AN INVESTIGATION OF RNA INDUCTION IN AMPHIBIAN TISSUES

by

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We accept this thesis as conforming to
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ABSTRACT

Ribonucleic acid (RNA) from calf spleen tissue was isolated and purified by a modified Kirby phenol procedure. The absorption maximum of the isolate occurred at 260 mμ indicating the presence of nucleic acids and the absorption minima recorded at 230 mμ and 280 mμ indicated the absence of peptides and proteins. Colorimetric analyses indicated the presence of RNA and the absence of peptide, protein, DNA and carbohydrate contamination. Chromatographic analysis indicated the absence of carbohydrate contamination only after the purification with 2-methoxyethanol. The spleen RNA prepared by the phenol method was undegraded and demonstrated three distinct molecular species when analysed with the ultracentrifuge; a 27S fraction, an 18S fraction and an 8S fraction. Competent early gastrula ectoderm and embryos of Xenopus laevis exposed to undegraded spleen RNA demonstrated no tissue-specific induction. However, in both the in vitro and in vivo experimental series an enhancement of development was observed. A possible explanation of this phenomena was discussed.

ACKNOWLEDGMENT

The author wishes to acknowledge the guidance and encouragement of Dr. C.V. Finnegan throughout the current investigation. I am indebted to Dr. W.S. Hoar and Dr. P. Ford for their comments. Appreciation is expressed to Dr. M.E. Reichmann of the Agricultural Research Station of the Government of Canada for technical assistance and the provision of research facilities.

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INTRODUCTION

Niu and Twitty (1953) demonstrated that when amphibian organizer material (dorsal lip of blastopore-axial mesoderm) was explanted into a non-nutrient medium, substances which diffused from the explant were capable of initiating differentiation in exposed competent ectoderm. Niu (1956) characterised the material as ribonucleoprotein and suggested that the RNA fraction was the active component. Yamada and Takata (1955) obtained differentiation using guinea pig kidney and reported that the inductive fraction was rich in ribonucleoprotein.

Niu (1958a,b) using calf thymus RNA, reported successful tissue-specific induction in amphibian tissue and concluded that thymus RNA resulted in thymus-like histogenesis. Saxen and Toivonen (1962) in discussing Niu's report considered the differentiation to be poor. Yamada (1961) repeated Niu's thymus RNA experiments using identical isolation procedures and was unable to show any tissue-specific effect on amphibian tissues with calf thymus RNA.

More recently, Hillman and Niu (1962a,b) using RNA isolated from embryonic chick brain and notochord reported specific effects on neural and notochordal histogenesis both *in vivo* and *in vitro* but no effect was observed with chick liver RNA. In the *in vivo* experiments an enlargement or duplication of brain and notochordal structures was observed.

Butros (1963) found that RNA isolated from embryonic chick brain and adult rat heart resulted in hyperplasia

of the epidermis while adult rat liver had no effect. On the otherhand, an unidentified adult pancreatic RNA enhanced the development of the endodermal epithelium.

Yamada (1961, 1962) and Yamada and Takata (1961) have reported definite and specific induction of neural and mesodermal tissue when competent ectoderm was exposed to protein fractions isolated from guinea pig bone marrow. However, Hillman and Niu (1963b) were unable to detect any specific histogenesis in tissues which had been exposed to isolated embryonic chick brain protein.

In the present investigation an attempt was made to isolate calf spleen ribonucleic acid (RNA) by a modified Kirby procedure (1962), and to remove protein and other contaminants from the isolate. To test the purity of the isolate ultraviolet (UV) absorption spectra were measured, and qualitative tests for protein, carbohydrates and desoxyribonucleic acid were applied. Sedimentation analyses of the samples were made to determine the degree of possible degradation of the RNA. Finally, the biological activity of the isolated and purified spleen RNA was tested with Xenopus laevis tissues in both in vivo and in vitro systems.

MATERIALS AND METHODS

I. Preparation of calf spleen ribonucleic acid (RNA).

Calf spleen was chosen as the source of ribonucleic acid since this organ, actively synthesizing protein, has a high RNA content, and is relatively free of connective tissue, thereby reducing the polysaccharide contamination of the isolated material.

Fresh spleen tissue was frozen by being placed on solid carbon dioxide immediately following removal from slaughtered calves. Upon return to the laboratory, samples of the frozen tissue were separated from the connective tissue capsule and sliced into small pieces (approximately 1cm. x 2cm.). Ribonucleic acid was isolated from these tissue slices by a modified Kirby procedure (1962) as shown in Table I (see, also, Appendix I). The tissue was homogenized in 2.5ml. of 0.015 M. napthalene-1,5-disulfonate and 2.5 ml. of 88-90% phenol (Mallinckrodt liquified phenol)/gram wet weight of tissue simultaneously for 2 minutes in a Waring blender at room temperature. Three (3) extractions with phenol were employed for the isolation of spleen RNA used in the culture experiments. The cloudy supernatant layers from the original centrifugate and washings were carefully suctioned off to insure against contamination from the intermediate layer (see figure 1). When preparations were purified by extraction with redistilled 2-methoxy ethanol the time of dialysis was 6 - 12 hours in the cold at 4°-6° C. with 2 changes of cold saline (pH at 7).

Table I. The isolation and purification of calf spleen ribonucleic acid (RNA).

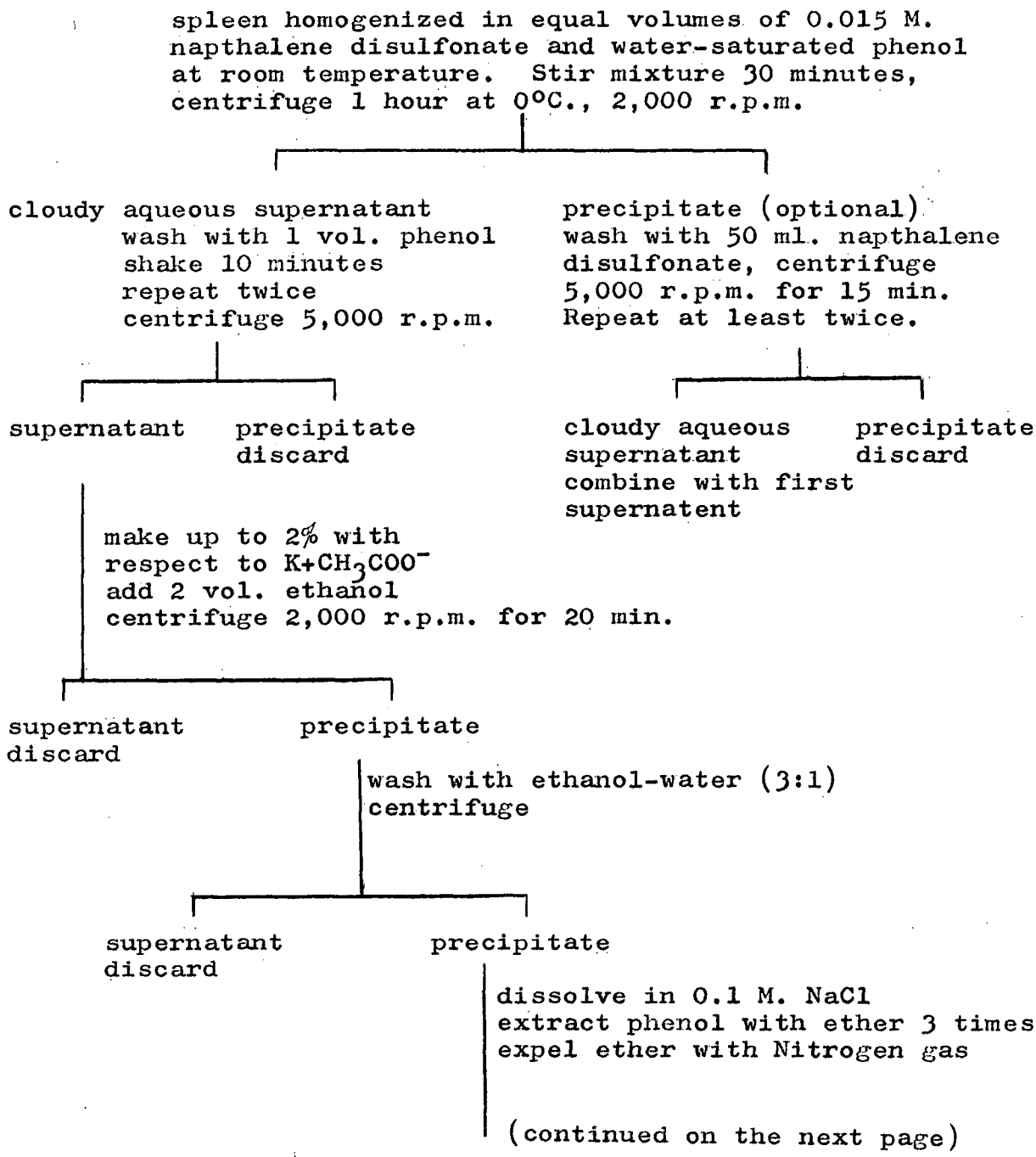

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Table I. (continued)

