In vitro studies of mouse embryos bearing mutations in the T complex: effects of culture in suboptimal medium upon \( t^6/t^6 \) and normal embryos

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SUMMARY

We have compared the behaviour of normal and \( t^6/t^6 \) embryos in PCMF, a suboptimal 'delay' medium which arrests normal development. Morphologically, the two types of embryos are indistinguishable in this medium. Although \( \beta \)-glucuronidase activity in embryos does not rise as quickly in delay medium as in cNCTC, a nutritive medium, the rate of increase is similar in \( t^6/t^6 \) and normal embryos. We conclude from these observations that the lethality of \( t^6/t^6 \) embryos is not a consequence of their reaching a given absolute age. Together with previous studies, our data suggest that embryo lethality correlates more closely with metabolic state than with morphological stage.

Blastocysts maintained in PCMF are unable to give rise to trophoblast outgrowths but do so upon transfer into cNCTC medium. When a mixture of fourth-day \( t^6/t^6 \) and normal embryos are transferred to cNCTC medium after lengthy pre-incubation periods in PCMF, trophoblast outgrowth is initiated from both types of embryos at approximately the same time. However, when embryos are removed from the genital tract on the second or third day of gestation, \( t^6/t^6 \) embryos are slower to produce trophoblast outgrowths than are normal embryos upon transfer from PCMF to cNCTC medium. Although the reason for this differential behaviour is not yet clear, it is hypothesized that some product(s) required for the outgrowth process is (are) more unstable in third-day \( t^6/t^6 \) embryos than in normal third-day embryos or fourth-day \( t^6/t^6 \) embryos.

Our ability to separate \( t^6/t^6 \) from normal embryos by their delayed initiation of trophoblast outgrowth provides us with a convenient way to identify, and to isolate for analysis, enriched populations of homozygous mutant embryos prior to the time at which they show gross morphological abnormalities.

INTRODUCTION

The \( t^6 \) mutation is one of a series of recessive lethal mutations of the T complex in mice (see Sherman & Wudl, 1977). \( t^6/t^6 \) embryos in utero show overt morphological abnormalities between the short egg-cylinder and the elongated...
egg-cylinder stages (sixth to seventh day of gestation). Characteristically $t^6/t^6$ embryos are recognized at this time by aberrantly arranged ectodermal and endodermal cells which contain excessive cytoplasmic lipid and mitochondria with crystalline inclusions (Nadijcka & Hillman, 1975). Because this $t$ mutation and others act during embryogenesis, they have been classified by some as developmental mutations (Gluecksohn-Waelsch & Erickson, 1970; Bennett, 1975).

It appears from recent studies that during early mammalian embryogenesis there are different thresholds for the expression of 'development-related' gene products; that is, by manipulating either the embryos or the formulation of their culture media, it is observed that some developmental markers are expressed whereas others are suppressed (see, for example, Johnson, Handyside & Braude, 1977; Braude, 1979; Sellens & Sherman, 1980; Schindler & Sherman, 1981). It is also evident that the appearance of some indicators of embryonic development is actually time-dependent rather than stage-dependent; these markers are detectable when the embryo reaches the appropriate absolute age regardless of the extent of overt development (Sellens & Sherman, 1980; Johnson, Pratt & Handyside, 1981; Van Blerkom, 1981). In view of this new information, it becomes pertinent in attempting to understand the nature of embryolethal mutations such as $t^6$ to determine whether lethality is a consequence of developmental stage or absolute age.

Preliminary reports have been published which bear upon the question of whether the lethality of $t^6/t^6$ embryos is age-dependent or stage-dependent (DiZio & Hillman, 1978; Sherman et al., 1981). In these experiments, $t^6/t^6$ embryos, along with their normal counterparts, were blocked in their development at a stage morphologically resembling the blastocyst either by ovariectomy of the mother or by placement under suboptimal culture conditions (Sellens & Sherman, 1980). Under these conditions, $t^6/t^6$ embryos seemed to remain viable and morphologically indistinguishable from their $+/+$ and $+/t^6$ counterparts despite reaching the absolute age at which lethality would normally occur (DiZio & Hillman, 1978; Sherman et al. 1981). These same studies suggested that after the embryos were released from delay conditions, putative $t^6/t^6$ embryos in some cases underwent implantation-related events more slowly than $+/+$ and $+/t^6$ embryos (DiZio & Hillman, 1978; Sherman et al. 1981). Accordingly, in this article we have examined more extensively the behaviour of $t^6$-mutant embryos during, and following release from, developmental delay.

