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Lethality of a tritiated amino acid in early mouse embryos

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SUMMARY

2- to 4-cell and morula- to blastocyst-stage mouse embryos were cultured for 1 h in tritiated leucine at two specific activities and their subsequent development followed *in vitro* and *in vivo* (after transfer to recipients), respectively. 2- to 4-cell embryos that incorporated an average of 42 d.p.m. per embryo were impaired in their ability to develop to the morula and blastocyst stage. Recipients receiving morulae and blastocysts that had incorporated an average of 384 d.p.m. per embryo failed to produce young. Reduction of the specific activity improved the viability of embryos both *in vitro* and *in vivo* but development was still less than that of unlabelled embryos. Protein degradation curves were different for both 2- to 4-cell and morula- to blastocyst-stage embryos labelled at the two different specific activities. Most studies using tritiated amino acids have employed higher specific activities than those used here and they may have to be reevaluated due to the possibility of radiation-induced artifacts.

INTRODUCTION

Radioactive amino acids have become an invaluable tool in the study of the developmental biology of preimplantation mammalian embryos. However, few data are available to determine whether physiological events detected through their use are real or an artifact of radiation damage to the embryos. Macqueen (1979) found that 2- to 4-cell mouse embryos incubated for 2 days in medium containing [^{35}S]methionine at a specific activity of 12 Ci mmol^{-1} did not survive to the 8-cell stage. Embryos cultured in [^{3}H]methionine at 12 Ci mmol^{-1} did survive to the 8-cell stage. Macqueen attributed the difference in lethality of the radioisotopes to the greater energy and path length of the electron emitted from the ^{35}S. She concluded that beta emitters such as ^{35}S or ^{14}C can be lethal wherever they are located in the cell, because the electrons they produce can reach the nucleus from any part of the cell. Weak emitters such as ^{3}H could be lethal only if located in the nucleus. This explains the lethality of [^{3}H]thymidine, which is incorporated into the nuclear DNA (Snow, 1973*a*,*b*).

Forty-four to 55 % of TCA-precipitable counts appear in nuclear proteins when embryos are cultured in [^{35}S]methionine for 5 h (Howe & Solter, 1979). Thus,

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tritiated amino acids could concentrate in the nucleus. Although most (>97 %) of the 2- to 4-cell embryos in Macqueen's experiments survived to the 8-cell stage after 2 days of culture in [^{3}H]methionine, this criterion for survival is not a stringent one. In culture, 2- to 4-cell mouse embryos will reach the morula or blastocyst stage after 2 days in culture (Wiebold, 1982). In Macqueen's culture system, unlabelled embryos had reached only the 8- to 16-cell stage after 2 days of incubation. This is especially important because evidence of radiation damage may be delayed for some time after exposure. Irradiation of 2- to 4-cell embryos with X-rays results in apparently normal development to the morula stage, but embryos then fragment as blastomeres die and blastocysts fail to form (Fisher & Smithberg, 1973; Alexandre, 1974, 1978; Kirkpatrick, 1974; Goldstein, Spindle & Pedersen, 1975). We undertook a study to critically examine the effect of tritiated leucine incorporation on mouse embryo viability both *in vitro* (for embryos labelled at the 2- to 4-cell stage and then remaining in culture) and *in vivo* (for embryos labelled at the morula and blastocyst stages and then transferred to recipients). We also examined whether use of differing specific activities of tritiated leucine affected protein degradation curves.

MATERIALS AND METHODS

Collection and labelling of embryos

Embryos were flushed from the oviducts and uteri of naturally mated Line C mice (Bradford, 1968) at 15.00–16.45 h on Day 2 of pregnancy (2- to 4-cell stage) or 10.00–11.45 h on Day 4 of pregnancy (morula and blastocyst stages). Day 1 of pregnancy was the day of the vaginal plug. Embryos were flushed with BMOC-2 (Brinster, 1965) but all subsequent rinses and cultures were in BMOC-3 (Brinster, 1971). Culture drops (50–100 μl) were layered with paraffin oil and incubated at 37 °C in a 5 % CO_2 in air atmosphere and 100 % humidity. Embryos were pooled, rinsed three times, and then transferred to drops of medium containing [4,5-^{3}H]leucine (ICN Pharmaceuticals, Inc.) at 17.00 h (2- to 4-cell embryos) or 12.00 h (morulae and blastocysts). Unlabelled leucine was previously added to the medium to produce two labelling media, hereafter designated as the high and low specific activity (s.a.) media: high s.a., 1·5–1·6 Ci mmol^{-1}, 31–33 μCi ml^{-1}, 0·02 mM-leucine; low s.a., 0·4 Ci mmol^{-1}, 12 μCi ml^{-1}, 0·03 mM-leucine. After 1 h of labelling, embryos were passed through three rinses of BMOC-3 to which 1 mM-unlabelled leucine had been added. A sample of 8 (high s.a., morulae and blastocysts), 16 (high s.a., 2- to 4-cell), or 24 (low s.a., morulae and blastocysts and 2- to 4-cell) embryos was removed, added to 200 μl water in a test tube, mixed, and frozen at −70 °C (0 h sample). Embryos in this sample were chosen to be representative of all the embryos in the particular drop as to stage of development (for example, 50 % morulae, 25 % early blastocysts, 25 % blastocysts).