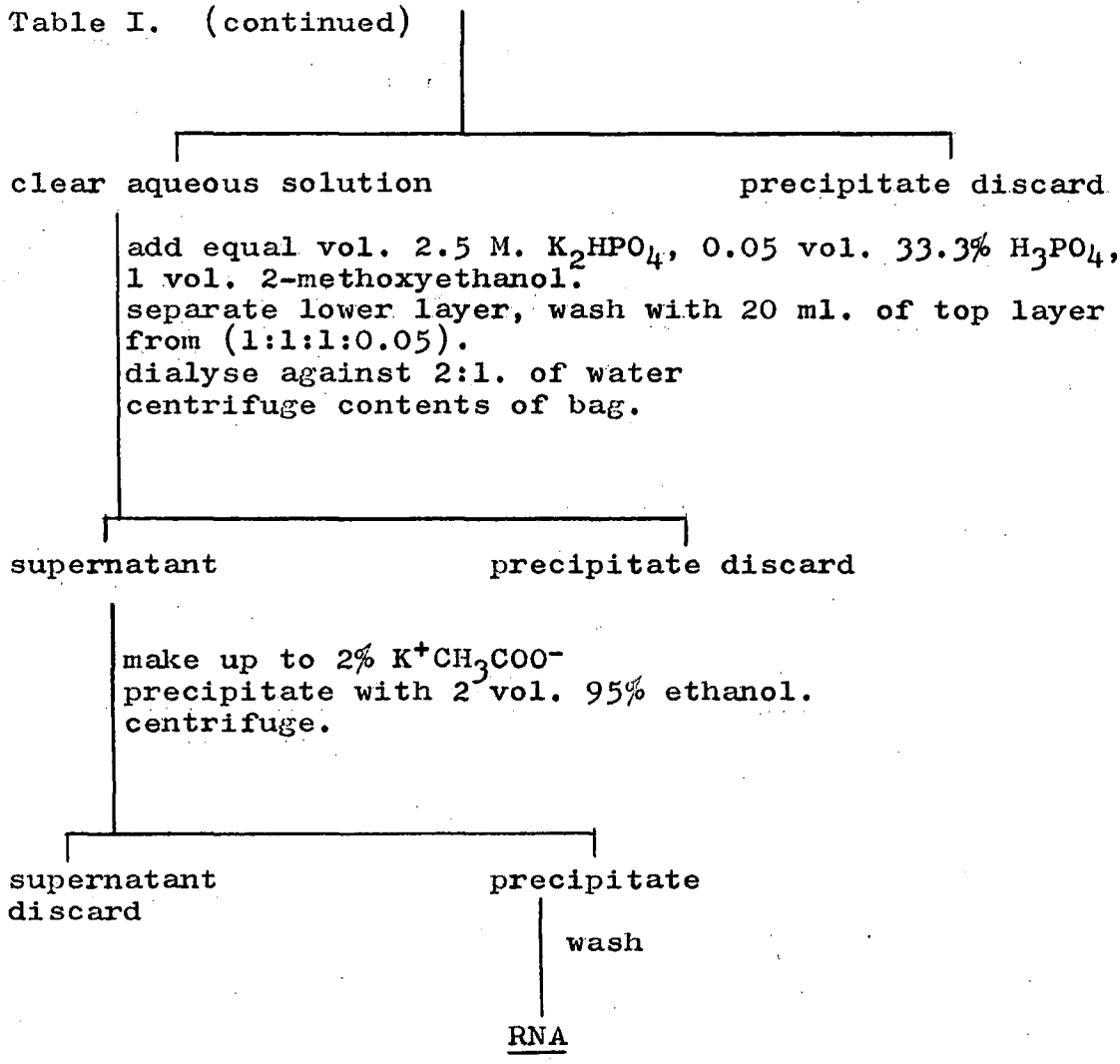
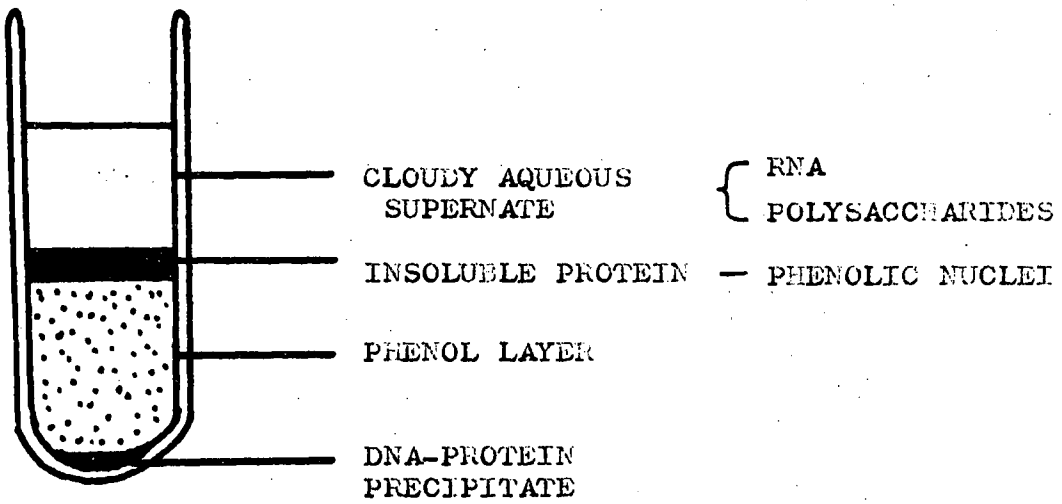
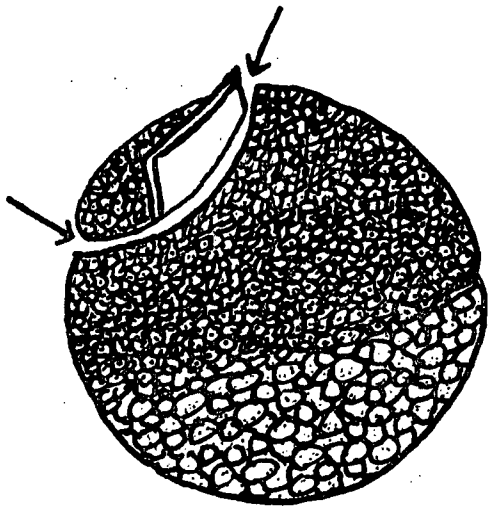


Figure 1. Sedimentation of the various layers of calf spleen tissue following homogenization in napthalene disulfonate and phenol.

Figure 2. A stage 9+ Xenopus laevis. The dotted area represents the competent ectodermal region which was removed for the in vitro study.


CLOUDY AQUEOUS SUPERNATE
RNA
POLYSACCHARIDES
INSOLUBLE PROTEIN
PHENOLIC NUCLEI
PHENOL LAYER
DNA-PROTEIN PRECIPITATE



To assure the removal of all previous contaminants and especially ribonuclease (RNase) both glassware and equipment were washed with 1N. NaOH. Versene[1], a chelating agent, was used in 2% concentrations to remove heavy metal ions which would precipitate the biological macromolecules during isolation procedures.

In the above isolations the samples were centrifuged in an International high speed centrifuge model HR-1 with an #856 head. All readings were in revolutions per minute (r.p.m.).