**MATERIALS AND METHODS**

*Collection of embryos*

Embryos in the experimental cross were obtained by mating $+/t^6$ mice *inter se* (for information concerning the generation of $+/t^6$ mice, see Wudl, Sherman & Hillman, 1977 and Wudl & Sherman, 1978). Because of trans-
mission frequency distortion, the expected genotypes of experimental cross embryos were as follows: $t^6/t^6$, 40%; $+/t^6$, 50%; $+/+$, 10% (Wudl et al. 1977). Similarly, the distribution of control cross ($+/+\times +/t^6$) embryos was 80% $+/t^6$ and 20% $+/+$. Embryos were collected on the second (2-cell), third (4- to 8-cell) and fourth (late morulae to mid blastocyst) day of pregnancy from superovulated females (Runner & Palm, 1953). Oviducts and/or uteri were flushed with preimplantation culture medium (PCM; Goldstein, Spindle & Pedersen, 1975) on the second and third days or phosphate-buffered saline (solution A of Dulbecco and Vogt) on the fourth day. The day of observation of the sperm plug is considered the first day of pregnancy.

**Culture of embryos**

Embryos were cultured either in PCMF or cNCTC medium. PCMF, a medium which does not permit trophoblast outgrowth and maintains embryos in a state resembling delay *in vivo*, consists of PCM (made with dialyzed bovine serum albumin) supplemented with dialyzed fetuin at a final concentration in the medium of 0-05% (Sellens & Sherman, 1980). PCMF is also referred to in the text as ‘delay medium’. In some experiments, second- or third-day embryos were cultured through preimplantation stages in PCM instead of PCMF. cNCTC, a medium supporting trophoblast outgrowth, consists of NCTC-109 medium (Microbiological Associates, Bethesda, Md.) supplemented with 10% heat-inactivated fetal calf serum and antibiotics (Sherman, 1976). Embryos were cultured in microtitre dishes (Microtest I, Type 3034, Falcon, Oxnard, Calif.). Periods of culture in the various media are indicated in the text.

**Scoring for trophoblast outgrowth and statistical analyses**

Embryos in cNCTC medium were scored for trophoblast outgrowth at least twice daily by inspection with an inverted microscope equipped with phase-contrast optics. Outgrowth was considered to have occurred when at least one cell with its nucleus could be seen flattened on the substratum. Four days after transfer to cNCTC medium, homozygous mutant ($t^6/t^6$) embryo outgrowths were distinguished from those of normal phenotype ($+/t^6$ and $+/+$) by a paucity of ICM cells and by possession of trophoblast cells with smaller nuclei, less prominent nucleoli and birefringent cytoplasmic inclusions (Fig. 1).

For statistical analyses, embryos were assigned ‘outgrowth times’. Since we could not establish the exact time at which embryo outgrowths began, the outgrowth time was considered arbitrarily to be the midpoint of the interval between the time at which outgrowth was first observed and the preceding scoring time (for example, an outgrowth time of 40 h was assigned to a blastocyst that had failed to outgrow by 30 h but had outgrown by 50 h). Comparisons were made between the mean outgrowth times for normal v. presumptive homozygous mutant embryos in a given population by a two-sample *t* test. Mean outgrowth times from experiments involving the same culture regimens were then averaged.
Fig. 1. Comparison of morphologies of normal and t<sup>6</sup>/t<sup>6</sup> embryos. Embryos were removed from uteri on the third day of gestation and cultured in PCMF for six days prior to transfer to cNCTC medium. (A) Morphology of an embryo (later phenotyped as t<sup>6</sup>/t<sup>6</sup>) approximately 48 h after transfer to cNCTC medium. (B) Morphology of a wild-type embryo after the same period of culture. (C) Morphology of a t<sup>6</sup>/t<sup>6</sup> embryo approximately 96 h after transfer to cNCTC medium. (D) Morphology of a wild-type embryo after the same period of culture. Note that the t<sup>6</sup>/t<sup>6</sup> outgrowth in (C) has lost its inner cell mass; trophoblast nuclei are substantially smaller than those in (D) and nucleoli are less prominent. Also, several cells in the mutant embryo outgrowth contain highly birefringent cytoplasmic droplets which are distinguishable by phase microscopy from the vacuoles seen in normal trophoblast cells shortly after the onset of outgrowth (B). All figures were photographed at the same magnification (scale marker in D = 50 μm). Hoffmann modulation-contrast optics were used in (A) and phase-contrast optics were used in the other figures.

