

Mediated Na^+-independent transport of L-glutamate and L-cystine in 1- and 2-cell mouse conceptuses

Lon J. Van Winkle, David F. Mann, Howard G. Wasserlauf and Meghana Patel

Department of Biochemistry, Chicago College of Osteopathic Medicine, Downers Grove, IL (USA)

(Received 25 November 1991)

Key words: Membrane transport; L-Glutamate; L-Cystine; Preimplantation conceptus; (Preimplantation embryo)

L-Glutamate and L-cystine appeared to compete for transport via a mediated Na^+-independent transport process in 1- and 2-cell conceptuses. Not only did these substances competitively inhibit each others' uptake by conceptuses, but their K_i values for inhibition were about equal to their K_m values for transport in 1-cell conceptuses. Moreover, the transport process interacted strongly with L-amino acids that had 3–6 atoms in a chain between their negatively charged groups, whereas it interacted weakly or not at all with amino acids that did not have these characteristics or that were N-methylated. Transport of anionic amino acids was not altered greatly by pH in the range 4.5–8.0, but transport of L-cystine was much faster at higher pH values. The slower cystine transport at lower pH values was due primarily to protonation of its second amino group. A small increase in the degree of deprotonation of cystine's carboxyl groups also probably contributed slightly to its faster transport at higher pH values. By all of these criteria, the transport process in conceptuses appears to be a form of amino acid transport system x_c^-. System x_c^- activity was several-fold higher in 1- than in 2-cell conceptuses. Similarly, L-glutamate uptake by unfertilized eggs was relatively rapid, whereas it was much slower in immature, fully-grown oocytes. System x_c^- activity in 1-cell conceptuses also appeared to increase in response to the oxidative stress of culture, whereas no such increase was observed for 2-cell conceptuses. We suggest that this transient increase in the activity of system x_c^- activity during development of 2-cell conceptuses from immature, fully-grown oocytes could help protect unfertilized and fertilized eggs from oxidative stresses in situ.

Introduction

At least ten transport systems for α-amino acids have been delineated in preimplantation mouse conceptuses (reviewed in Ref. 1). Several of these ten systems had been described previously in other types of cells, but half of the systems were first well characterized in early conceptuses. Subsequently, some of the latter novel systems were also detected in other tissues [1]. For example, system $b^{0,+}$ appears to be present in the human placental trophoblast [2]. The activities of most of the amino acid transport systems in conceptuses are developmentally regulated [1]; system X_{AG}^- and the novel systems $B^{0,+}$, B, $b^{0,+}$ and b_2^+ increase greatly in activity between the 8-cell and blastocyst stages of development. Conversely, system Gly and the novel system b_1^+ seem to disappear during development of blastocysts [1]. In contrast to the timing of the changes in the activities of the transport systems that are associated with formation of the blastocyst, development of 2-cell conceptuses from fertilized eggs is associated with a large decrease in the activity of another as yet only poorly characterized Na^+-independent glutamate transporter [1].

Na^+-independent glutamate transport is known to occur via system x_c^- in several types of mammalian cells [3–9]. System x_c^- prefers as substrates anionic amino acids with three to six atoms in the chain between the anionic groups. Moreover, it transports L-cystine when cystine bears a net -1 charge [8,9]. The activity of system x_c^- has been shown to increase greatly in several types of mammalian cells when they are placed in culture in vitro [3–7]. The latter increase allows cells to take up more cystine in exchange for intracellular glutamate that had been derived from glutamine previously taken up from the medium. The cystine that is taken up in this manner is reduced and utilized for the synthesis of glutathione. This mechanism for glutathione production appears to help cells resist oxidative stress in vitro [3–7]. It is not known whether system x_c^- activity can increase to help cells resist oxidative stress in vivo, although the latter possibility seems likely in light of the ease with which the

Correspondence to: L.J. Van Winkle, Department of Biochemistry, Chicago College of Osteopathic Medicine, 555 31st Street, Downers Grove, IL 60515, USA.

increase occurs in culture. Because the activity of a Na^+-independent glutamate transporter decreases greatly in conceptuses between the 1- and 2-cell stages of development, and because this change could have important physiological consequences, we determined whether this transport activity is system x_c^- or another system(s).