In some of the control biochemical and physical tests commercial liver RNA, consisting of the s-RNA[2] fraction, was utilized but this material (s-RNA) was not used in the tissue culture or <u>in vivo</u> experiments concerned with the possible biological function (induction) of tissue-specific RNA.

II. UV absorption spectra

Spectrophotometric readings of both the commercial (liver) RNA and the isolated (spleen) RNA solutions were made using the Unicam Sp. 200 spectrophotometer and the Beckman spectrophotometer. From this data the ratio of

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1. versene = E.D.T.A. or ethylene diamine tetracetic acid.
2. s-RNA = soluble or transfer RNA.
3. S = Svedberg unit or S value = 10^{-13} m./sec./dynes.
4. Cetavlon = cetyltrimethyl ammonium bromide.

the absorption values at 230 mμ, to 260 mμ and 280 mμ to 260 mμ was calculated to indicate the presence or absence of nucleic acids (260 mμ), proteins (280 mμ), and peptides (230 mμ) (Morton, 1962).

III. Colorimetric and chromatographic tests.

Solutions containing isolated spleen RNA were tested to determine the presence of contaminating substances. The Biuret test (Schneider, 1957) was applied to determine the presence of protein. Dische's diphenylamine test Schneider, 1957; Chargaff and Davidson, 1955) was utilized to demonstrate the presence or absence of DNA. The Bial's orcinol method (Chargaff and Davidson, 1955) was used to confirm the presence of RNA in solution (see Appendix II). Dreywood's anthrone test (Morris, 1948) was used to demonstrate the presence of carbohydrates. A chromatogram was prepared to demonstrate the presence or absence of carbohydrates (ie. polysaccharides) before and after extraction with 2-methoxyethanol (see Appendix III).

IV. Ultracentrifugal analyses.

Sedimentation analyses were carried out in a Spinco Model E analytical ultracentrifuge. UV optics were employed for an examination of the commercial s-RNA preparation from liver and Schleiren optics were employed for examination of the isolated calf spleen RNA. Photographs were taken after the speed of 44,770 r.p.m. was attained in the case of spleen RNA and 55,770 r.p.m. in the case of liver s-RNA, at 4 minute intervals. Sedimentation coefficients

were determined by the method of Markham (1960, 1962) (see Appendix IV).

V. Preparation of biological materials.

Eggs of Xenopus laevis (Daudin, 1802) were obtained with the method of Brown and Littna (1964) modified by using a "breeding medium" containing NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and streptomycin sulfate but not penicillin. The pH of the medium was maintained at 7 and the light and dark periods were alternated every 12 hours. Eggs were removed from the breeding medium when at stages 9 and 9+ (very early gastrula) according to Nieuwkoop and Faber (1956).

A modified Niu-Twitty medium (Douglas and Finnegan, unpublished) was used to culture whole animals in vivo and ectodermal tissue cultures in vitro (see Appendix V). This medium was "conditioned" by the addition of 50 µg/ml. of purified spleen RNA, added immediately before use in order to minimize any possible RNA degradation effects.

Competent ectoderm (see figure 2) was isolated from stage 9+ animals with care taken to remove any clinging material from the ectodermal isolate. Sterile procedures were used throughout the experiments. Explants developed in either control or conditioned media for 5 days before they were fixed in Carnoys, embedded in paraffin wax and sectioned at 5 µ for histological examination. Similar culture and histological procedures were followed for the whole embryos though the latter were sectioned at 8 µ.

The whole embryos were stained with methyl-green Pyronin, Toluidine blue and Haematoxylin and Eosin. The tissue culture balls were stained with Chromotrope.

RESULTS

Part I. Isolation, purification and characterisation of calf spleen RNA.

Samples of ribonucleic acid (RNA), isolated and purified by the modified Kirby procedure, were dissolved in o.1 M. NaCl solution and were examined with the spectrophotometer in the ultraviolet regions to determine the presence of nucleic acids, proteins and peptides (Morton, 1962). Absorption spectra for a commercial preparation of liver s-RNA as well as the isolated calf spleen RNA are represented in figures 3 and 4. It can be seen from figure 3 that the liver s-RNA demonstrated an absorption peak at 265 mμ and as seen in Tables 2a, 2b, and 2c the ratio of the absorption values 260 mμ/230 mμ was 2.20 and the ratio of the values 260 mμ/280 mμ was 1.82. The former ratio of 2.20 demonstrates the relative freedom of the s-RNA from peptide contamination and the latter ratio demonstrates freedom from protein contamination.

When calf spleen RNA was isolated by the Kirby procedure with a single phenol extraction an absorption curve as represented in figure 4 was produced in which the ratio of the absorption values 260 mμ/230 mμ was 1.80 and the ratio of 260 mμ/280 mμ was 1.82 (see Table 2b). As is characteristic of nucleic acids, the maximum absorption occured at 260 mμ and the minimum absorption values were recorded at 230 mμ and 280 mμ, typical of peptides and proteins respectively (Morton, 1962). When calf spleen

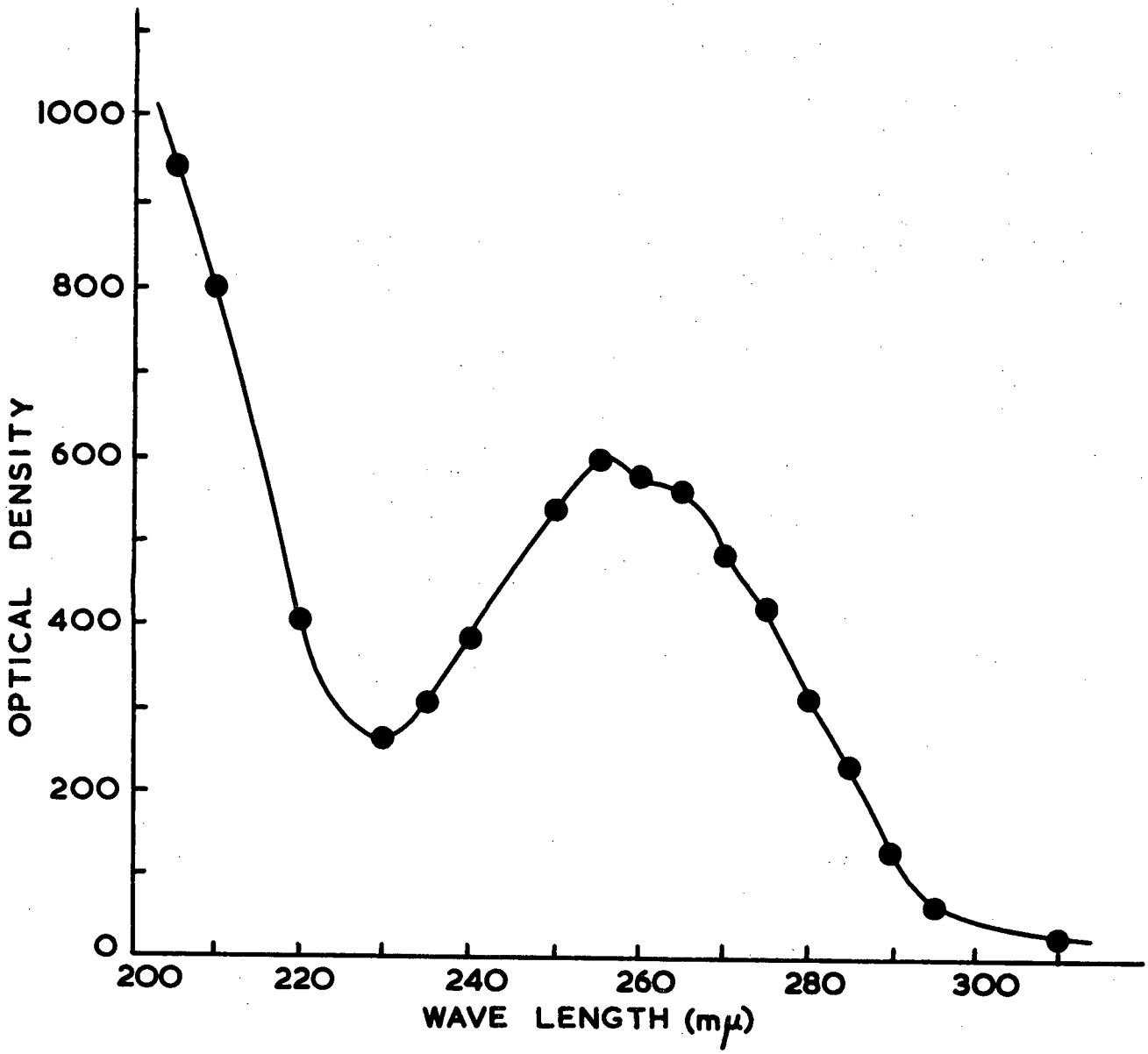

1000
800
600
400
200
0
OPTICAL DENSITY
200
220
240
260
280
300
WAVE LENGTH (mμ)

Figure 3. The ultraviolet absorption spectrum of commercial liver s-RNA.

