The results with buffer resembled those to which we have already referred. For sulfur mustard, medium at 37°C as a condition of pretreatment led to the same survival for subsequent treatment in room-temperature buffer or 37°C medium; similarly for x-rays, as indicated by the open circles. However, when the incubation after treatment was in room-temperature medium (that is, medium, 37°C; sulfur mustard; medium, ~24°C), a survival curve results (data not presented) having a slope equal to the steepest in Fig. 1. Thus, in contrast with the lack of effect after x-rays (closed circles), after a dose of sulfur mustard that would produce the same survival (about 17 percent) for optimal conditions throughout, medium at room temperature effected a 100-fold decrease.

The curve traced by the triangles in Fig. 3 shows that progressive fall in survival results from incubation, before irradiation, in buffer at 23°C. For sulfur mustard, the same pretreatment results in tenfold drop in survival in 1 hour (left ordinate in Fig. 2). A twofold drop results in the same period for x-rays. The dose used for the latter results was chosen to yield survival, after pretreatment with buffer for 1 hour, about equal to that at zero hour in Fig. 2 (open circle). The open squares in Fig. 3, obtained with the same conditions of post-treatment as for the upper curve in Fig. 2, show that survival after x-irradiation can be increased by room-temperature buffer but only about twofold. The closed squares refer to incubation after treatment in room-temperature medium. (This set of data was obtained about 1 hour after the open squares. The small difference between the first points on each curve probably reflects small differences in the compositions of the populations.) Compared to the situation after treatment with sulfur mustard, here too a decrease in survival results, but at most amounting to a factor of 2. $\bar{D}_0$ shifts of less than 25 percent can account for the survival changes in Fig. 3.

In summary: Simple alterations in pre- or post-treatment medium or temperature, or in both, can effect large changes in the survivability of surface-attached Chinese hamster cells after their exposure to sulfur mustard. Survival after x-ray exposure may be similarly affected, but the changes are much smaller. If this rough parallelism is evidence of the production of identical primary injuries by both agents, there must be significant dissimilarities in their secondary effects. The number and types of potentially lethal lesions may be the same, but a cell's ability to cope with them may reflect the agent as well as the conditions used. Alternatively, the quantitative differences in expression of lethal damage may indicate only a small degree of overlap of primary lesions in respect to number or type, or both.

In addition to radiomimetic properties, our results with sulfur mustard prompt us to consider the question of damage expression after treatment with such an agent. Generally speaking, we would expect optimal growth conditions throughout a treatment course to promote a considerable amount of repair of lethal damage. That incubation in buffer after treatment gives the same dose-effect curve as in medium at 37°C, when the pretreatment also is in medium at 37°C, is not inconsistent with this expectation. We need only assume either that active metabolism is unnecessary for the repair process or that repair is initiated, and is equally effective, after the buffer is removed. To explain the results in Fig. 1, however, we must also assume that: (i) pretreatment with buffer reduces the effectiveness of the repair system, and (ii) ability to repair can be reinstated by subsequent treatment with buffer (even at room temperature) but not with medium at room temperature. Thus our results with mammalian cells may reflect the same rescue processes that are thought to be active in bacteria (*4*) in which incubation in buffer, after exposure to a drug, also causes increases in survival, although over longer periods (2 to 6 hours versus 30 minutes).

While the foregoing may be essentially correct, on purely logical grounds the large fluctuations involved prompt us to mention another view. The degree of damage expressed after treatment with a given agent may involve *degradative* as well as *reconstructive* processes. Cell survival may reflect not only the number of initial lesions and the proportion of these that are repaired. The fate of a cell may also depend on whether or not the initial damage that can be expressed (amount or type) is changed by some treatment so that survivability is altered, even though ability to repair may not be. For example, in Fig. 1 post-treatment with medium leads to more fixation of damage, perhaps not because repair is inhibited but rather because medium increases the amount of damage requiring repair.