Materials and Methods

Conceptuses were obtained as described previously (e.g., Refs. 10–12) from sexually mature 8–11-week-old ICR mice (Harlan Sprague-Dawley, Inc.). Mice were induced to ovulate and mate utilizing gonadotropins [13]. 1- and 2-cell conceptuses were obtained from oviducts about 19 and 43 h, respectively, after administration of human chorionic gonadotropin. Most conceptuses were washed and stored for 6 h or less in Brinster's medium [14] in a humidified atmosphere of 5% CO_2 in air at 37°C (pH 7.4). Some 1-cell conceptuses were incubated in Brinster's medium for 24 h to obtain 2-cell conceptuses.

Conceptuses were incubated with L-[^{3}H]glutamate (45 Ci/mmol) or L-[^{35}S]cystine (143 mCi/mmol before decay) (Amersham) and one of several concentrations of various nonradioactive amino acids in phosphate-buffered media at 37°C (pH 7.1; Refs. 10, 11, 15, 16 and see figure legends). In some cases, the pH was adjusted and also buffered with Tris, citrate and ϵ-amino-*N*-caproate to values between 4.5 and 8.0 as described in the appropriate figure legend [17]. Amino acid transport was frequently studied at substrate concentrations near 1 μM for reasons discussed more completely elsewhere [18,19] and because some of the K_m values for transport of anionic amino acids by blastocysts were near or below 10 μM [17]. Incubations were short enough to estimate initial velocities of uptake (i.e., 10 min or less), and no saturable binding of amino acids to plasma membranes has been detected in preimplantation conceptuses (Refs. 12, 17–19, and data not shown). The extracellular concentrations of amino acids seemed to remain virtually constant during the course of experiments as discussed previously [15,16]. All nonradioactive amino acids and other chemicals and hormones were purchased from Sigma Chemical Company or Behring Diagnostics. After incubation with a radiolabeled substrate, conceptuses were processed to determine how much of the substrate they had taken up [10]. Where appropriate, data were assessed statistically utilizing analysis of variance [20,21].

Results

Uptake of anionic amino acids by 1- and 2-cell mouse conceptuses was shown previously to be entirely Na^+-independent (Refs. 1, 22, and data not shown).

TABLE I

Effect of various amino acids on L-glutamate uptake by 1- and 2-cell conceptuses

Conceptuses were incubated with 0.89 or 2.22 μM L-[^{3}H]glutamate (45 Ci/mmol) and the indicated non-radioactive amino acid for 5 min at 37°C in phosphate-buffered LiCl (pH 7.1). The mean uptake ± S.E. was calculated from 4–19 replicate determinations (2–7 conceptuses/determination) obtained in 2–10 replicate experiments (two determinations and one experiment for *N*-methyl amino acids; S.E. not reported). Inhibition to less than 60% of uptake in the control (none) group was statistically significant ($P < 0.01$).