Embryo development in vitro

After rinsing and removal of the 0 h sample, labelled 2- to 4-cell embryos were cultured in BMOC-3 + 1 mM-leucine for 2·5 days. Unlabelled embryos from the same collection were cultured in a separate drop of BMOC-3. Labelled and unlabelled embryos were examined twice daily and the percentage of embryos reaching various stages of development, based on morphological appearance, determined at each observation. The development of labelled and unlabelled embryos was compared in three high s.a. and four low s.a. experiments and differences tested for significance by Student's t-test.

Embryo development after transfer

After rinsing and removal of the 0 h sample, labelled morulae and blastocysts (n = 8) were surgically transferred to the left uterine horn of Day 3 pseudopregnant Line C females. Unlabelled embryos were transferred in the same manner to recipients matched for age with recipients of labelled embryos. The proportion of morulae and blastocysts transferred was roughly the same for labelled and unlabelled transfers. Each transfer of labelled embryos had at least one unlabelled transfer done on the same day, with embryos originating from the same pool. Five separate pools of embryos were used to test *in vivo* viability of both high s.a. and low s.a. embryos. Recipients were allowed to go to term and the number of young was noted soon after parturition. Differences in the proportion of recipients that became pregnant and the number of transferred embryos represented as young born were tested for significance by Chi-square analysis.

Protein degradation

Following the labelling period, rinsing, and removal of the 0 h sample, embryos were transferred to BMOC-3+1 mM-leucine and returned to the incubator. At regular intervals, the embryos were rinsed and representative samples removed for determination of radioactivity remaining in the protein. The number of embryos in each sample was the same as the 0 h sample and they were treated similarly. Samples were not taken at each of the intervals in every experiment and sometimes more than one sample was taken at a particular time, but a 0 h sample was always taken. 27 and 14 experiments were carried out to determine the protein degradation rate in high s.a. and low s.a. morulae and blastocysts, respectively. 8 and 10 experiments were conducted to determine the protein degradation rate in high s.a. and low s.a. 2- to 4-cell embryos. Least squares regression analysis of log % recovery of incorporated [^{3}H]leucine over time was carried out (0 h = 100 % recovery) and a mean half-life ($t_{1/2}$) of the labelled proteins was calculated. Confidence intervals were calculated to determine if half-life was significantly different for high s.a. and low s.a. embryos at the 2- to 4-cell stage or morula to blastocyst stages. Confidence intervals were also used to determine if differences in % recovery of incorporated [^{3}H]leucine at the various sampling times were significant.

Measurement of incorporated radioactivity

Samples were frozen and thawed four times to disrupt the cells. Acid-insoluble extraction was similar to that of Anderson & Foote (1975), except that 5 ml chloroform: methanol: ether (1:2:1, v/v/v) was used to rinse the fibre-glass filter before the final ethanol rinse. Filters were dried in a 60 °C oven and counted in a Packard Tri-Carb liquid scintillation spectrometer, using a premixed scintillation solution (Econofluor, New England Nuclear). Samples from the same experiment were extracted together. Blanks were run to verify that there was no carry over between samples. External and internal standards were used to determine the efficiency of counting and disintegrations per minute (d.p.m.) were determined after subtraction of blanks.

