Discussion. From these results the protective action of selenium against nutritional encephalomalacia was shown to be small but significant when the dietary lipid stresses were borderline. The failure of other investigators(8-10) to observe protection may have been due to their use of larger overwhelming levels of the stressor lipid, thereby masking whatever possible protection the added selenium might afford. In the present study, feeding an 8% corn oil diet also appeared to overwhelm the protective effect of selenium. Higher levels of selenium in the diet, up to 1.56 ppm, did not overcome the effect of feeding more corn oil.

In our studies, the omission of supplementary methionine from the diet did not prevent the effect of selenium in lowering the incidence of encephalomalacia. Possibly the present diet, which contained 18% casein and 10% gelatin, furnished enough methionine, although borderline, so that sufficient selenomethionine could be formed *in vivo* when selenium was added to the diet. The role of selenomethionine(18,19) as an *in vivo* antioxidant is not established.

Summary. Addition of 0.13 ppm of selenium as sodium selenite to a semisynthetic diet containing 4% corn oil freed of tocopherol reduced the incidence of encephalomalacia. On the other hand, when 8% corn oil was fed, selenium in levels up to 1.56 ppm had no effect on the proportion of chicks with encephalomalacia. Growth was not affected by addition of selenium.

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Penetration of the Placental Barrier by the Lactate Dehydrogenase-Elevating Virus (Riley) and its Behavior in Mouse Embryo Cultures Following Infection *in utero.* * (29571)

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There are contradictory findings on the behavior of Riley's lactate dehydrogenase elevating agent(1) (LDH agent) in tissue cultures. Although some authors have reported successful *in vitro* propagation(2,3,12), others have found the LDH agent to disappear from tissue cultures after a few days(4,5). In attempts to adapt the LDH agent to host cells

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we have infected murine embryo cells *via* the placenta by inoculation of pregnant mice. This report deals with the results of transmitting the LDH agent *in utero,* and its effect on tissue cultures prepared from such infected cells.

Materials and methods. Virus. The LDH-agent used was obtained from mice bearing Sarcoma I(6) maintained in our institute during the last 8 years by standard transplant passages. An agent pool was prepared as previously described(7) with an ID 50 titer of $1 \times 10^4/0.5$ ml. Virus titers were determined on the 4th day after infection of normal mice.

Virus inoculation. Pregnant NMRI mice were inoculated with 0.5 ml of the virus pool intraperitoneally at 24, 72, and 96 hours prior to embryo harvest for tissue culture on the 16th day of gestation. Embryos were aseptically transferred, within the uterus, from anaesthetized mice to Petri dishes. Upon opening of the uterus and removal of embryos, contact with the mothers' blood was carefully avoided. After 2 washings in Hanks' solution containing Penicillin (2,500 U/ml) the embryos were minced with scissors and then trypsinized. A superimposed second virus inoculation was applied to half of the tissue cultures by addition of 0.5 ml of the same LDH agent pool to compare the competence of the cultures under the two conditions.

Tissue cultures and medium. Mouse embryo cultures (MECs) were prepared in the usual way by suspending 25×10^6 embryo cells in 10 ml of a medium containing chick-embryo extract, inactivated calf serum, and Hanks' solution in a 1:2:7 ratio. The medium was changed twice a week.

Virus assay. Samples of the MECs medium, without cells, were tested at various periods by IP injection of 0.5 ml into normal random bred mice as shown in Table I. A single dose was given to a group of 7 mice and their level of LDH ascertained by 5 to 6 measurements during the following 14 days. Quantitative virus determinations were obtained by injecting ten-fold dilutions of MEC supernatant into 5 normal mice per group, with LDH determinations made on the following 4th day. The ID_{50} was calculated by the method of Reed and Muench.

Enzyme assay. Serum from peripheral blood was obtained by the orbital bleeding technique (Riley(8)), but without heparin. LDH activity was measured at 366 $m\mu$ as previously described(6,7) and average LDH values of the test animals were expressed in Buecher units per ml.