Inhibitor	L-Glutamate uptake (% of control)			
	1-cell		2-cell	
	10 mM	1 mM	10 mM	1 mM
None	100.0 ± 9.3	100.0 ± 17.6	100.0 ± 10.2	100.0 ± 8.7
L-Aspartate	45.0 ± 6.7	81.6 ± 9.5	35.1 ± 5.5	83.5 ± 16.1
L-Glutamate	1.9 ± 0.4	9.5 ± 1.6	4.8 ± 0.7	12.2 ± 1.9
L-Alanine	109.0 ± 15.6	101.4 ± 14.4	91.6 ± 13.7	131.1 ± 17.5
L-Lysine	98.0 ± 6.4	115.8 ± 10.9	124.2 ± 25.1	125.8 ± 16.6
L-Tryptophan	41.6 ± 6.1	99.8 ± 11.5	64.9 ± 10.3	84.2 ± 5.5
Taurine	114.3 ± 8.8	102.6 ± 14.2	103.1 ± 18.4	119.2 ± 15.7
L-Homocysteate	1.1 ± 0.2	6.3 ± 1.4	4.4 ± 0.7	5.7 ± 1.2
D-Glutamate	27.3 ± 3.2	93.1 ± 14.2	18.5 ± 2.8	51.4 ± 9.0
DL-Aminopimelate	3.6 ± 0.7	32.6 ± 4.8	6.3 ± 1.2	11.4 ± 2.1
L-Aminoadipate	1.1 ± 0.1	6.3 ± 1.2	3.6 ± 1.2	6.6 ± 2.7
L-Cysteate	16.8 ± 3.1	82.6 ± 12.8	13.9 ± 2.9	47.4 ± 7.2
L-Cysteine sulfinate	19.4 ± 3.5	98.2 ± 11.7	12.9 ± 2.2	33.3 ± 4.5
L-Glutamine	59.0 ± 5.8	132.8 ± 20.0	66.3 ± 11.3	81.4 ± 15.6
N-Methyl-L-Aspartate	92	–	79	–
N-Methyl-D-Aspartate	128	–	79	–
N-Methyl-L-Glutamate	107	–	90	–
L-Cystine	–	21.1 ± 3.7 (0.5 mM)	–	12.1 ± 2.1 (0.5 mM)

Na^+-independent L-glutamate uptake was a little more than 2-fold higher in 1-cell conceptuses that had been in culture for 6 h than in conceptuses that were studied as quickly as possible (i.e., < 15 min) after they were removed from the reproductive tract ($P < 0.01$). In contrast, uptake decreased slightly although not in a statistically significant manner in 2-cell conceptuses incubated in vitro for the same time period (data not shown). For these reasons, uptake was studied in 1-cell conceptuses that had been in vitro for < 2 h or for 4–6 h, whereas no such distinction was made for 2-cell conceptuses.

Uptake of L-[^{3}H]glutamate was inhibited relatively strongly by L-amino acids with 3–6 atoms in the chain between their anionic groups (i.e., L-homocysteate, DL-aminopimelate, L-aminoadipate, L-cystine and nonradioactive L-glutamate; Table I). In contrast, uptake was inhibited relatively weakly by D-glutamate and smaller anionic amino acids (i.e., L-aspartate, L-cysteate and L-cysteine sulfinate), and inhibition was not usually statistically significant for amino acids with only one anionic group (Table I). Moreover, N-methylation of anionic amino acids greatly reduced their abilities to inhibit [^{3}H]glutamate uptake (Table I).

L-Glutamate uptake and its inhibition by L-homocysteate were not greatly influenced by pH in the range 4.5–8.0 in either 1- or 2-cell conceptuses (Fig. 1a). Nevertheless, L-glutamate uptake in the presence of 0.5 mM L-homocysteate was somewhat faster at lower than at higher pH values ($P < 0.01$). In contrast, uptake of L-cystine and inhibition of L-glutamate uptake by L-cystine were influenced greatly by pH in the range 4.5–8.0 (Figs. 1a and b). L-Glutamate and L-cystine appeared to compete for transport at the same sites as indicated by competitive inhibition of each other's transport (e.g., Fig. 2), and by K_i values that were about the same as their K_m values for transport (Table II).

Discussion

A single, substrate-saturable system in 1- and 2-cell mouse conceptuses appears to transport both L-glutamate and L-cystine. These two substances competitively inhibited each others transport in conceptuses with K_i values approximately equal to their K_m values for transport ('AB' portion of the 'ABC' test for competition of substrates for the same enzyme; e.g., Ref. 23).