RESULTS

Embryo development in vitro

The development of labelled and unlabelled 2- to 4-cell embryos *in vitro* was examined over a 2·5-day incubation period. Fewer embryos labelled in the high s.a. medium had attained the morula stage by Day 4 A.M. (67 *versus* 78 %, $P < 0{\cdot}05$) or the blastocyst stage by Day 4 P.M. (7 *versus* 33 %, $P < 0{\cdot}05$) as compared with unlabelled embryos (Table 1). In contrast, embryos labelled in the low s.a. medium developed successfully to the morula stage (98 %), but were impaired in their ability to reach the blastocyst stage at Day 4 P.M. (39 *versus* 62 %, $P < 0{\cdot}05$) or Day 5 A.M. (65 *versus* 95 %, $P < 0{\cdot}05$) as compared to

Table 1. *Comparison of the development of 2- to 4-cell embryos after [^{3}H]leucine labelling at different specific activities*

Treatment	No. of embryos	d.p.m. per embryo*	Time of observation and criterion: Day 2 P.M. % > 2-cell	Day 3 A.M. % > 2-cell	Day 4 A.M. % ≥ morula	Day 4 P.M. % blastocysts	Day 5 A.M. % blastocysts
Unlabelled	42	0	10 ± 1	93 ± 1	78 ± 2[a]	33 ± 5[b]	52 ± 20
1·6 Ci mmol^{-1}, 33 Ci ml^{-1} [^{3}H]leucine	46	42 ± 5	13 ± 2	90 ± 5	67 ± 3[a]	7 ± 5[b]	10 ± 5
Unlabelled	42	0	7 ± 4	100 ± 0	95 ± 3	62 ± 5[c]	95 ± 3[d]
0·4 Ci mmol^{-1}, 12 Ci ml^{-1} [^{3}H]leucine	41	11 ± 2	24 ± 4	100 ± 0	98 ± 2	39 ± 4[c]	65 ± 3[d]

* TCA insoluble d.p.m./embryos based on three to four aliquots of embryos.
Values are mean ± S.E.M.
Mean with common letter superscripts are different ($P<0·05$).

unlabelled embryos. Embryos that had not reached the blastocyst stage by day 5 A.M. were usually degenerating. Although the unlabelled embryos were not exposed to the 1 mM-leucine chase added to the labelled embryo cultures, preliminary experiments had shown that embryo development in BMOC-3+1 mM-leucine is identical to that in BMOC-3 alone.

Embryo development in vivo

Embryos labelled with high s.a. or low s.a. medium at the morula and blastocyst stages appeared normal for at least 12 h after labelling. Embryos labelled in high s.a. medium were not viable, however, when transferred to suitable recipients and allowed to develop to term (Table 2). After the transfer of 56 high s.a. embryos to seven recipients, none of the females gave birth although 69 % of the unlabelled embryos (55/80) resulted in full-term young and all recipients receiving unlabelled embryos were pregnant (10/10). Some embryos labelled in low s.a. medium were viable. Embryo survival to birth was not different in those females that were pregnant (29 %, labelled *versus* 52 %, unlabelled, $P > 0{\cdot}10$) but was different when expressed as embryo survival for all recipients (9 % labelled *versus* 35 % unlabelled, $P < 0{\cdot}01$). The seven liveborn young that developed from the low s.a. labelled embryos were normal in appearance at birth and at maturity.

Protein degradation

Protein degradation curves (Fig. 1) revealed that there were at least two major classes of embryonic proteins labelled at the 2- to 4-cell stage. The majority of the label was in long-lived proteins while a smaller portion was in short-lived proteins (average half-life <1 h). Because the percentage recovery of incorporated [^{3}H]leucine was not different ($P > 0{\cdot}10$) at 1 h for 2- to 4-cell embryos labelled in high s.a. (61·1 % retention) or low s.a. (80·9 % retention) media (Table 3), it

Table 2. *Viability of radioactively-labelled morulae and blastocysts after transfer to pseudopregnant recipients*

Treatment	d.p.m. per embryo*	Pregnancy rate	(%)	Young born/embryos transferred (%)			
				All recipients		Pregnant recipients	
Unlabelled	0	10/10	(100)[a]	55/80	(69)[b]	55/80	(69)
1·5 Ci mmol^{-1}, 3L Ci ml^{-1} [^{3}H]leucine	384 ± 29	0/7	(0)[a]	0/56	(0)[b]	—	
Unlabelled	0	6/9	(67)	25/72	(35)[c]	25/48	(52)
0·4 Ci mmol^{-1}, 12 Ci ml^{-1} [^{3}H]leucine	64 ± 10	3/10	(30)	7/80	(9)[c]	7/24	(29)

* TCA-insoluble d.p.m./embryo based on five aliquots of embryos (mean ± S.E.M.).
Ratios with common superscripts are different ($P < 0{\cdot}01$).

appears the mean half-life of the short-lived proteins is unaffected by the specific activity of the labelling medium. The mean half-life of the long-lived proteins was calculated by linear regression from 1 to 24 h. Long-lived embryonic proteins had a mean half-life of 63·1 h when labelled in high s.a. medium but mean half-life of 63·1 h when labelled in high s.a. medium but mean half-life decreased to 20·4 h ($P < 0{\cdot}01$) when 2- to 4-cell embryos were labelled in low s.a. medium.