and denoted T<sub>3</sub> in Table 1. The differences in T<sub>3</sub> values are denoted ΔT<sub>3</sub> in the same table. The significance of the ΔT<sub>3</sub> values, when these were averaged from replicate experiments, was assessed by the use of a χ<sup>2</sup> method for combining probability estimates [Σ − 2 ln P (Fisher, 1946)].

Embryos failing to outgrow (approximately 5% of all cultured fourth-day embryos, 10% of all cultured third-day embryos and 25% of all cultured second-day embryos) were excluded from analysis since their phenotypes could not be determined.
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Table 1. Time required for trophoblast outgrowth following culture of embryos at different stages and in different media

<table>
<thead>
<tr>
<th>Time of initiation of culture (day)</th>
<th>Days in delay medium</th>
<th>Number of normal $t^6/t^6$ embryos</th>
<th>Number of $t^6/t^6$ embryos</th>
<th>$T_4$ for outgrowth (h)</th>
<th>Normal embryos</th>
<th>$t^6/t^6$ embryos</th>
<th>$\Delta T_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>116</td>
<td>82</td>
<td>39.9</td>
<td>41.0</td>
<td>+1.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1†</td>
<td>18</td>
<td>14</td>
<td>46.9</td>
<td>46.7</td>
<td>-0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>88</td>
<td>61</td>
<td>30.7</td>
<td>34.9</td>
<td>+4.2**</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>75</td>
<td>53</td>
<td>24.9</td>
<td>29.1</td>
<td>+4.2*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>123</td>
<td>61</td>
<td>26.4</td>
<td>38.2</td>
<td>+11.8**</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>122</td>
<td>82</td>
<td>41.7</td>
<td>70.5</td>
<td>+28.8**</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3†</td>
<td>73</td>
<td>52</td>
<td>26.2</td>
<td>31.3</td>
<td>+5.1**</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>111</td>
<td>93</td>
<td>29.6</td>
<td>36.1</td>
<td>+6.5**</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>929</td>
<td>623</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>60</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number of embryos and $T_4$ values given in these experiments refer to those which were capable of producing trophoblast outgrowths, since the phenotypes of those failing to outgrow could not be determined. The approximate percentages of second-, third- and fourth-day embryos giving rise to trophoblast outgrowths were 75, 90 and 95%, respectively. The $T_4$ values represent mean outgrowth times from the time of transfer to cNCTC medium. $\Delta T_4$ is the difference between the $T_4$ for normal embryos and the $T_4$ for $t^6/t^6$ embryos. Where indicated by asterisks, the outgrowth initiation times for trophoblast cells from $t^6/t^6$ embryos were significantly slower than those from normal embryos at the 0.05 (*) or 0.01 (**) levels (see Materials and Methods for a description of statistical analyses). $\Delta T_4$ values without asterisks were not significantly different at the 0.05 level.

† In these experiments, embryos were cultured in PCM instead of PCMF.

$\beta$-Glucuronidase analyses

Embryos were assayed for $\beta$-glucuronidase activity by measuring the release of 4-methylumbelliferone from 4-methylumbelliferyl-$\beta$-D-glucuronic acid by the microfluorometric method described by Wudl & Sherman (1976).