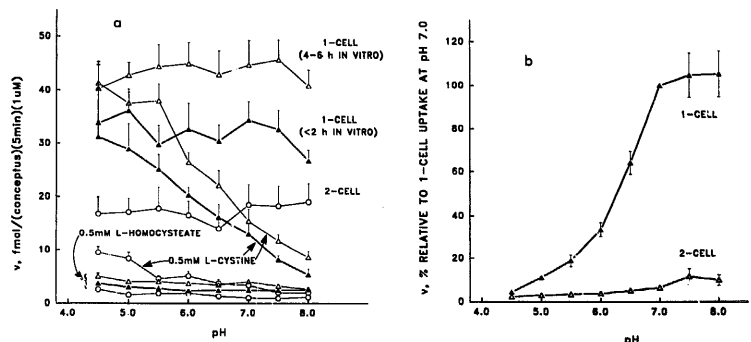

Fig. 1. Effect of pH on uptake of L-glutamate (a) and L-cystine (b) by 1- and 2-cell mouse conceptuses. Conceptuses were incubated with 0.59 (1-cell) or 2.22 (2-cell) μM L-[^{3}H]glutamate (45 Ci/mmol) or 42 μM L-[^{35}S]cystine (143 mCi/mmol) for 5 (glutamate) or 10 (cystine) min at 37°C in phosphate-buffered LiCl also buffered with ϵ-amino-*N*-caproate, Tris and citrate (Ref. 17). The pH was adjusted to the indicated values with HCl or KOH. L-Glutamate uptake was also measured in the presence of 0.5 mM L-cystine or L-homocysteate as indicated for lines with points marked by the same symbols as for L-glutamate uptake in the absence of other amino acids. Since L-glutamate uptake increases in 1-cell conceptuses when they are incubated in vitro (e.g., see Table II), data is shown for conceptuses that had been in vitro either for < 2 h (▲) or for 4–6 h (△). Data for L-[^{35}S]cystine are reported as the % of uptake at pH 7.0 in order to pool data for 1-cell conceptuses that had been in culture for < 2 h or for 4–6 h. Cystine uptake by 2-cell conceptuses is reported as a percentage of the average uptake by 1-cell conceptuses at pH 7.0. The mean ± S.E. uptake was calculated from 8–15 (a) or 5–11 (b) replicate determinations (4 or 5 conceptuses/determination) obtained in 5–7 (a) or 2–6 (b) independent experiments. The S.E. was not calculated for cystine uptake by 1-cell conceptuses at pH 7.0 since this uptake was arbitrarily set at 100% in each experiment. When the S.E. is not shown in other cases it fell within the symbol. L-Glutamate uptake was influenced significantly by pH in the presence of L-cystine or L-homocysteate ($P < 0.01$) but not in the absence of these inhibitors (a), whereas L-cystine uptake was influenced significantly by pH in the absence of inhibitors (b, $P < 0.01$).

The system in conceptuses is also Na^+-independent and inhibited relatively strongly by L-amino acids with 3–6 atoms in a chain between their anionic groups. In contrast, the system is inhibited weakly by D-glutamate and smaller anionic amino acids and usually not at all by amino acids with only one negatively charged group. Weak apparent inhibition of [^{3}H]glutamate uptake by glutamine (Table I) is probably due to slight contamination of the glutamine preparation with glutamate (Sigma Chemical Co.). N-methylation of L-glutamate also greatly reduces its interaction with the system in conceptuses.

The greatly reduced interaction of cystine with the system at lower pH values in the range 4.5–8.0 (Figs. 1a and b) is probably due mainly to protonation of cystine's second amino group [9]. A small increase in the degree of deprotonation of cystine's carboxyl groups at higher pH values also probably contributes somewhat to the effect of pH on its transport. Similarly, slightly greater deprotonation of the negatively charged groups of L-homocysteate at higher pH values appears to increase its ability to inhibit L-glutamate uptake somewhat (Fig. 1a). By all of these criteria, the system in 1- and 2-cell mouse conceptuses appears to be a form of system x_c^- [8,9].