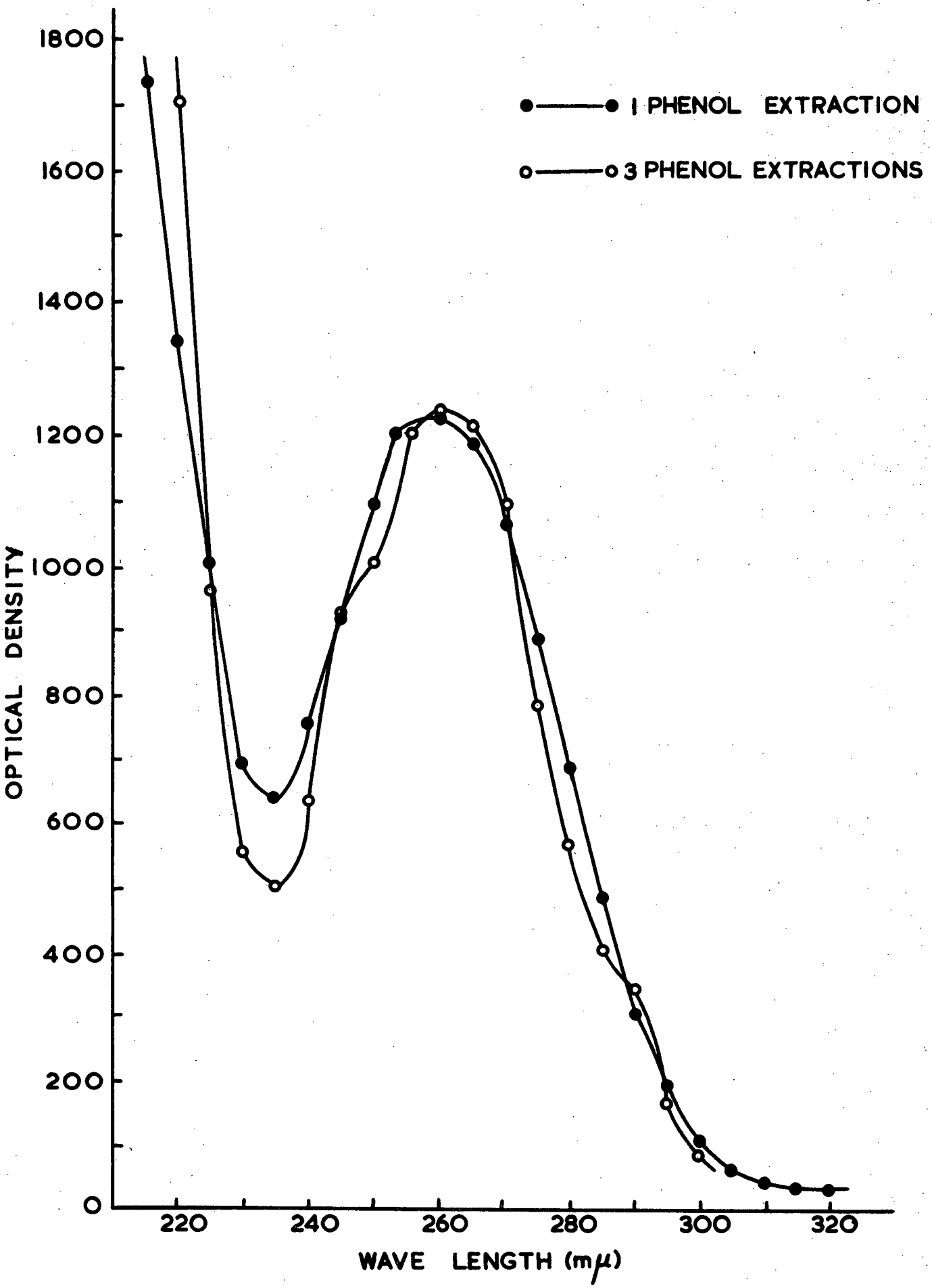

1800
1600
1400
1200
1000
800
600
400
200
0
OPTICAL DENSITY
220
240
260
280
300
320
WAVE LENGTH (mμ)
1 PHENOL EXTRACTION
3 PHENOL EXTRACTIONS

Figure 4. The ultraviolet absorption spectra of isolated calf spleen RNA following one phenol and three phenol extraction(s).

Table 2a. Spectrophotometric analysis of commercial liver s-RNA.

wavelength (mμ)	reading in O.D.	ratio 260/230	260/280
230	0.266		
260	0.588		
280	0.421		
		2.20	1.82

Table 2b. Spectrophotometric analysis of calf spleen RNA after one phenol extraction.

wavelength (mμ)	reading in O.D.	ratio 260/230	260/280
230	0.690		
260	1.240		
280	0.680	1.80	1.82

Table 2c. Spectrophotometric analysis of calf spleen RNA after three phenol extractions.

wavelength (mμ)	reading in O.D.	ratio 260/230	260/280
230	0.550		
260	1.230		
280	0.560		
		2.23	2.20

RNA was extracted with three phenol washings an absorption spectrum was produced as seen in figure 4 in which the ratio of the absorption value 260 mμ/230 mμ was 2.23 and the ratio of the absorption value 260 mμ/280 mμ was 2.20. (See Table 2c). These ratios indicate that protein and peptide contamination was more effectively removed with the additional phenol extraction.

The uv absorption at 260 mμ indicates the presence of all nucleic acids (DNA as well as RNA). Thus it is necessary to determine more specifically the nature of the constituents of the isolate particularly since contamination by substances other than ribonucleic acid in isolations using the Kirby procedure has been reported by various workers (Kirby, 1956, 1960, 1962; Laskov et al., 1959; Huppert and Pelmont, 1962; and, Ralph and Bellamy, 1964). To this end various qualitative tests were employed to indicate the presence or absence of specific molecular constituents. The results of these tests are represented in Table 3.

The Biuret test, to indicate the presence of protein, when applied to samples both of one and three phenol extractions yielded negative results. When the biuret-RNA solution complex was observed on the spectrophotometer at 550 mμ no blue colour developed which would be indicative of peptides or proteins. However, the Biuret method cannot be considered as sensitive as an analysis of the constituents of the RNA solution with the spectrophotometer (see Tables 2b and 2c).

Table 3. Qualitative experiments demonstrating the presence or absence of various materials in the isolated sample.

Test	Result	Comment
1. Biuret	(a) <u>one phenol</u>	
	no color change	absence of proteins and peptides
	(b) <u>three phenols</u>	
	no color change	absence of proteins and peptides
2. Orcinol (Bial's test)	(a) <u>one phenol</u>	
	very positive green color	indicates the presence of RNA in large quantity
	(b) <u>three phenols</u>	
	very positive	RNA present
3. Diphenylamine (Dische's test)	(a) <u>one phenol</u>	
	slight blue color developed	presence of small quantity of DNA
	(b) <u>three phenols</u>	
	no color change	no DNA detectable
4. Anthrone (Dreywood's)	(a) <u>before methoxy</u>	
	slight change of colour from yellow to blue-green	carbohydrate present
	(b) <u>after methoxy</u>	
	no colour change	no detectable carbohydrate

The presence of ribonucleic acid (RNA) could be confirmed by the method of Bial, which employs orcinol reagent and is specific for purine ribonucleotides (Chargaff and Davidson, 1955). After the solution containing isolated spleen RNA and orcinol reagent was heated for 20 minutes at 100°C a definite green colour developed, characteristic of solutions containing RNA. The isolate following three phenol extractions gave a reading on the spectrophotometer of 0.30.