Protein degradation curves for proteins labelled at the morula and blastocyst stages (Fig. 2) were analysed assuming a single major class of labelled proteins. Regression analysis revealed a mean half-life of 8·1 h for embryos labelled in high s.a. medium and 12·2 h for embryos labelled in low s.a. medium (Table 4). Although mean half-life was not different ($P > 0{\cdot}10$) after labelling in high s.a. or low s.a. medium, the protein degradation curves appeared to be diverging. At 12 h, the percentage recovery of incorporated [^{3}H]leucine was different ($P < 0{\cdot}02$) for high s.a. (35·9) and low s.a. (50·7) embryos.

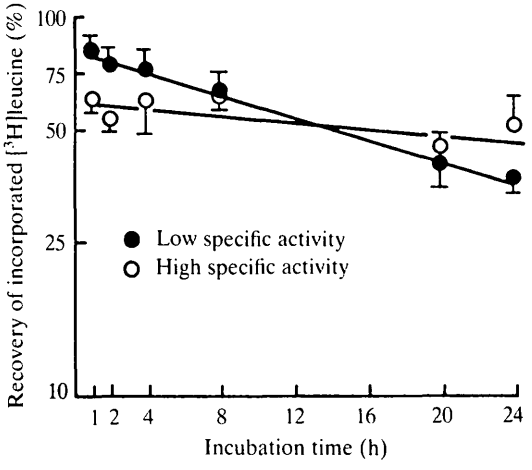

Fig. 1. Protein degradation in 2- to 4-cell embryos *in vitro* after radioactive labelling at low and high specific activity.

Table 3. *Protein degradation in 2- to 4-cell embryos after labelling at different specific activities*

	% Recovery (mean ± S.E.M.) of incorporated [^{3}H]leucine* after:						
Treatment	1 h	2 h	4 h	8 h	20 h	24 h	$t_{1/2}$
High s.a.	61·1 ± 5·6 (n = 5)	53·6 ± 3·8 (n = 5)	59·7 ± 12·2 (n = 5)	60·8 ± 5·1 (n = 5)	45·1 ± 2·3 (n = 5)	51·0 ± 10·4 (n = 5)	63·1 h[a]
Low s.a.	80·9 ± 8·8 (n = 6)	74·8 ± 8·2 (n = 5)	71·9 ± 10·3 (n = 5)	63·3 ± 7·3 (n = 5)	40·3 ± 5·4 (n = 5)	37·4 ± 3·6 (n = 5)	20·4 h[a]

* d.p.m. of embryos at given time/d.p.m. of 0 h embryos.
$t_{1/2}$ determined by least-squares regression analysis of log % recovery *versus* time, 1 to 24 h.
Means with common superscripts are different ($P < 0{\cdot}01$).

DISCUSSION

Labelling of embryonic proteins with tritiated leucine at a specific activity of 1·5–1·6 Ci mmol^{-1} inevitably resulted in the death of most or all of the embryos. This was true for both embryos labelled at the 2- to 4-cell stage and embryos labelled at the morula to blastocyst stage. At the earlier stage, radiation damage was expressed as a decreased ability to reach the morula and blastocyst stages. At the later developmental stage, radiation damage resulted in a loss of viability after transfer to recipient females. These results agree with those of previous researchers documenting the effects of acute X-irradiation on embryo development (Fisher & Smithberg, 1973; Alexandre, 1974, 1978; Kirkpatrick, 1974; Goldstein *et al.* 1975). In the case of [^{3}H]leucine labelling, however, a large part of the label is probably retained in the nucleus (Howe & Solter, 1979) in long-lived proteins and is thus having a chronic rather than an acute effect on the cellular DNA. The tritiated leucine incorporated into cytoplasmic proteins would be

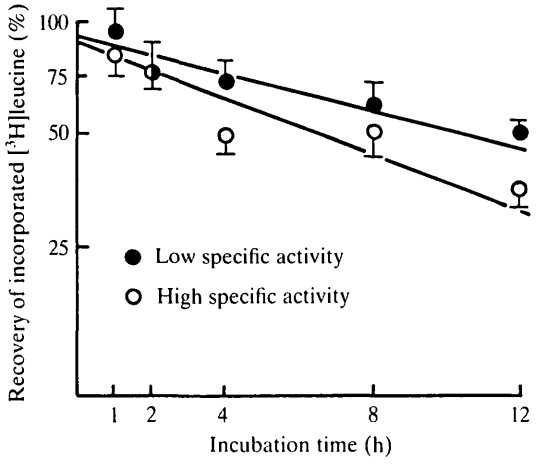

Fig. 2. Protein degradation in morulae to blastocysts *in vitro* after radioactive labelling at low and high specific activity.