F. MAURO*
M. M. ELKIND
Laboratory of Physiology,
National Cancer Institute,
Bethesda, Maryland 20014

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Competent Chick Ectoderm: Nonspecific Response to RNA

Abstract. *Presumptive chick neuroectoderm responds to RNA from brain and heart by forming neural tubes, but it does not respond to liver RNA. This differential response can be correlated with the presence of Folin-positive material in those RNA preparations which elicit the formation of neural structures.*

There are divergent claims concerning the effects of RNA (extracted with phenol) on intact embryos and competent tissues. It has been stated that RNA acts as an "inducing" agent resulting in the formation of structures specific to the organ source of the RNA (*1–3*). Other workers, utilizing similar test systems, find no specificity related to tissue source (*4, 5*).

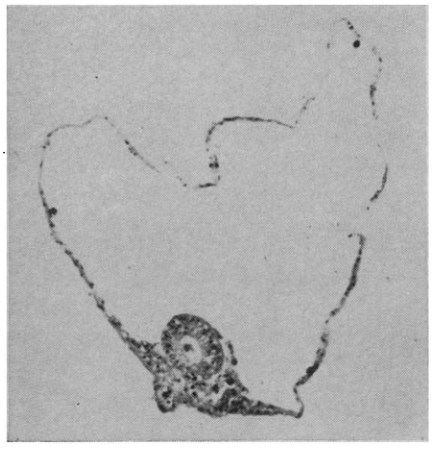

Fig. 1. Cross section of explant treated with unwashed B-RNA. Note presence of neural tube.

A tissue not previously used for testing the inductive capacity of RNA is presumptive cephalic neuroectoderm isolated from stage 3+ chick embryos (*6*). This ectoderm is competent to respond to cephalic chordamesoderm taken from chick embryos that have developed to the early stages of cephalogenesis. In combination with such chordamesoderm in coelomic grafts, the ectoderm forms neural tubes. No structures form, however, when this ectoderm is grafted alone (*7*). We have been able to culture stage 3+ neuroectoderm for a period of 2 days in Tyrode's solution. When cultured in this medium, the ectoderm rounds up, and the cut edges fuse within 4 hours. The fusion of the edges results in the formation of a hollow ball of a single layer of cells. We have used this technique to grow competent ectoderm for testing the hypothesis that RNA is organ specific. When the ectoderm is cultured alone, no neural tubes develop. In accordance with the hypothesis, RNA from embryonic chick brains should induce the ectoderm to form neural tubes, whereas RNA extracted from other organs should not.

Table 1. Responses of explanted chick ectoderm to control and experimental medium and effects of zero and five ether washes of the RNA's. All data are given as numbers.

Medium	Explants	Surviving explants	Explants with nonspecific tubules	Explants with neural tubes
Tyrode's (T)	35	15	7	0
T + nucleotides	28	22	10	0
T + boiled B-RNA	26	23	5	8
No ether washes				
T + B-RNA	40	38	13	17
T + H-RNA	34	34	6	14
T + L-RNA	42	40	7	0
Five ether washes				
T + B-RNA	37	33	8	0
T + H-RNA	35	32	5	0
T + L-RNA	38	35	11	0

The operations, in which ectodermal pieces were extirpated from stage 3+ embryos, were done on a fortified 0.5 percent agar medium. Each piece (0.4 by 0.4 mm) was removed with tungsten needles. The experimental pieces were immediately placed into ½ ml of Tyrode's medium to which was added ½ ml of sterile 0.9 percent saline containing 2 mg of RNA. Other pieces, which served as controls, were placed into (i) ½ ml of the medium plus ½ ml of sterile 0.9 percent saline, (ii) ½ ml of medium plus ½ ml of 0.9 percent saline containing 2 mg of brain RNA that was previously boiled, or (iii) ½ ml of medium plus ½ ml of sterile 0.9 percent saline containing 1×10^{-6} mole of each of the four major 2′(3′)-ribonucleotides (Calbiochem).

The RNA used was extracted from 10- to 12-day-old embryonic chick hearts (H-RNA), brains (B-RNA), and livers (L-RNA) by the modified Kirby technique (*1*). The extracted RNA was subjected to either zero or five ether washes, and the ether was removed under negative pressure. The RNA was stored in aliquots of 4 mg/ml of sterile 0.9 percent saline at −20°C. In all cases, the ectodermal cultures were maintained for 2 days at 37°C. At the termination of the culture period, the ectoderm was fixed, sectioned, and stained for histological examination. The results are summarized in Table 1.

