A study of the influence of isolated calf spleen RNA on amphibian histogenesis

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INTRODUCTION

In the cell-free inductor demonstrated by Niu & Twitty (1953) the effective material was later characterized by Niu (1956) as ribonucleoprotein with the suggestion that the ribonucleic acid (RNA) fraction was the active component. Subsequently Niu (1958a, b) reported tissue-specific (thymus-like) induction in amphibian material exposed to calf thymus RNA, though Yamada (1961), using identical procedures, was unable to repeat the results, and Saxén & Toivonen (1962) considered the thymus-like histogenesis in Niu's results to be poor.

In the present investigation calf spleen ribonucleic acid was isolated so as to ensure removal of contaminants from the isolate prior to testing for inductive capacity with competent amphibian ectoderm and embryos. Sedimentation analysis with the ultracentrifuge indicated that the isolated spleen ribosomal RNA was undegraded when introduced into the culture medium. In a limited series of test cases neither embryos nor ectodermal isolates of *Xenopus laevis* demonstrated any tissue-specific inductive response (i.e. erythropoiesis), though a general enhancement of epithelial development was obvious. Further information on such enhancement of epithelial histogenesis was obtained when post-gastrula stages of *Taricha torosa* and *Ambystoma gracile* were cultured in a commercial liver s-RNA preparation.

MATERIALS AND METHODS

Calf spleen was used as the source of RNA since it has a high RNA content, a low connective tissue polysaccharide content and its specific histogenesis (erythropoiesis) is easy to see in cultured tissues. The freshly frozen spleen tissue was sliced into pieces approximately 1 × 2 cm and then homogenized, in 2-5 ml. of 0.015M naphthalene-1,5-disulfonate and 2.5 ml. of 88–90 % phenol per gram weight of tissue, for 2 minutes in a Waring Blendor at room tempera-

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ture (Kirby, 1956, 1962). The cloudy supernatant was carefully sucked off and subjected to a total of three extractions with phenol (Biggin, 1964). Glassware and equipment were washed with 1N-NaOH and with versene to remove RNase and heavy metal contaminants. The purity of the isolate was indicated with the Biuret test (Schneider, 1957) for protein, Dreywood’s anthrone test (Morris, 1948) for carbohydrate, Dische’s diphenylamine test (Chargaff & Davidson, 1955; Schneider, 1957) for DNA and Bial’s orcinol method (Chargaff & Davidson, 1955) to estimate RNA. Following extraction of the isolate with 2-methoxyethanol, no polysaccharide contamination was demonstrable either with the anthrone reagent or by chromatography.

Spectrophotometric analysis was made of the isolates in 0.1M-NaCl solution with the Unicam SP500, and from these data the ratio of absorption values for 260/230 m\(\mu\) and for 260/280 m\(\mu\) were calculated to indicate, according to Morton (1962), the relative amounts of peptide (230 m\(\mu\)) and protein (280 m\(\mu\)) present with the nucleic acid (see Table 1). Sedimentation analysis was conducted with a Spinco model E analytical ultracentrifuge, employing Schleiren optics with photographs taken at 4 min intervals at 44,770 r.p.m. The sedimentation coefficients of the isolated RNA were determined according to Markham (1960, 1962).

The method of Brown & Littna (1964) was employed to obtain eggs of *Xenopus laevis* which were removed for use at late blastula to early gastrula stages (stages 9–9+, Nieuwkoop & Faber, 1956). Sterile techniques were used throughout. Isolated gastrula ectoderm (six cases) and embryos from stage 9+ (eight cases) were cultured as controls in Niu-Twitty medium for 5 days with daily observations. Similarly, isolated ectoderm (eighteen cases) and embryos (eight cases) were cultured as experimental cases in Niu-Twitty medium to which 50 \(\mu\)g/ml. (Niu, 1958b; Yamada, 1961) of isolated spleen RNA was added immediately before use to minimize possible RNA degradation effects. All embryos (sixteen) and a few randomly selected control and experimental explants (four) were fixed in Carnoy’s acetic-alcohol, paraffin-embedded, sectioned at 5–8 \(\mu\)m and stained with haematoxylin-eosin or Chromotrope 2R for histological examination.

Late gastrula of *Taricha torosa* (stage 13), from California, and *Ambystoma gracile* (stage 12), obtained locally, were freed from the egg jelly and (the majority) from vitelline membranes before being placed in a medium produced by adding Reagent Grade Beef liver RNA (Nutritional Biochemical Company) at a concentration of 25 mg/ml. to Niu-Twitty solution. Ten animals of each species and five control animals (in salt solution) were grown until stage 39 (2–3 weeks), with the media replaced at least twice per week, after which they were fixed in Carnoy’s and stained with the modified Mallory’s (Liisberg, 1962).