Since an absorption maximum at 260 mμ could represent unprecipitated DNA as well as the demonstrated RNA, the Dische diphenylamine test, which is specific for DNA, (2-desoxyribose and 2-deoxyxylose) was employed. When the cloudy aqueous layers resulting from one phenol extraction were tested with diphenylamine reagent a positive blue colour developed indicating that DNA was present. The spectrophotometric reading of this mixture at 595 mμ was 0.026. Since little care was taken to remove the upper layer without contamination from the interfacial layer, the small quantity of DNA which reacted with the reagent could be attributed to the phenolic nuclei which have been demonstrated in this intermediate layer by Georgiev *et al.*, (1960). On the other hand after three phenol extractions and complete removal of the interfacial material the same test was negative indicating the absence of DNA contamination in any detectable quantity (see Table 3).

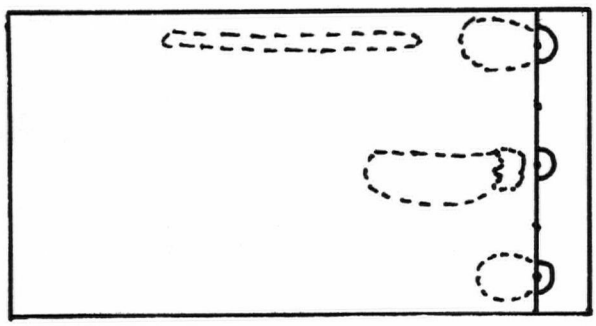
Since any possible role of carbohydrates such as mucopolysaccharides in induction is unknown it is considered imperative to remove all traces of these substances. That polysaccharide contamination exists, even after extensive

phenol extractions has been demonstrated by Kirby (1956, 1962) and Ralph and Bellamy (1964). Two methods were employed to indicate the presence of contaminating carbohydrates. One method was the Anthrone test of Dreywood (Morris, 1948), which is specific for carbohydrates. The RNA solution, after three phenol extractions, developed a blue-green colour with anthrone reagent at room temperature. The reading on the spectrophotometer at 620 mμ was 0.065 indicating the definite presence of carbohydrates. After methoxyethanol extraction the RNA solution demonstrated no colour change with the anthrone reagent. Thus the carbohydrate contamination appears to have been drastically reduced by the purification procedure.

A second method of demonstrating carbohydrate in the isolate was accomplished with paper chromatography. A chromatographic study of the isolated RNA before purification with 2-methoxyethanol demonstrated traces of polysaccharide and one spot corresponded to the control spot containing glucose (see figure 5). The second spot in the isolate may be ribose. Following extraction with methoxyethanol no trace of carbohydrate could be detected.

These results indicate that the isolate contains RNA in the absence of DNA, peptide, protein and carbohydrate contamination only after three phenol extractions and purification with methoxyethanol extraction. However, these data have given no clue as to the size or condition (degraded) of the molecules of isolated RNA.

To obtain information on the size and condition of both commercial liver s-RNA and Kirby isolated calf spleen



BEFORE METHOXYETHANOL EXTRACTION

GLUCOSE CONTROL

AFTER METHOXYETHANOL EXTRACTION

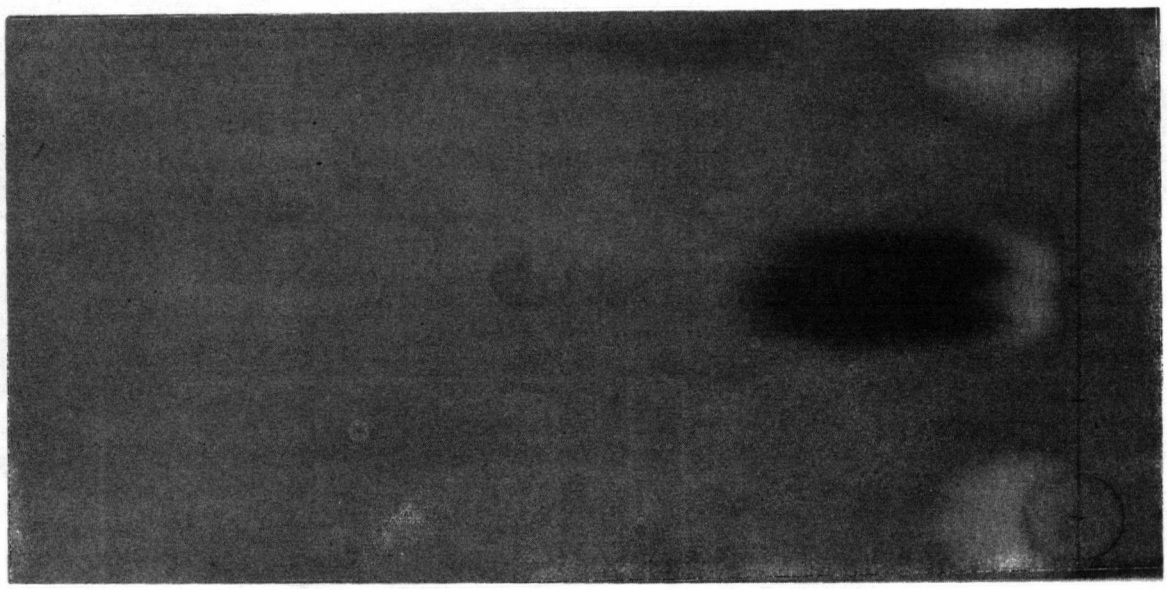
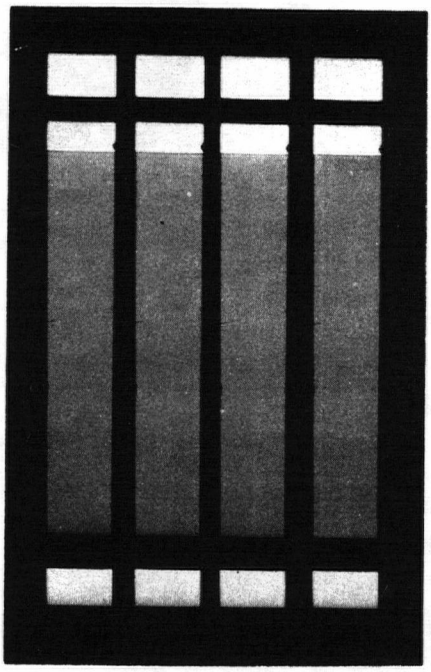


Figure 5. Chromatographic analysis of purified RNA solution before 2-methoxyethanol extraction. After the extraction no spot was observed.

RNA, analyses of these molecules were carried out using the analytical ultracentrifuge. The sedimentation patterns obtained by this procedure are shown in figures 6, 7, and 8.

The quantity of commercial s-RNA was small and thus uv optics were employed. The diagram obtained in figure 6 represents the liver RNA in 0.1 M. NaCl at 55,770 r.p.m. for 20 minutes. As can be seen from figure 6, there was no apparent movement of the molecules. This indicates either that the molecule of s-RNA was a large molecule which had become degraded, possibly by the isolation procedures employed or by methods of storage, or that the s-RNA molecule was quite small. The latter explanation would appear to be consistent with the present knowledge concerning the size of soluble RNA (Spirin, 1963).

The analysis of isolated calf spleen RNA using Schlieren optics demonstrated a definite molecular species (figure 7). The sedimentation coefficients expressed in Svedberg or S units for these molecules were calculated from the graph (figure 8) by the method of Markham (1960). The values for the 3 species of RNA were 27S, 18S, and 8S respectively, ($S_{20,w}$) for water at 20°C. The fastest moving component, the 27S fraction, was also present in the highest concentration. This sedimentation pattern for spleen RNA served to demonstrate that the isolated molecules were relatively intact and not degraded by the isolation procedure. It is this material which was used in the induction experiments.