As for system x_c^- in other types of cells [3–7], system x_c^- activity increases by about 2-fold when 1-cell conceptuses are cultured in Brinster's medium for 6 h. This increase in system x_c^- activity could be a response to increased oxidative stress in culture (see Introduction). This response would be futile in media that do not contain L-cystine (i.e., in media that are usually utilized to culture preimplantation mouse conceptuses; e.g., Refs. 14, 22, 24–26). Nevertheless, the increase in system x_c^- activity in 1-cell conceptuses in culture may reflect a response to oxidative stresses that also occur in vivo. Such a response by 1-cell conceptuses in vivo probably would not be futile since the extracellular cystine/cysteine in oviductal fluid probably is present mainly as cystine (e.g., Ref. 27). Interestingly, 1-cell mouse conceptuses do not accumulate H_2O_2 when they are subjected to the oxidative stress of culture [28], perhaps because they had synthesized sufficient stores of glutathione from cystine in vivo, whereas 2-cell conceptuses do accumulate H_2O_2 in culture [28]. 2-cell conceptuses have a relatively low capacity to take up cystine via system x_c^- (e.g., Fig. 1b), and the activity in 2-cell conceptuses does not increase during their culture in vitro for 6 h. Moreover, we have shown in preliminary studies that Na^+-independent glutamate

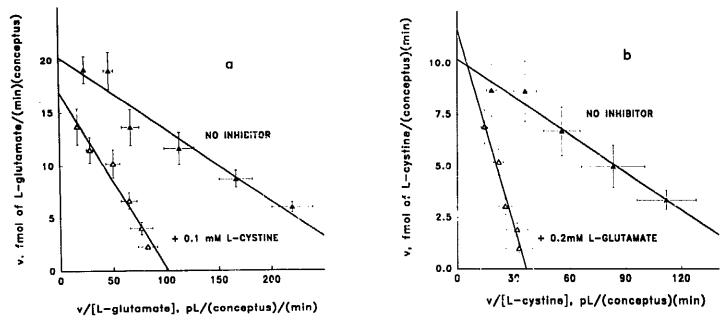

Fig. 2. Competitive Inhibition between L-glutamate and L-cystine for uptake by 1-cell conceptuses. To generate Hofstee plots, conceptuses were incubated with the indicated concentrations of L-glutamate (27–802 μM; 2.2 μM L-[^{3}H]glutamate; 45 Ci/mmol) or L-cystine (30–480 μM; 143 mCi/mmol) for 5 (glutamate) or 10 (cystine) min at 37°C in phosphate buffered LiCl (pH 7.1). Nonsaturable uptake was equivalent to less than 11% of total uptake even at 802 μM glutamate, and nonsaturable uptake was subtracted from total uptake to generate the data presented. The mean uptake ± S.E. was calculated from five (cystine, b) or ten (glutamate, a) replicate determinations (3–6 conceptuses/determination) obtained in three (cystine) or nine (glutamate) independent experiments. Because glutamate transport activity increases in 1-cell conceptuses during incubation in vitro (e.g., see Table II), uptake at each glutamate concentration was determined at various times between four and six h in culture so that the average time in vitro of conceptuses was about the same (approx. 5 h) at each concentration of glutamate studied. Similarly, uptake at each cystine concentration was determined at various times between zero and two h in culture so that the average time in vitro of conceptuses was about the same (approx. 1 h) at each concentration of cystine studied. Regression lines were calculated from the five or six mean values for each line as described elsewhere (e.g., Ref. 20). The values of the kinetic parameters were (a) K_m = −slope = 68 μM, V_{max} = y intercept = 20 fmol of L-glutamate per conceptus per min and K_i = [L-cystine]/((apparent K_m/K_m) − 1) = 70 μM; (b) K_m = 61 μM, V_{max} = 10 fmol of L-cystine per conceptus per min and K_i = 47 μM.