The spectrophotometric data for the commercial-liver s-RNA were obtained as were the calf spleen RNA data and are presented with the latter in Table 1 and Fig. 1. In the sedimentation analysis with the Spinco model E analytical ultra-
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When calf spleen RNA isolated with a single phenol extraction was examined spectrophotometrically (see Fig. 1) the ratio of absorption values 260/230 m\(\mu\) (1.80) and the ratio of 260/280 m\(\mu\) (1.82) indicated protein and peptide contamination of the isolate (see Table 1). On the other hand, after three phenol extractions, these same ratios were 2.23 and 2.20, respectively, indicating a more effective removal of proteins and/or peptides from the isolates. The Biuret test

![Optical density vs. wave-length graph](image)

Fig. 1. The ultraviolet absorption spectra of isolated calf-spleen RNA, after one and after three phenol extractions, and of reagent-grade beef-liver RNA. ●—●, One phenol extraction; ○—○ three phenol extractions; ▲—▲, commercial RNA.
was negative in all cases. The commercial liver RNA had an absorption peak at 265 m\(\mu\) (see Fig. 1) and, while the peptide removal seemed effective, the protein/nucleic acid absorption ratio was the same as for the single phenol calf spleen isolation (see Table 1).

A spectrophotometrically measurable positive response was obtained with the diphenylamine test for DNA with the single phenol extract which was not detectable in the isolate after three phenol extractions. It is possible that the small quantity of DNA observed in the first case is the result of the nuclei demonstrated by Georgiev, Mantirva & Zbarsky (1960) to be present in the intermediate layer after centrifugation.

Table 1. Spectrophotometric analysis of isolated calf-spleen RNA and reagent-grade liver RNA

<table>
<thead>
<tr>
<th></th>
<th>Optical densities (m(\mu))</th>
<th>Ratios</th>
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<tbody>
<tr>
<td>One phenol extraction</td>
<td>0.690</td>
<td>1.240</td>
</tr>
<tr>
<td>Three phenol extractions</td>
<td>0.550</td>
<td>1.230</td>
</tr>
<tr>
<td>Reagent-grade liver RNA</td>
<td>0.266</td>
<td>0.588</td>
</tr>
</tbody>
</table>

Polysaccharide contamination, which could be demonstrated chromatographically and with the anthrone reagent even after three phenol extractions, and previously shown by Kirby (1956, 1962) and by Ralph & Bellamy (1964), was effectively removed by methoxyethanol. Thus, the isolate from calf spleen contained RNA in the apparent absence of peptide, protein, DNA and carbohydrate. However, these data gave no clue as to the physical condition of the isolated RNA molecule and Laskov, Margoliash, Littauer & Eisenberg (1959) and Ralph & Bellamy (1964) have reported the RNA molecule to be degraded when isolated with the original Kirby (1956) method.

Information on the size and condition of the isolated RNA molecules was obtained with the analytical ultracentrifuge and the resulting sedimentation patterns are shown in Figs. 2 and 3. Three definite molecular species of RNA were present in the calf-spleen isolate, whose sedimentation coefficients (in Svedberg units) were calculated to be 27S, 18S and 7–8S (S_{20,w}), and with the fastest moving component, the 27S fraction, being present in the highest concentration. These results indicate that the isolated molecules were not degraded by the procedure (Darnell, Penman, Scherrer & Becker, 1963; Ralph & Bellamy, 1964) and it is this RNA which was used in the induction experiments with *Xenopus* tissues. With the commercial liver RNA in the ultracentrifuge there was no apparent movement of the molecules, which may be taken to indicate that these s-RNA molecules are quite small; an explanation consistent with present knowledge concerning the size of soluble RNA (Spirin, 1963).

The excised *Xenopus* gastrula ectoderm remained free of the glass coverslip
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Fig. 2. Representation of the data obtained for the isolated calf-spleen RNA with the analytical ultracentrifuge. The slope of the curves is a measure of the sedimentation coefficients.