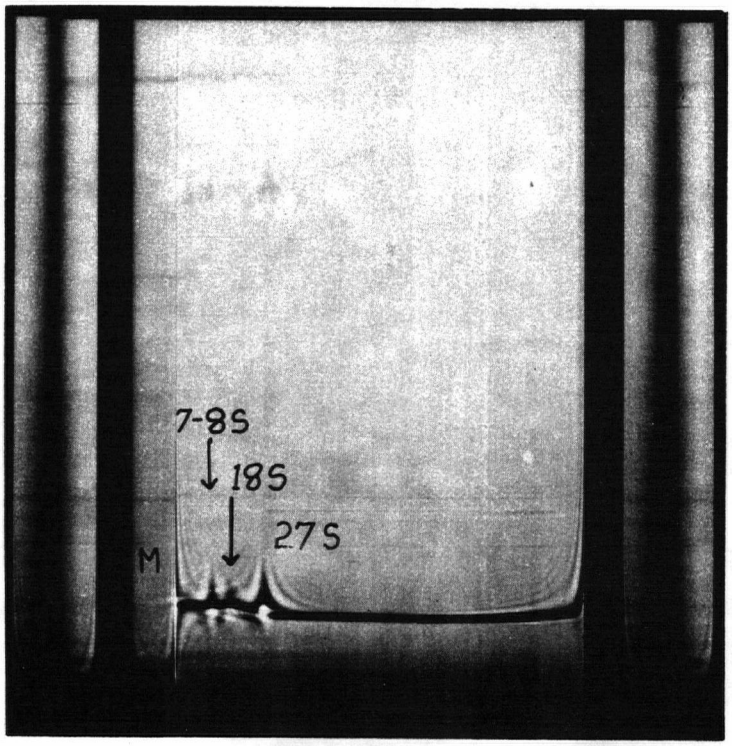

7-8S
18S
27S
M

Figure 6. Sedimentation diagram of commercial liver-RNA.

Figure 7. Schlieren photograph of a 0.1M 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 8S, 18S, and 27S.

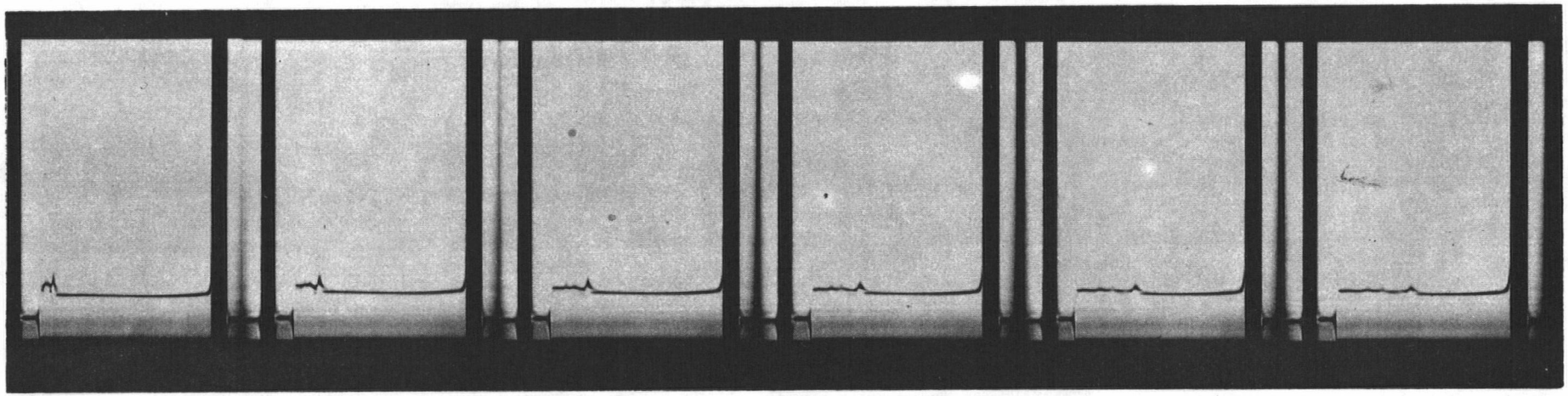


Figure 8. Sedimentation diagram of calf spleen RNA.

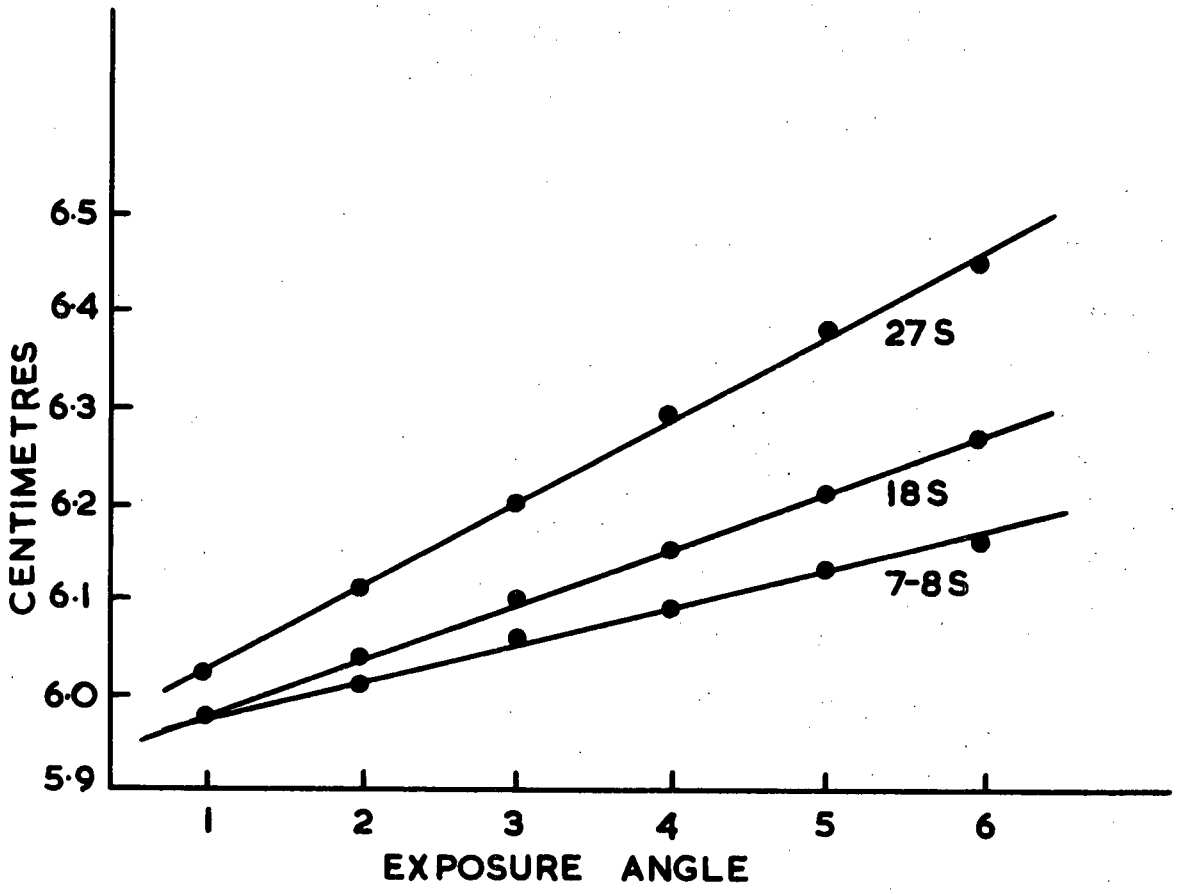

6·5
6·4
6·3
6·2
6·1
6·0
5·9
CENTIMETRES
27 S
18 S
7-8 S
1
2
3
4
5
6
EXPOSURE ANGLE

Figure 9. The combined graphs from the ultracentrifuge analysis of calf spleen RNA (fig. 7). The slope of the curves is a measure of the sedimentation coefficients, which is read off on a special transparent protractor designed by Markham (1960).

Part II. The *in vitro* and *in vivo* effects of calf spleen RNA on competent ectoderm and embryos of *Xenopus laevis*.

The isolated, undegraded and uncontaminated calf spleen RNA was tested for specific biological activity (i.e. induction) by exposing the tissues of *Xenopus laevis* embryos under both *in vitro* and *in vivo* conditions to the RNA at a concentration of 50 µg/ml. (Niu, 1958; Yamada, 1961).

1. *In vitro* studies.