TABLE II

Values of kinetic parameters for L-glutamate or L-cystine transport and competitive inhibition of transport in 1- and 2-cell conceptuses

The values of K_m, V_{max} and K_i were determined in a manner similar to that described in the legend of Fig. 2. K_i values are for inhibition of glutamate uptake by cystine and for inhibition of cystine uptake by glutamate. When a S.E. is shown for 1-cell conceptuses the mean value ± S.E. was calculated from two (cystine) or four (glutamate) replicate determinations. Values of kinetic parameters were determined for 1-cell conceptuses that had been in vitro for < 2 h vs. 4–6 h, since uptake increased in 1-cell conceptuses during incubation in vitro ($P < 0.01$, data not shown), whereas such was not the case for 2-cell conceptuses. The V_{max} value for glutamate uptake was significantly higher for 1-cell conceptuses that had been in vitro for the longer time period ($P < 0.01$). Although the increase in the mean of the V_{max} value with time in vitro appeared to be larger for glutamate uptake than for cystine uptake, the difference between the V_{max} values for glutamate uptake was not statistically significantly greater than the difference between the V_{max} values for cystine uptake. Kinetic constants for L-cystine uptake were not determined in 2-cell conceptuses because of the relatively low specific activity of the radiolabeled form of this substance, and because of its relatively slow uptake by 2-cell conceptuses (e.g., Fig. 1b).

Stage of development	Substrate	V_{max} (fmol conceptus^{-1} min^{-1})	K_m (μM)	K_i (μM)
1-cell	L-glutamate	18.4 ± 1.3 (4–6 h) 11.1 ± 0.7 (< 2 h)	70 ± 8	100 ± 31
	L-cystine	12.7 ± 0.3 (4–6 h) 10.9 ± 0.7 (< 2 h)	67 ± 6	85 ± 38
2-cell	L-glutamate	4.1 ± 0.3	54 ± 1	~ 180

transport is nearly as rapid in unfertilized eggs as it is in fertilized ones, whereas it is less than 1/5 the latter rate in fully grown but immature denuded oocytes (unpublished data). Thus, system x_c^- activity, which may increase in response to the level of oxidative stress, seems to appear transiently during development of 2-cell conceptuses from immature, fully-grown oocytes. We suggest that this transient increase in system x_c^- activity may help protect unfertilized and fertilized eggs from oxidative stresses in situ. In this regard, fertilization of sea urchin eggs is associated with considerable oxidative stress [29], although it is apparently not yet known whether such is also the case for mammalian eggs. We are currently attempting to determine when during maturation or ovulation of oocytes a relatively high level of system x_c^- becomes active in their plasma membranes, and whether the ability to increase this activity in response to in vitro culture develops at the same time.

The decrease in system x_c^- activity, and the loss of the ability to increase the activity upon in vitro culture, occurs in association with the switch from maternal to embryonic mRNA utilization in 1- and 2-cell conceptuses. The latter switch, which appears to lead to extensive remodelling of the protein synthetic pattern in conceptuses, is largely complete by 15 h following cleavage of 1-cell conceptuses [30]. We have observed in preliminary studies (not shown) that system x_c^- activity also decreases during development of 2-cell from 1-cell conceptuses in Brinster's medium ($P < 0.01$). Therefore, it should be possible to study the mechanism by which system x_c^- activity decreases under controlled conditions in vitro. Moreover, the average decrease in system x_c^- activity that occurred in 2-cell conceptuses that developed in vitro was not as great as the average decrease that occurred in vivo. Only about 1/3 of the 1-cell conceptuses that we have observed to cleave in vitro have been able subsequently to develop into blastocysts (unpublished data). Thus, it should be interesting to learn whether development is merely somewhat slower in vitro or whether a greater decrease in system x_c^- activity is associated with higher subsequent viability. In this regard, it is now possible to assay individual preimplantation conceptuses for their ability to take up glutamine or glutamate from media and then to test the ability of the same conceptuses to develop in vitro or in vivo [31]. If viability of conceptuses is related to the extent of the change in their system x_c^- activity, then we might gain insight into the importance of this aspect of amino acid homeostasis to early development.