Fig. 3. A drawing made from a Schlieren photograph of a 0.1 M-NaCl solution of spleen RNA after reaching the speed of 44,770 r.p.m. in the analytical ultracentrifuge. From the meniscus, M, the peaks have sedimentation coefficients of 7-8S, 18S, and 27S.
and rolled into a ball in approximately 30 min in both control and RNA-containing media. Daily examination during the culture period, in which control *Xenopus* embryos reached swimming larval stages, showed no tissue-specific induction (i.e. erythropoiesis) or, indeed, any histogenesis in either control or experimental cases. In preliminary experiments it was found that the control explants would not survive much beyond a 5-day culture period whereas the explants in RNA medium would survive well into the second week. At the same time, in all experiments, the explants in RNA-medium were less susceptible to disintegration during the 5-day culture period than were the control explants.

Thus, it was apparent that the maintenance and the survival of the explants were enhanced in the RNA-enriched medium as compared with the control medium. Since the daily observations for evidence of erythropoiesis in the explants showed none, only two of each series, randomly selected from the experimental and control explants, were sectioned for histological examination. Again no erythropoiesis was seen, but in the similar-sized explants the single layered squamous-cuboidal ectoderm of the control explants contrasted with the 2- to 4-layered, frequently columnar, epithelium of the experimental explants.

The intact *Xenopus* embryos cultured in the medium containing calf-spleen RNA showed what appeared to be an accelerated histogenesis as compared with the controls, for when sectioned after 5 days' culture it could be seen that an
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increase in epithelial cell number and/or size was present which was non-specific since it involved such diverse epithelial systems as that of the otic vesicles, the intestine and some glandular epithelia (see also Taricha torosa results below).

In the commercial liver RNA medium the T. torosa embryos all developed posterior dorsal head lesions and excessive cranial flexion during the culture period. It was apparent in the sectioned material that a very definite hyperplasia had occurred in the mesencephalon roof, i.e. the tectum (see Fig. 4), which continued posteriorly in the rhombencephalon (see Figs. 5, 6). This neural hyperplasia was rather unorganized in the mesencephalon and anterior rhombencephalon but became less chaotic posteriorly in the hind brain (see Fig. 7). Although the magnitude of the chaotic histogenesis varied it was present in all experimental animals (see Fig. 8).

Table 2. Auditory vesicle size in Taricha torosa embryos grown in commercial liver-RNA medium

<table>
<thead>
<tr>
<th></th>
<th>Length (µ)</th>
<th>Cross-sectional diameters* (µ)</th>
<th>No. of cells per section*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Min.</td>
</tr>
<tr>
<td>Control†</td>
<td>210</td>
<td>21-3</td>
<td>27</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>259</td>
<td>24-3</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>287</td>
<td>33-8</td>
<td>43-8</td>
</tr>
<tr>
<td>3</td>
<td>245</td>
<td>25-0</td>
<td>27-5</td>
</tr>
<tr>
<td>4</td>
<td>231</td>
<td>23-8</td>
<td>28-8</td>
</tr>
<tr>
<td>5</td>
<td>217</td>
<td>23-8</td>
<td>30</td>
</tr>
</tbody>
</table>

* The vesicle diameters and the number of nuclei per section were measured at several identical anterior-posterior levels in all animals, namely the endolymphatic duct, the auditory ganglion and the largest, or a larger, section. The diameter values for this table are those of the endolymphatic duct level.
† A single control animal, which approximated the average control length, was chosen for this comparison since all control measurements were less than the experiments.

Auditory vesicles of these T. torosa embryos were measured as to total anterior-posterior length, as to both dorsal-ventral and medial-lateral width and as to cell population (number of nuclei per section at various levels along the anterior-posterior axis). An example of the data obtained is given in Table 2. All experimental auditory vesicles were larger than the controls in length and width but no variation in cell population could be demonstrated (see Table 2), which implies that an increase in cell size (not readily apparent histologically) was responsible for, or accompanied, the increase in volume.

Cell counts made of the mesenchyme cells present dorsal to the rhombencephalon indicated an increase in this population which is probably of neural crest origin, but the hyperplasia did not involve the ganglia or the melanophores. The increase in dorsal mesenchyme cells was continued in the anterior trunk, being
reflected there by the more lateral or ventro-lateral positioning of the dorsal somite tips and the increase in size of the basal portion of the dorsal fin. This hyperplasia did not continue into the posterior trunk though the base of the fin appeared to remain large. In the posterior trunk the cloacal endodermal epithelium was enlarged in thickness, apparently because individual cells were columnar and not the normal cuboidal shape, for no increase in cell number could be shown in this tissue.