Results. If pregnant mice were inoculated with the agent only 24 hours before removing the embryos for tissue culture, the culture media contained no active agent, as shown by samples tested at successive intervals (Table I). However, when the agent was inoculated either 72 or 96 hours prior to removal of the embryos, all culture flasks contained the LDH-elevating agent for 10 to 16 days. During this time the plasma LDH in recipient test mice was stimulated to about a 5-fold increase. Either infected or non-infected embryo cultures that received a second 0.5 ml inoculum of virus after 24 hours, showed the same plasma LDH elevating effect. The enzyme values corresponded closely, and no tangible difference was found between cell cultures with the agent added *in vivo* or those with the second *in vitro* virus inoculum (Table I). LDH-elevating agent has been maintained in normal MECs for 8 days, and on MECs from *in vivo* fetus-infected cells for 16 days. These represent average values from several experiments. When this survival time is compared with periods of preservation of the LDH agent in cell-free tissue culture medium(10) the periods differ significantly. There is only a borderline significance in the difference between the agent preservation time in virus-inoculated MECs and *in vivo* fetus-infected cells. In all tissue cultures older than 96 hours the culture medium had been changed. Nevertheless, the virus titer appeared unchanged as demonstrated by enzyme elevation in the inoculated recipient host mice. Determination of the infective titers of the virus following 7 or more days of *in vitro* incubation, at which time 2 or 3 tissue medium renewals had been made, were much be-

TABLE I. Time Required for the Riley Agent to Pass the Placental Barrier in Pregnant Mice, and Its Subsequent Survival Time in Mouse Embryo Tissue Culture.

Time between inj of pregnant mice with virus and harvest of embryos (hr)*	Incubation time of mouse embryo cultures: Days 3	4	5	6	7	8	10	14	16	20	25	28
	Hours 72	96	120	144	168	192	240	336	384	480	600	672
	Serum LDH level in test mice†											
24	13 (—)	23 (—)	20 (—)			30 (—)						
72					175 (+)		207 (+)	202 (+)	196 (+)	36 (—)	43 (—)	51 (—)
96		164 (+)	205 (+)	193 (+)	175 (+)		152 (+)					
24‡	136 (+)	219 (+)	216 (+)			105 (+)						
72‡					212 (+)		218 (+)	126 (+)	93 (+)	29 (—)	63 (—)	72 (—)
96‡				270 (+)	230 (+)							

* All embryos harvested on 16th day of gestation.

† Buecher LDH units. Normal values (non-infected) 10-75 units, elevated (infected) levels 90-300 units.

‡ A second *in vitro* inoculation of virus made directly into the mouse embryo cultures at the time they were initiated.

low the titer of the original virus pool. The ID_{50} of the culture fluid after 144 and 240 hours was $10^1/0.5$ ml, while the starting virus pool had an ID_{50} of $10^4/0.5$ ml inoculum.

The LDH enzyme activity of the media in the tissue culture flasks was measured at various intervals during agent incubation. However, there was no detectable change of LDH-level as compared with flasks free of the agent. Both were about 40 Buecher-units. Such enzyme activity is similar to that of the plasma or serum LDH of our normal stock mice.

Discussion. The results demonstrate the transmission of the LDH-elevating agent to mouse embryos *in utero* across the placenta of infected pregnant mice. The time of agent transmission from female host to embryos, as shown by the assay of embryo cultures, takes more than 24 but not over 72 hours. Agent infection of embryo cells, after passing the placenta is in accord with the findings that the LDH agent is present in all adult host tissues(4,7).

Our findings demonstrate, for the first time, the passage of the LDH agent across the placental barrier. A similar suggestion has been brought forward by Notkins and Scheele(9) after showing a 90% infection of the offspring subsequent to virus inoculation between the 7th and 18th day of gestation. In these experiments, however, the possibility of contact infection by mother's milk, feces or saliva cannot be excluded. Our method of *in vivo* infection followed by Cesarian section and *in vitro* isolation of the LDH agent gives stronger evidence for placental passage.

The *in vivo* transmitted agent can be maintained in the *in vitro* grown embryo cells only over the limited period of 10 to 16 days. The period corresponds to that of agent preservation seen after simple inoculation of the virus directly into the culture flasks(2). Incubation of the agent at 38°C in buffered salt solutions, without cells, preserves its activity for a maximum of only 72 hours(10). Although a continuous propagation was not realized the experiments show "preservation" of the agent in the tissue cultures described.