RESULTS

Trophoblast outgrowth analyses with normal and $t^6/t^6$ embryos

When fourth-day blastocysts from the experimental ($+/t^6 \times +/t^6$) cross are placed in cNCTC medium, the $T_4$ for the outgrowth is approximately 40 h and no significant difference is observed in the outgrowth time of embryos which are subsequently phenotyped as either mutant ($t^6/t^6$) or normal ($+/t^6$ or $+/+$) (Table 1). If blastocysts are placed in PCMF for three to six days, they may attach transiently to the culture dish, but outgrowth of trophoblast cells does not occur. Outgrowth does occur, however, if these blastocysts are subsequently
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Fig. 2. Typical trophoblast outgrowth curves for normal and presumptive $t^6/t^6$ embryos. (A) A total of 122 blastocysts were removed from $+/t^6$ females on the fourth day after mating with $+/t^6$ males. The embryos were maintained in PCMF for three days. The embryos were then transferred to cNCTC medium and scored for the time of trophoblast outgrowth. After four days in cNCTC medium, the embryos were categorized as (O) normals ($+/+$ or $+/t^6$) or (●) homozygous mutants ($t^6/t^6$). (B) A similar experiment was carried out with 51 8-cell embryos removed from uteri on the third day of pregnancy and cultured in PCMF for four days prior to transfer to cNCTC medium. Symbols are as in (A).

transferred from delay medium to cNCTC medium (Table 1). The $T^*$ for outgrowth of trophoblast cells in cNCTC medium is shorter for embryos previously maintained in delay medium for three days than for six days, although in both cases the $T^*$ values are lower than those of embryos placed directly into cNCTC medium. Once again, there are no significant differences in the time required for trophoblast outgrowth from normal v. $t^6/t^6$ embryos (Fig. 2; Table 1).

Third-day embryos do not develop well when placed directly into cNCTC medium (unpublished observations). If they are placed in PCM (or PCMF) for 24 h, they reach the late morula or early blastocyst stage, after which they can develop satisfactorily upon transfer to cNCTC medium. The $T^*$ values for trophoblast outgrowth from such normal and $t^6/t^6$ embryos are similar and in each case greater than those of fourth-day embryos (Table 1). If embryos are maintained in delay medium for two days, that is until they reach the late blastocyst stage, the $T^*$ values for trophoblast outgrowth are shorter than those of fourth-day blastocysts placed directly into cNCTC medium (Table 1). There is a progressively increasing difference in the time required for trophoblast outgrowth between normal and $t^6/t^6$ embryos with longer periods of culture in
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PCMF prior to transfer to cNCTC (Table 1 and Fig. 2). Some embryos (presumably t6/t6) fail to outgrow after more than 180 h in cNCTC following a 6-day pre-incubation period in PCMF: during incubation in cNCTC they retain the morphology of expanded blastocysts (Fig. 1).

When embryos removed from oviducts at the 2-cell stage are cultured for three days in PCM or PCMF, they reach the late blastocyst stage. Upon transfer to cNCTC medium, normal embryos give rise to trophoblast outgrowths in less than 30 h. Trophoblast cells from homozygous t6-mutant embryos are significantly slower to outgrow than those from normal embryos. A similar result is observed when 2-cell embryos are maintained in delay medium for six days prior to transfer to cNCTC.

Table 1 illustrates that of the total of 1552 embryos subjected to analysis in these experiments, 40% were phenotyped as t6/t6 embryos, consistent with the proportion expected from transmission frequency studies with our stocks of t6-bearing animals (Wudl et al. 1977).

β-Glucuronidase analyses of normal and t6/t6 embryos

We have previously measured β-glucuronidase activities of individual normal and t6-mutant embryos cultured in cNCTC medium (Wudl & Sherman, 1978). Since we could not distinguish morphologically between normal and t6/t6 embryos at early stages, we reasoned that so long as t6-mutant embryos were keeping pace with normals, the ratio of enzyme activities in the upper 60% group (proportion of normals) to the low 40% group (proportion of mutants) would remain approximately constant. The data obtained (Wudl & Sherman, 1978), replotted in Fig. 3, indicate that β-glucuronidase activity in the low 40% group of embryos fails to rise as rapidly as the upper 60% group beyond the first day of culture. The upper 60%:low 40% enzyme activity ratio rises steadily from 2.0 after one day of culture to 8.6 after five days (Wudl & Sherman, 1978). Figure 3 also illustrates that when experimental cross embryos are maintained in PCMF, the average values of β-glucuronidase activity after 3–5 days of culture for the entire population are very close to those observed for the low 40% of embryos cultured in cNCTC medium. Furthermore, the β-glucuronidase levels reached by embryos after five days of culture in PCMF are the same whether the embryos are obtained from experimental or control crosses (Fig. 3), despite the fact that the former population contains 40% t6-homozygous mutant embryos whereas the latter population contains none.