No external evidence of an effect of the commercial-liver RNA was shown by the *Ambystoma gracile* embryos during the culture period but, in the sectioned

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**Fig. 6.** A section of a *Taricha torosa* (animal no. 4) grown in a commercial liver RNA medium which shows hyperplasia in the dorsal postotic rhombencephalon. Again, no increase in the ventral neural fiber material was demonstrated. (Traced from a projection.)

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**Fig. 7.** (A) A section of a *Taricha torosa* (animal no. 2) grown in a commercial liver RNA medium and showing the reduced neural hyperplasia found in these animals at the anterior trunk level of the neural tube. (Traced from a projection.) (B) A section of a control *Taricha torosa* animal, grown in a salt solution, at approximately the same anterior trunk level as in the above (A). (Traced from a projection.)
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material, the dorsal neural hyperplasia was definitely present though much less pronounced than it had been in *Taricha torosa*. There was some indication of chaotic development in the anterior rhombencephalon roof in only two of the experimental animals. No increase in the auditory vesicles could be demonstrated and it was evident that the more subtle hyperplasia of the neural tissue had

Fig. 8. Sections of *Taricha torosa* embryos grown in a commercial liver RNA medium which show less extensive hyperplasia in the dorsal postotic rhombencephalon than that seen in Fig. 6. A, Animal no. 7; B, animal no. 9. (Traced from a projection.)

not interfered with neural organogenesis in this species as it had in *T. torosa*. The dorsal mesenchyme population was increased about one-third in the hindbrain–anterior trunk region of the experimental animals resulting in a broad base to the dorsal fin. This increase in fin size was continued into the posterior trunk, in the absence of any mesenchyme increase, and in the presence of a similar, though more loose, fiber content: this is taken to indicate a probable increase in ground substance, particularly hyaluronic acid which is so highly concentrated in the dorsal fin (C. V. Finnegan, unpublished). The cloacal endodermal epithelium was enlarged as had been seen in *T. torosa*.

DISCUSSION

The procedure for the isolation of ribonucleic acid as employed by Niu (1958a, b); Niu, Cordova & Niu (1961); Hillman & Niu (1963a, b); Yamada (1961) and Butros (1963) was the Kirby (1956) method reported by Laskov *et al.* (1959) to yield a degraded product. When the methoxyethanol purification was deleted to reduce this degradation (Niu *et al.* 1961), it would seem that the polysaccharide contamination might have become significant, particularly since liver and muscle tissues were often the source material used. With the single phenol extraction used it is possible that the protein and/or peptide contamination also might have been significant, since Huppert & Pelmont (1962) were able to demonstrate with the sensitive Lowry method the presence of between 20 and 50 µg/ml.
protein following three phenol extractions of the isolated RNA. Thus it is diffi-
cult to consider, as do many workers (Gierer, 1957; Gierer & Schramm, 1956;
Kirby, 1962; Niu, 1958a, b; Niu et al. 1961; Hillman & Niu, 1963a, b; Butros,
1963), that protein and/or peptide contamination is completely absent from the
Kirby-isolated RNA. However, after the additional phenol washings such con-
tamination would appear to be reduced to a concentration which does not act
inductively in the experimental system used in this investigation.

Analysis with the ultracentrifuge demonstrated that undegraded ribosomal
RNA (Spirin, 1963), namely the 27S and the 18S fractions, and possible mes-
senger RNA (Spiegelman & Hayashi, 1963; Darnell et al. 1963), namely the 8S
fraction, were present initially in the isolate, but the instability of isolated RNA
molecules at room temperature has been shown by Huppert & Pelmont (1962),
Kubinski & Koch (1963) and Amos & Moore (1963). Degradation is initiated
before 130 min and is drastic within 24 h. However, Amos & Moore (1963) and
Amos, Askonas & Soeiro (1964) reported that the biological activity of isolated
RNA is retained over a period of several weeks if stored at $-20^\circ C$ and thus the
RNA of the present study was either employed immediately following isolation
or after being stored at $-20^\circ C$ for no more than 7 days. Once exposed in the
culture medium it is probable that the rate of RNA degradation in the present
investigation would have been less than that reported by Huppert & Pelmont
(1962) since the embryos and explants were cultured at 12$^\circ C$ rather than at room
temperature ($20^\circ C$). It would seem, then, that a large concentration of intact
ribosomal and possible messenger RNA molecules would have been present in
the conditioned medium during the early hours of exposure of the competent
amphibian embryos and ectodermal isolates.