It seems of interest that virus inoculation in tissue culture caused no change of enzyme-elevating activities comparable to the synergistic effect demonstrated by Riley(3,11)

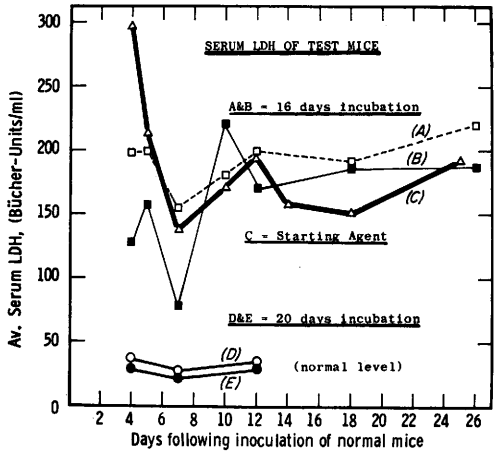

FIG. 1. Serum LDH of mice inoculated with Riley agent-infected mouse embryo tissue culture fluids after various periods of *in vitro* incubation. (A) MEC incubated for 16 days with the LDH-elevating Riley agent. (B) Same as (A) plus an early inoculation of agent directly into the MEC. (C) Mice injected with Riley agent from the original mouse serum pool having a titer of approximately 10^4 ID_{50} infectious units. (D) Same as (A) but MEC incubated for 20 days before testing in normal mice. (E) Same as (D) plus an early inoculation of Riley agent directly into the MEC.

with spontaneous or chemically induced tumors.

Serum LDH enzyme levels in test mice following inoculation of infected culture fluids after various incubation periods were equally elevated. The mouse serum LDH was between 180 and 225 Buecher-units (that is 800 to 1500 Wroblewski units), and thus corresponds to LDH values observed following inoculation of the original virus pool (Fig. 1). However, the degree of plasma or serum LDH elevation is not necessarily a quantitative reflection of the amount of virus injected. To assess its titer the agent must be serially diluted and the infective ID_{50} determined. By comparing infectious titers we found a significant decrease from 10^4 to 10^1 ID_{50} during tissue culture incubation. While this indicates a diminished quantity of active virus, the continued infectivity produced an LDH-elevation in mice with the usual 5-fold increase over normal values. These findings confirm our suggestion that the agent can be maintained for a short period in the mouse embryo cultures described, but probably not propagated. This was not altered by "adaptation" of the agent to the embryo cells *in utero*.

Our findings contradict the results of Yaffe. With the agent which he employed, it was possible to demonstrate agent propagation indefinitely by serial passages on fresh primary mouse embryo cultures. With this same technique our results were negative. Nevertheless, we did find a significant prolongation of preservation time, which indicates some effect of mouse embryo cells upon the LDH agent. In attempting to reproduce the technique and results of serial culture passages described by Yaffe(2), we lost the active LDH-elevating agent after the 3rd or 4th *in vitro* transmission, with a corresponding decrease of infective titer after each passage. To explain the contradictory findings, two possibilities might be considered. There could be differences of methods or unknown influences upon immunological or other biological factors. On the other hand, one might postulate the existence of various LDH agents differing in their biological behavior. Riley(3) first discussed the question of different agents, referring to variations observed upon the enzyme levels of host mice inoculated with agents from different sources.

Our findings of limited survival of the LDH agent in tissue cultures are supported by results of others. Adams *et al*(4), have also lost the LDH-agent through tissue culture transmissions, and Tennant and Ward (5) separated pneumonia virus from the LDH agent by passages through hamster kidney cells. Further experiments on the behavior of LDH agent *in vitro* therefore seem to be of interest.

Summary. The LDH-agent passes the placenta of pregnant NMRI mice and appears in the mouse embryo tissues within 72 hours post-injection. Although there is a prolongation of agent maintenance in cultivated embryo cells as compared to cell-free incubation, it was not possible to propagate the agent indefinitely *in vitro*. Unlike its behavior in living mice, the infectivity of the agent is lost after a maximum period of 16 days incubation *in vitro*.

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