In Table 2, average β-glucuronidase levels are given for embryos from the experimental cross cultured in PCMF from the third or the fourth days of gestation. Enzyme levels of cultured third-day embryos, even after four days, fail to reach those of fourth-day embryos after only one day in vitro. When enzyme activities are separated into the high 60% and low 40% categories and averaged, the ratios between the values obtained, unlike those for experimental cross embryos in cNCTC medium (Wudl & Sherman, 1978), remain similar
DISCUSSION

In previous studies with $t^{12}$-, $t^6$- and $t^{w5}$-mutant embryos, we have demonstrated that the embryos degenerate \textit{in vitro} at times which are in reasonable agreement with the embryolethal periods \textit{in utero} (Wudl & Sherman, 1976, 1978; Wudl \textit{et al.} 1977; Sherman & Wudl, 1977). In the present investigation,
In vitro studies on $t^6/t^6$ mouse embryos

Table 2. $\beta$-Glucuronidase activities of embryos placed into culture on the third or fourth gestation days

<table>
<thead>
<tr>
<th>Time of initiation of culture (day)</th>
<th>Days of culture in PCMF</th>
<th>Number of embryos</th>
<th>Average $\beta$-glucuronidase activities</th>
<th>High 60%</th>
<th>Low 40%</th>
<th>High 60% Low 40%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>37</td>
<td>0.17</td>
<td>0.20</td>
<td>0.13</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>78</td>
<td>2.5</td>
<td>2.8</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>78</td>
<td>5.7</td>
<td>6.8</td>
<td>3.8</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>16</td>
<td>1.2</td>
<td>1.4</td>
<td>0.7</td>
<td>2.0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>40</td>
<td>6.8</td>
<td>8.5</td>
<td>4.2</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>92</td>
<td>10.6</td>
<td>12.8</td>
<td>7.6</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>56</td>
<td>13.6</td>
<td>16.5</td>
<td>9.1</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>31</td>
<td>18.2</td>
<td>20.9</td>
<td>14.1</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>59</td>
<td>22.3</td>
<td>27.0</td>
<td>15.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Embryos were removed from the genital tract on either the third or fourth day of pregnancy. They were either assayed for $\beta$-glucuronidase activity directly or cultured prior to assay as indicated. $\beta$-Glucuronidase activities are expressed in terms of pmoles product/h/embryo.

we have maintained embryos in delay medium for prolonged intervals such that after release from delay, the absolute age of the embryos is greater than that at which $t^6/t^6$ embryos would normally die. Based upon the proportion of embryo outgrowths typed as $t^6/t^6$ in these studies (Table 1), we can state that virtually all of the $t^6$-mutant embryos have remained viable through, and beyond, the delay period. We conclude, therefore, that the lethal period for $t^6/t^6$ embryos in culture, as in utero (DiZio & Hillman, 1978; Nadijcka, Morris & Hillman, 1981), is not predicated upon the absolute age of the embryos.

Is it then valid to conclude that the embryolethal period for $t$ mutations is stage-specific? Traditionally, the stage of an embryo is based upon morphologic observation. We do not believe that the death of homozygous $t$-mutant embryos is related to morphologic stage. This is because many embryos fail to recapitulate morphologic development in cNCTC medium beyond the early egg-cylinder stage; whereas cultures of wild-type embryos generally remain viable despite this limitation, presumptive $t^6/t^6$ and $t^{w5}/t^{w5}$ embryos, as mentioned, degenerate in vitro near the times expected from their behaviour in utero (Wudl & Sherman, 1976, 1978; Wudl et al. 1977).