It is probable that any induction of the *Xenopus* tissue, under the present test
conditions, would have had to occur during the first hour(s) of exposure, if at all,
since some time later, and certainly after 24 h, there would have been a solution
of nucleotides and nucleosides rather than intact RNA molecules. But it is pre-
cisely during this period when any inductive activity would have occurred, since
Yamada (1962) has shown that a 55% inductive response was achieved in
amphibian ectoderm exposed for 30 min to a protein medium and that this
could be increased to a 94% response with an exposure of 180 min. In the event,
no induction was evidenced but there was observed an enhancement of develop-
ment (namely an increase in epithelial cell number and/or size) in the RNA-
conditioned medium. This effect appears to be similar to results described by
Ambellan (1955, 1958) and Ambellan & Webster (1962) in which solutions of
various nucleotides did not induce, but did accelerate, the formation of neural
tubes in amphibian embryos.

In the test series using commercial-liver RNA it was initially considered that
while the surface coat (particularly in *Ambystoma gracile*) would probably
function to prevent most, if not all, RNA uptake by the superficial cells, suffi-
cient uptake would take place in the various invaginated epithelia (after their
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invagination) to demonstrate an effect if one were to occur. The obvious enhancement of anterior neural histogenesis observed in both T. torosa and A. gracile would probably have been the result of tissue exposure, immediately following neurulation, to both intact RNA and nucleotides. Butros (1965) noted an enhancement of neural fiber histogenesis (which was not seen in the present work) when chick-heart RNA was applied to explanted segments of chick blastoderm, which he attributed to the versatility of the responding cells in using the foreign RNA for their own purposes (i.e. the synthesis of neurofibrils). He had previously called attention to epidermal hyperplasia which resulted when chick tissues were exposed to specific foreign RNA, namely brain, heart and pancreas (Butros, 1963), and it would seem probable that the suggestion of Butros (1965), that in these test systems the added RNA is depolymerized and the nucleotides then used in the synthesis of homologous RNA, best conforms to the reported observations. The suggestion has the added merit of emphasizing that the resulting histogenesis in the experimental tissue is that which the assimilating tissue is already competent to express.

Finally, it must be noted that Amos & Moore (1963) and Amos et al. (1964) have suggested that the absence of any specific inductive effect with isolated RNA in experimental procedures of this type may be due to a failure to obtain a biologically 'active' preparation.

SUMMARY

1. Ribonucleic acid (RNA) was isolated from calf spleen tissue by the new Kirby procedure followed by three phenol washings to reduce the peptide–protein contamination. Ultraviolet absorption spectra and colorimetric analyses indicated the reduction or absence of protein, peptide, DNA and, after methoxyethanol purification, polysaccharide from the isolated RNA.

2. Ultracentrifuge sedimentation analysis showed three components in the undegraded RNA isolate: a 27S fraction, an 18S fraction and a 7–8S fraction. This ribosomal-messenger RNA was added to non-nutrient medium for induction testing.

3. Neither embryos nor competent gastrula ectoderm of Xenopus laevis demonstrated, in a limited series, any tissue-specific inductive response, but both experimental systems in the RNA-conditioned medium did demonstrate enhanced epithelial development as compared with the control systems.

4. Postgastrula stages of Taricha torosa and Ambystoma gracile exposed during development to reagent-grade beef liver s-RNA demonstrated hyperplasia in the dorsal mesencephalon–rhombencephalon and the anterior neural crest mesenchyme population along with an enhancement of auditory and posterior entodermal epithelial development.
RÉSUMÉ

**Etude de l'influence d'ARN isolé de la rate du veau sur l'histogenèse des Amphibiens**

1. On a isolé de l'acide ribonucléique à partir de tissu splénique de veau à l'aide du nouveau procédé de Kirby suivi de trois lavages au phénol pour réduire la contamination par les peptides et les protéines. Les spectres d'absorption dans l'ultraviolet et les analyses colorimétriques ont indiqué la diminution ou l'absence de protéines, de peptides, d'ADN et, après purification au méthoxy-éthanol, de polysaccharides, dans l'ARN isolé.

2. L'analyse par sédimentation et ultracentrifugation a décelé trois composants dans l'ARN isolé non dégradé: une fraction 27 S, une fraction 18 S et une fraction 7-8 S. Ces ARN ribosomique et messager ont été ajoutés à un milieu non nutritif pour des essais d'induction.

3. Dans une série limitée, ni les embryons ni l'ectoderme compétent de gastrula de *Xenopus laevis* n'ont manifesté de réponse inductive spécifique du tissu, mais les deux systèmes expérimentaux, dans le milieu conditionné par l'ARN, ont présenté un développement épithéial accru par rapport aux systèmes témoins.


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