When excised competent ectoderm from stage 9+ embryos was placed either in RNA-conditioned or control media there was no adhesion of the gastrula ectoderm to the glass coverslips in the culture dishes and within one-half an hour of the operation the ectodermal explant had rolled into a ball. These ectodermal explants cultured in the RNA-conditioned medium for 5 days demonstrated no specific induction. Neither were any indications of histogenesis evident in the control series cultured in the balanced salt solution which incidentally is deficient in both protein and RNA. However, the enhanced development of the explants in the conditioned medium was most evident (see figure 10 and Table 4a). When the sectioned materials were compared, the single-layered ciliated ectodermal cells of the explants in unconditioned medium contrasted with the superficial cells of the RNA-enhanced explants which were two to four layers in thickness and with certain of the cells appearing columnar rather than cuboidal. In addition ectodermal explants in RNA-medium were observed to survive better than those grown in the unconditioned medium.

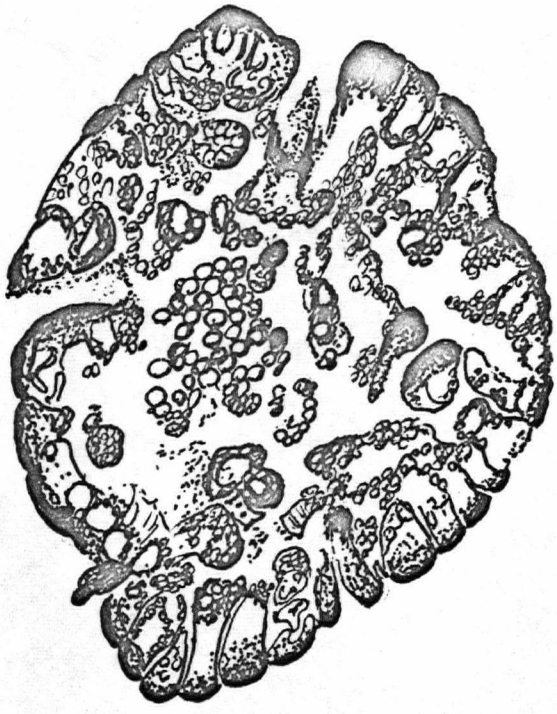


Figure 10. Isolated Xenopus ectoderm which had been cultured in a solution of calf spleen RNA for 5 days. No tissue-specific induction can be seen, but in contrast to the control series the cells are better developed and more numerous.

Table 4a. Effect of isolated calf spleen RNA on amphibian competent ectoderm *in vitro*.

	number of cases	inductive effect	no effect
controls	6	0	6
experimentals	18	0	18

N.B. Although 6 control animals and 18 experimental animals were observed throughout the experiment, only 2 of each series, chosen at random, were sectioned.

Table 4b. Effect of isolated calf spleen RNA on whole embryos of *Xenopus laevis in vivo*.

	number of cases	inductive effect	no effect
controle	8	0	8
experimentals	8	0	8

N.B. All animals in this series were sectioned.

2. *In vivo* studies.

Stage 9+ embryos were placed in RNA-medium and control medium and allowed to develop for 5 days. Embryos developed well in both control and conditioned media. However, the experimental animals were at all times slightly, but consistently, accelerated in organogenesis as compared to the control animals (see figures 11 and 12 and Table 4b). Also, while there was no increased histogenesis of spleen-like or mesodermal tissue in the experimental animals at the termination of the culture period, the otic vesicles demonstrated a more pronounced differentiation of the ventromedial epithelium and were becoming divided into the pars inferior and the pars superior, the torsion of the intestine was more obvious and the histological development of the gastric glands more distinct than in the controls.

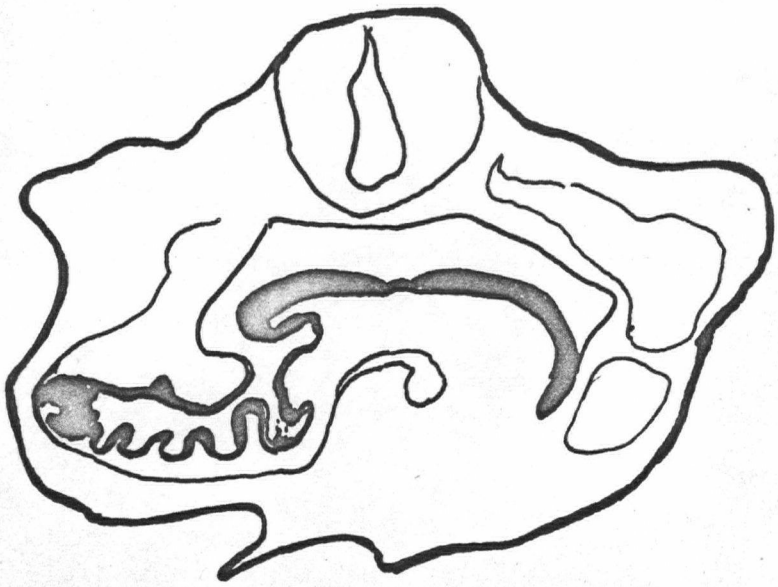


Figure 11. Section through control *Xenopus* embryo cultured in an "unconditioned" medium.

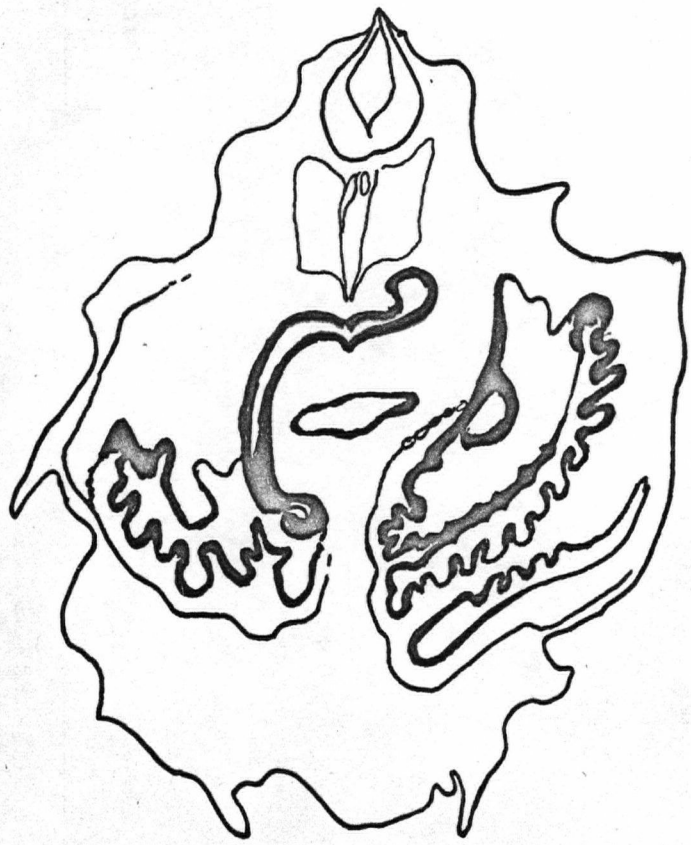


Figure 12. Section through experimental Xenopus embryo cultured in an RNA-enriched medium.

DISCUSSION

The isolation of calf spleen RNA by the Kirby two-phase partition system (1956) required modification to insure less contaminated and undegraded RNA molecules for the subsequent induction experiments since RNA isolated by this method has been shown to be contaminated with other components (Kirby, 1956, 1960, 1962; Ralph and Bellamy, 1964). Following the first phenol washing the aqueous phase can be demonstrated to contain RNA, polysaccharides (Kirby, 1956; and Table 4), protein (Ralph and Bellamy, 1964), amino acids (Niu et al, 1961), and DNA (Table 4). Any lipids which may be present in the isolate have been shown by Hayashi (1959) to be unimportant in induction and are not considered in this work.