As embryogenesis proceeds, cells undergo shifts in their metabolic patterns (as reflected by alterations in metabolic requirements; see Sellens & Sherman, 1980) and, in the case of ICM derivatives, divide at increasingly rapid rates (see Snow, 1976). We have argued previously that cells from $t^6/t^6$ embryos die because they possess a metabolic lesion and cannot, therefore, survive as metabolic demands increase to some particular level (Wudl & Sherman, 1978). In other words, the lethal period of $t^6/t^6$ embryos would be related to a metabolic
state as opposed to a morphologic stage. Our present findings reinforce this view. The data in Fig. 2 and Table 2 demonstrate that at least with respect to levels of β-glucuronidase activity, we can cause normal embryos to resemble \(t^6/t^6\) embryos by having their metabolism restricted through culture in suboptimal medium. Furthermore, the observation that \(t^6/t^6\) embryos do not possess more β-glucuronidase activity in cNCTC medium than in PCMF medium (Fig. 2) suggests that they are unable to respond in a positive manner to conditions supportive of an advanced metabolic state. In fact, these nutritive culture conditions lead to the marked negative response of \(t^6/t^6\) embryos (that is, death) which can be avoided for relatively long periods by culture in delay medium, which presumably protects the embryos by suppressing their progression to a more advanced metabolic state.

The data in Table 1 are complex, and an earlier explanation based on preliminary results with \(t^6/t^6\) embryos released from delay (Sherman et al. 1981) is likely to have been too simplistic. The present study and other recent ones (Sellens & Sherman, 1980; Sherman & Matthaei, 1980; Sherman et al. 1981; Schindler & Sherman, 1981) indicate that events leading to trophoblast outgrowth occur in at least two synthetic phases. Although we do not know which gene products are responsible for trophoblast outgrowth, the aforementioned studies allow us to make the following statements about the two phases: (a) the early phase occurs at or before the expanded blastocyst stage, whereas the later phase takes place within the period 20–40 h before outgrowth; (b) early phase events can take place in nutrient or delay medium whereas the occurrence of the later phase, which appears to involve protein synthesis, requires nutrient medium; and (c) the later phase cannot take place until early phase events have been completed. These conclusions explain why normal embryos pre-incubated in PCMF require less time in cNCTC for outgrowth than do embryos placed directly into cNCTC medium (Table 1). On the other hand, the observation that the \(T_4\) for normal embryos increases when pre-incubation in PCMF exceeds three days (Table 1) suggests that early phase products are eventually lost in suboptimal medium and must be replenished upon transfer to cNCTC medium prior to the onset of the later phase. If the latter proposal is correct, the longer periods required for outgrowth of \(t^6/t^6\) embryos relative to normal embryos following culture in PCMF from the third day (Table 1) can be attributed to more rapid destabilization of early phase products and/or a protracted period required for resynthesis of these products following transfer to cNCTC medium.

Why, then, is there no significant difference between the trophoblast outgrowth times when fourth-day normal and mutant embryos are cultured in PCMF for as long as six days prior to transfer to cNCTC (Table 1)? We can offer no ready explanation for this observation. However, from a comparison of β-glucuronidase activities between embryos placed into culture on the third or the fourth day of gestation (Table 2), it is clear that the latter embryos always
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contain substantially higher activities than the former during culture in PCMF. It is possible, therefore, that \( t^6/t^6 \) embryos placed in delay medium from the fourth day possess other metabolic differences which render them more capable of stabilizing early phase products than \( t^6/t^6 \) embryos cultured in PCMF from the third day. Finally, it should be stated that in preliminary results we have observed that embryos homozygous for other \( t^6 \)-complementation group mutations (\( t^{db} \)) show delayed trophoblast outgrowth times relative to normal embryos when culture is begun on the third or the fourth day of gestation (Sherman & Pai, unpublished observations). We are currently investigating possible reasons for this difference.

Our data suggest a convenient method for obtaining populations enriched for, or depleted of, \( t^6/t^6 \) embryos. For example, after maintaining third-day embryos in delay medium for four days (Fig. 2), it should be possible, upon transfer to cNCTC medium, to obtain populations containing a large proportion of normal embryos (those outgrowing at early times) or of \( t^6/t^6 \) embryos (those failing to outgrow after half or more of the embryos have already done so). We must stress that, for reasons described above as well as on the basis of ultrastructural studies (Nadigcka & Hillman, 1975), the homozygous mutants so obtained would not be expected to be completely normal. Nevertheless, they would be likely to contain a more restricted array of biochemical defects than they would when selected at later morphological stages on the basis of overt abnormalities; this might, therefore, provide us with a better opportunity than in the past to distinguish between the primary \( t^6 \) lesion and secondary effects in molecular terms.

REFERENCES


*(Received 27 November 1980)*