These experiments show that: (i) the survival of the explants is enhanced in medium containing either nucleotides, boiled RNA, or one of the extracted RNA's, (ii) nonspecific tubular arrangements of cells can be found in both the control and experimental explants, (iii) definitive neural tubes develop only in those explants cultured in medium containing either B-RNA, H-RNA, or boiled B-RNA, and (iv) this effect of the RNA is lost if the RNA is washed five times with ether before being stored.

The first observation, the enhancement of survival, is consistent with the findings of Finnegan and Biggin (*5*) who noted that the addition of RNA to Niu-Twitty solution enhanced the survival of competent ectoderm taken from gastrulae of *Xenopus laevis*. This observation may also explain the findings of Sanyal and Niu (*3*) who noted that

Table 2. Amount of Folin-positive material per stored RNA sample (milligrams of Folin-positive material per 4 mg of RNA). Each number represents the mean of five samples plus or minus the standard deviation.

RNA	No ether washes	Five ether washes
Brain	1.35 ± .14	0.93 ± .07
Heart	1.28 ± .08	0.84 ± .17
Liver	0.86 ± .08	0.30 ± .10

explants of the chick definitive primitive streak treated with RNA in Pannet-Compton saline survived after being grafted into older host embryos. The majority of control explants, treated only with Pannet-Compton saline before being grafted, degenerated.

In our experiments those explants that survived but did not form neural tubes developed in one of two directions. They either remained as a single-layered hollow ball, which continued to enlarge through cell division, or they became a solid ball as a result of proliferation of cells which ultimately filled in and obliterated the central cavity. These proliferated cells arranged themselves into a pattern resembling tubules. The nonspecific tubular groupings were found in both control and experimental explants.

The only definitive morphological structures formed in the explants were neural tubes, and these vesicles formed in explants treated with B-RNA, H-RNA, or boiled B-RNA (Fig. 1). Since these neural tubes were found not only in those explants treated with B-RNA but also in those maintained in medium containing either H-RNA or boiled B-RNA, it would appear that they formed as an ectodermal response to a nonspecific stimulus. The ribonucleotides did not, on the other hand, affect the explant. No structures characteristic of heart or liver could be found in the treated ectoderm, and, while the ectoderm responded to H-RNA by forming neural tubes, no morphological change could be seen in explants treated with L-RNA. The effects elicited by the B-RNA and H-RNA were lost when these RNA's were repeatedly washed with ether prior to storage. After five washes with ether, these RNA's were similar to L-RNA in that the survival of the explants was enhanced but no neural tubes were formed in the treated ectoderm. These cumulative results may be interpreted as indicating that the ectoderm responded not to the RNA itself but rather to a possible contaminant present in the H-RNA, B-RNA, and boiled B-RNA but absent or reduced

in concentration in L-RNA and commercially obtained ribonucleotides. This contaminant appears to be removed by repeated ether washes of the RNA sample.

Ether is used in the modified Kirby extraction method to remove contaminating phenol. We routinely check the purity of the RNA samples by subjecting the extracted RNA to tests for DNA (diphenylamine), for protein (biuret), and for protein and phenol (Folin). The Folin test alone shows consistent and significant differences among the preparations (Table 2). There is always a higher concentration of Folin-positive material in the unwashed samples of B-RNA and H-RNA than there is in L-RNA. After five ether washes, the concentration of Folin-positive material decreases in the H-RNA and B-RNA and approaches that routinely found in L-RNA that has not been washed with ether. These differences are consistent and are found among all of the tissue extractions.

The experimental results reported above have led us to reevaluate the role of RNA in the induction of specific morphological changes in competent ectoderm. Since the ectoderm responds to extracts from both heart and brain and since this response is also found in ectoderm exposed to boiled B-RNA, it appears that the specificity of the induction is open to question and that the role of RNA in the inductive process should be further tested experimentally. Our results further suggest that a Folin-positive material in the RNA preparation may be responsible for the nonspecific induction which we have observed.