Acknowledgements

We thank Barb Le Breton for helping to prepare the manuscript. This work was supported by USPHS grant HD21801.

References

1 Van Winkle, L.J. (1992) in Mammalian Amino Acid Transport: Mechanisms and Control (Kilberg, M.S. and Häussinger, D., eds.), Plenum Press, New York (in press).
2 Furesz, T.C., Moe, A.J. and Smith, C.H. (1991) Am. J. Physiol. 261, C246–C252.
3 Hishinuma, I., Ishii, T., Watanabe, H. and Bannai, S. (1986) In Vitro 22, 127–134.
4 Takada, A. and Bannai, S. (1984) J. Biol. Chem. 259, 2441–2445.
5 Watanabe, H. and Bannai, S. (1987) J. Exp. Med. 165, 628–640.
6 Bannai, S. and Ishii, T. (1988) J. Cell. Physiol. 137, 360–366.
7 Bannai, S., Sato, H., Ishii, T. and Sugita, Y. (1989) J. Biol. Chem. 264, 18480–18484.
8 Bannai, S. and Kitamura, E. (1980) J. Biol. Chem. 255, 2372–2376.
9 Makowske, M. and Christensen, H.N. (1982) J. Biol. Chem. 257, 5663–5670.
10 Van Winkle, L.J., Christensen, H.N. and Campione, A.L. (1985) J. Biol. Chem. 260, 12118–12123.
11 Van Winkle, L.J., Campione, A.L., Gorman, J.M. and Weimer, B.D. (1990) Biochim. Biophys. Acta 1021, 77–84.
12 Van Winkle, L.J. and Campione, A.L. (1990) Biochim. Biophys. Acta 1028, 165–173.
13 Fowler, R.E. and Edwards, R.G. (1957) J. Endocrinol. 15, 374–384.
14 Brinster, R.L. (1971) in Pathways to Conception: The Role of the Cervix and Oviduct in Reproduction (Sherman, A.I., ed.), Charles C. Thomas, Springfield.

15 Van Winkle, L.J., Campione, A.L. and Gorman, J.M. (1988) J. Biol. Chem. 263, 3150–3163.
16 Van Winkle, L.J., Haghighat, N., Campione, A.L. and Gorman, J.M. (1988) Biochim. Biophys. Acta 941, 241–256.
17 Van Winkle, L.J., Mann, D.F., Weimer, B.D. and Campione, A.L. (1991) Biochim. Biophys. Acta 1068, 231-236.
18 Van Winkle, L.J., Campione, A.L. and Gorman, J.M. (1990) Biochim. Biophys. Acta 1025, 215–224.
19 Van Winkle, L.J., Campione, A.L. and Farrington, B.H. (1990) Biochim. Biophys. Acta 1025, 225–233.
20 Woolf, C.M. (1968) Principles of Biometry, 1st Edn., D. Van Nostrand, London.
21 Kramer, C.Y. (1956) Biometrics 12, 307–310.
22 Van Winkle, L.J., Haghighat, N. and Campione, A.L. (1990) J. Exp. Zool. 253, 215–219.
23 Scriver, C.R. and Wilson, O.H. (1964) Nature 202, 92–93.
24 Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. and Torres, I. (1989) J. Reprod. Fert. 86, 679–688.
25 Spindle, A. (1990) In Vitro Cell. Dev. Biol. 25, 151–156.
26 Lawitts, J.A. and Biggers, J.D. (1991) Biol. Reprod. 45, 245–251.
27 Christensen, H.N. (1990) Physiol. Rev. 70, 43–77.
28 Nasr-Esfahani, M.H., Aitken, J.R. and Johnson, M.H. (1990) Development 109, 501–507.
29 Shapiro, B.M. (1991) Science 252, 533–536.
30 Latham, K.E., Garrels, J.I., Chang, C. and Solter, D. (1991) Development 112, 921–932.
31 Gardner, D.K., Clarke, R.N., Lechene, C.P. and Biggers, J.D. (1989) Gamete Res. 24, 427–438.