Table 4. *Protein degradation in morulae and blastocysts after labelling at different specific activities*

	% Recovery (mean ± S.E.M.) of incorporated [^{3}H]leucine* after:					
Treatment	1 h	2 h	4 h	8 h	12 h	$t_{1/2}$
High s.a.	82·5 ± 10·1 (n = 14)	74·5 ± 6·3 (n = 15)	49·8 ± 5·4 (n = 15)	52·0 ± 7·4 (n = 14)	35·9 ± 3·6^a (n = 15)	8·1 h
Low s.a.	93·9 ± 14·0 (n = 7)	73·0 ± 14·0 (n = 6)	69·5 ± 10·0 (n = 7)	60·5 ± 8·9 (n = 6)	50·7 ± 3·7^a (n = 6)	12·2 h

*d.p.m. of embryos at given time/d.p.m. of 0 h embryos.
$t_{1/2}$ determined by least-squares regression analysis of log % recovery *versus* time, 0 to 12 h.
Means with common superscripts are different ($P < 0·02$).

expected to have little or no effect on cell viability due to the short path length of the emitted electrons (Macqueen, 1979). It is unlikely that radiolytic degradation products or contaminants are responsible for the effects seen since different stocks of various ages were used in the experiments.

Reducing the specific activity of the labelling medium to 0·4 Ci mmol^{-1} resulted in a reduction of the lethal effects. Embryos labelled at the 2- to 4-cell stage in such media were capable of development to the morula stage, but showed impaired ability to form blastocysts. Morulae and blastocysts labelled in this low s.a. medium were, in some cases, capable of developing to normal, full-term foetuses. Since morulae have lower protein synthetic rates than blastocysts (Epstein & Smith, 1973), and thus would incorporate less [^{3}H]leucine, it is possible that the full-term foetuses developed from the transferred morulae. In that case, the d.p.m. per embryo compatible with viability may be lower than the value given (64 d.p.m.) in Table 2, which is the average of a mixture of morulae and blastocysts. In a similar manner, those labelled 2- to 4-cell embryos that developed to the blastocyst stage may have been at a particular stage of the cell cycle in which nuclear protein synthesis was minimal.

Tritiated amino acids are generally used for labelling embryonic proteins at much higher specific activities than those reported here (80 Ci mmol^{-1} [^{3}H]methionine, Brinster, Brunner, Joseph & Levey, 1979; Merz, Brinster, Brunner & Chen, 1981; 5–20 Ci mmol^{-1} [^{3}H]leucine, Epstein & Smith, 1973; Brinster, Wiebold & Brunner, 1976). The question that arises is whether the use of radioisotopes at these levels affects the results obtained, due to artifacts imposed by radiation damage. The answer depends, in part, on the specific use of the label and the experimental design. When embryos are radioactively labelled with tritiated amino acids and then maintained (*in vivo* or *in vitro*) for an extended length of time, deleterious effects are more likely to be seen and may compromise the results. This is especially true of protein degradation measurements where embryo sampling up to 24 h postlabelling is not unusual. In our experiments, calculated half-lives and/or protein degradation curves were quite different after labelling in high and low specific activity medium, even though embryos appeared morphologically normal over the time of sampling. Although the effect of decreased specific activity was not consistent between the two embryonic stages (decreased mean half-life of labelled embryonic proteins in 2- to 4-cell embryos, increased mean half-life in morulae and blastocysts), protein degradation systems (lysosomal, cytoplasmic) may vary at different stages of development and could be affected differently by radiation damage.

When embryos are labelled with tritiated amino acids for a short period of time and then immediately processed (for electrophoresis, for example), radiation damage may be minimal and have no effect on the results obtained. In light of the effects seen with low specific activities and short-term labelling in these experiments, however, it may be advisable to reevaluate previous studies that used much higher specific activity tritiated amino acids.

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