NINA HILLMAN
RALPH HILLMAN

Temple University,
Philadelphia, Pennsylvania

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Adaptation of an Insect Cell Line (Grace's Antheraea Cells) to Medium Free of Insect Hemolymph

Abstract. *Cultures of the insect cell line derived by Grace from* Antheraea eucalypti *Scott* [Austrocaligula eucalypti *(Scott)*] *were successfully adapted to medium supplemented with fetal bovine serum, whole-egg ultrafiltrate, and bovine plasma albumin instead of insect hemolymph. Cells, now in their 37th passage, have a population doubling time of 2.5 days; those of unadapted cultures, 4.2 days.*

The cell culture derived from ovarian tissues of *Antheraea eucalypti* Scott [*Austrocaligula eucalypti* (Scott)] (Lepidoptera: Saturniidae) by Grace (*1*) constitutes the first true line of arthropod cells and has considerable potential value for studies of insect and insect-borne viruses as well as for fundamental aspects of insect physiology. Although quickly disseminated to laboratories throughout the world, practical use of these cells has been limited because the medium must be supplemented with significant volumes of lepidopteran hemolymph, which is expensive and often difficult to obtain. The cell line was developed in a medium containing homologous hemolymph; later that of the related species *Antheraea pernyi* Guerin, *Austrocaligula helena* (White), and *Samia cynthia* (Drury) was found suitable (*2*). In the absence of hemolymph, cells of this line fail to grow, quickly undergo degenerative changes, and die within 3 or 4 weeks (*3*). We report here the rapid growth of a subline of these cells in medium free of hemolymph and supplemented only by readily available bovine and avian factors.

The adaptation experiments were done at the Rocky Mountain Laboratory. The original cell cultures and *A. pernyi* hemolymph were obtained from T. Grace, CSIRO, Canberra, Australia. Cells were cultivated at 28°C in 30-ml, disposable, tissue-culture flasks (Falcon Plastics) containing 4 ml of culture medium. Minimal culture medium used was Grace's insect tissue culture medium (Grand Island Biological Co.), which contains the antibiotics penicillin and streptomycin sulfate. Substances to be tested, over 60 kinds or combinations thereof, were empirically selected and added aseptically to make up the complete medium. Their effects on growth of cells were judged by inspection in comparison with control cultures grown continuously in the minimal medium enriched with 10 percent *A. pernyi* hemolymph. [Later it was shown that no more than 3 percent hemolymph was required by *Antheraea* cells (*3*) and the concentration of hemolymph in our controls was adjusted accordingly.]

Many of the additives did not permit survival or growth of the insect cells; those that did included fetal bovine serum, rabbit serum, and hemolymph of lobsters (*Homarus americanus* Milne-Edwards).

The most successful medium was one in which 10 percent heat-inactivated fetal bovine serum (Grand Island Biological Co.), 10 percent whole chicken-egg ultrafiltrate (Microbiological Associates, Inc.), and 1 percent bovine plasma albumin (fraction V; Armour Co.) were added to the minimal medium. When first transferred to this, cells survived, but increased only slowly until, after 49 days, their number was sufficient to warrant subcultivation. Growth rate increased with 12 successive passages until the cultures required subcultivation at weekly intervals. This subline has been established for 17 months and subcultivated 44 times.

At the 21st passage some of these cells were transferred to minimal medium containing 10 percent *A. pernyi* hemolymph instead of the substitute factors. They failed to grow and underwent degeneration over a period of a month. When offered bovine and egg factors, these cells resumed their vigorous growth and normal morphology.

Three other strains of *Antheraea* cells were induced to grow in minimal medium containing either 5 percent lobster hemolymph or 5 or 10 percent rabbit serum. The first was maintained for 13 months (24 passages) and the last two for 58 passages (17 months) and 34 passages (6½ months), respectively. Growth rate of these strains, however, was never as rapid as with the above-mentioned subline, and routine maintenance of the cultures proved difficult. A gradual accumulation of