In the present investigation an ultraviolet absorption analysis of isolates obtained with one phenol washing and with three phenol washings has demonstrated that protein and peptide c ontamination was considerably reduced with no significant reduction in RNA content (Table 2b and 2c). However, Huppert and Pelmont (1962), using the more sensitive method of Lowry for protein determination, found a protein content between 20 and 50 μg/ml. after three phenol extractions. Thus it is difficult to consider, as do many workers (Gierer, 1957; Gierer and Schramm, 1956a, b; and Kirby, 1962; Niu, 1958a, b; Niu et al, 1961; Hillman and Niu, 1963a, b; and, Butros, 1963), that protein or peptide contamination is completely absent for the isolated RNA even after three phenol washings. Very small quantities of protein would not be detected with the Biuret test and the ratios of the values

at 260 mμ/230 mμ and 260 mμ/280 mμ only serve to indicate the qualitative absence of peptides and proteins from the isolated sample. Certainly the low level of peptide and protein contamination possibly remaining after three phenol extractions in the current experiments was insufficient to act inductively. The DNA, present in the RNA solution after one phenol extraction and attributed to "phenolic nuclei" (Georgiev, 1960) in the interfacial material, is removed with the subsequent phenol extractions. Carbohydrates or polysaccharide contamination (see Figure 5) is not removed with phenol but with 2-methoxyethanol extraction.

Laskov et al (1959) found that RNA isolated by the phenol water method (Kirby, 1956; Gierer and Schramm, 1956a, b) resulted in degradation of the molecule. A higher molecular weight RNA was obtained if napthalene-1,5-disulfonate was employed in place of water. The instability of the isolated RNA molecule has been demonstrated by Huppert and Pelmont (1962), Kubinski and Koch (1963) and Amos and Moore (1963) who have shown that when the isolate was left at room temperature degradation began within 130 minutes and was drastic within 24 hours. After several days no sedimentable fractions were observable (Huppert and Pelmont, 1962). Equally pertinent, was the observation of Amos and Moore (1963) and Amos et al (1964) who reported that, while they were able to obtain an active proparation of RNA only irregularly, the active preparation retained its biological activity when stored at temperatures of -20°C. over a period of several weeks. As a result of these studies the spleen RNA utilised in the present experiments was employed

either immediately following isolation or within the next 7 days after storage at -20°C. on the assumption that the biological activity of the isolate, if existing, would have been maintained.

A possible source of RNA degradation is by ribonuclease activity during or after isolation procedures. Kirby (1956, 1960) demonstrated that ribonuclease was inhibited by phenol reagent, which apparently acts to denature the ribonuclease molecule. However, when the endogenous ribonuclease in the tissues has been destroyed by phenol activity there is still the danger of contamination from glassware used in the previous isolations. Extreme care was employed throughout the present isolations to reduce as much as possible any such sort of degradation. Since these experiments were terminated Ralph and Bellamy (1964) introduced a modification of the Kirby procedure in which RNA is recovered as the cetyltrimethylammonium salt. The modification is based on the finding of Dutta et al (1953) that a difference in solubility of the Cetavlon[4] salts of the nucleic acids permits the separation of RNA from DNA. This method of Ralph and Bellamy is rapid and removes the dialysis step which itself produces an opportunity for ribonuclease activity.

The sedimentation evidence, presented in figures 7, 8, and 9, demonstrates that the isolated spleen RNA was composed of three molecular species; a 27S fraction, an 18S fraction and an 8S fraction. The 27S and 18S fractions would appear to represent undegraded RNA molecules, the ribosomal fraction (Spirin, 1963). Huppert and Pelmont (1962) obtained RNA in two fractions when isolated from ascites tumor cells

and similar results were reported by Ralph and Bellamy (1964) with RNA isolated from rat liver, chinese cabbage, and tobacco cells; by Hall and Doty (1959) with calf liver microsomes; and by Cheng (1959, 1960) with mouse brain. Jiang (1964), isolating ribonucleic acid from mammalian pituitaries demonstrated three fractions, 28S, 18S, and 4S. These values correspond to those obtained in the present analysis so that the 8S fraction found here may not be a degradation product. The sedimentation coefficients of the ribosomal RNA (27S and 18S) appear to differ slightly depending upon the biological source of the preparation (Spirin, 1963). Demonstration of the two high molecular weight species of RNA from calf spleen isolate tends to support the idea that the RNA molecules obtained from the modified procedure were intact and undegraded when introduced into culture medium for the induction experiments.

The procedure for the isolation of ribonucleic acid as employed by Niu (1958 a,b), Niu et al (1961), Hillman and Niu (1963 a,b), Yamada (1961), and Butros (1963) were essentially the method proposed by Kirby in 1956, which has been demonstrated (Laskov et al., 1959) to yield a rather degraded product. The shorter procedure also used by these workers, in which the methoxyethanol purification procedure was deleted, would tend to reduce the amount of degradation of RNA but since only one phenol extraction was performed the protein and peptide as well as the polysaccharide contamination of their product might be significant. Niu (1958b) dialysed his sample against running water overnight, a procedure that may allow considerable ribonuclease

contamination, and apparently no attempt was made to remove polysaccharide material even though liver and muscle tissues were the source material used.

In the present experiments calf spleen tissue was utilized as the source tissue because it is an organ of completely mesodermal origin with a large quantity of nucleic acid and because Yamada (1961) and Yamada and Takata (1961) were able to obtain such definite inductions using the RNP and protein fractions of bone marrow tissue. However, both the *in vitro* and *in vivo* results have indicated the absence of any tissue-specific (i.e. mesodermal) inductive response when isolated and purified calf spleen RNA was presented to competent embryonic material. Niu *et al.* (1961) and Amos and Moore (1964) have demonstrated that RNA molecules are taken up by embryonic cells.

Since no inductive effect was observed it is necessary to consider as a possible factor the exposure time of the test material. In the first place, 5 days of culture appeared sufficient to result in any observable histogenesis since the tissues and embryos of *Xenopus laevis* are exceedingly rapid in their development. With regard to the initial exposure time, Yamada (1962) has demonstrated that a 55% inductive response was achieved in amphibian ectoderm after 30 minutes exposure to a protein medium and that this could be increased to 94% with a 180 minute treatment. Therefore, induction under these test conditions should occur within the first hour(s) of exposure of the competent tissue to the inducer. It seems likely that it is precisely during this time when intact RNA would be present in the test

medium since Huppert and Pelmont (1962) have shown that degradation of isolated RNA is only initiated within the first 130 minutes at room temperature (20°C) and these cultures were maintained at a temperature of 12°C. This would tend to decrease the rate of degradation so that total degradation of the RNA molecules in the test medium would not be complete for more than 24 hours. Thus, at some time after introducing the intact RNA molecules and the competent ectoderm into the medium, and after the time when the RNA induction could have occurred, there probably would be present a solution of nucleotides and nucleosides instead of the intact RNA molecules. While the presence of associated protein in the test media might function as suggested by Niu (1958b) to stabilize the RNA molecule for a sufficient time in an inductive system, it is also possible that the *in vitro* induction observed by Niu and Twitty (1953) and Niu (1958b) may have been the result of a specific response by competent tissue to a high concentration of oligonucleotides (Lash *et al*, 1962) or RNP (Yamada, 1958, 1961, 1962) present in the conditioned medium.

While no induction was evidenced in the present investigation, there was observed an enhancement of tissue development both in the ectodermal explants and in the whole embryos. This effect with spleen RNA *in vitro* and *in vivo* appears to be similar to the results described by Ambellan (1955, 1958, 1962. 1963) in which solutions of various nucleotides were found to accelerate neural-tube formation in amphibian embryos. A similar mechanism may be operating in the enhancement of differentiation achieved with pancreatic, heart and brain RNA by Butros (1963). This

enhancement of development may have been the result of tissue exposure to both intact RNA and RNA fragments (i.e. nucleotides) since both would be present in the early period of these experiments.

Finally it must be noted that the absence of specific inductive effects in any of these test media may have been due to the absence of a biologically "active" preparation as suggested by Amos and Moore (1963) and Amos et al.(1964).

SUMMARY

1. Ribonucleic acid (RNA) from calf spleen tissue was isolated and purified by a modified Kirby procedure.
2. The ultraviolet absorption spectrum of the RNA solution was measured. The absorption maximum occured at 260 mμ indicating the presence of nucleic acids while the absorption minima observed at 230 mμ and 280 mμ indicated the absence of peptides and proteins.
3. Colorimetric analyses indicated the absence of peptides, proteins, DNA, and carbohydrates as well as the presence of RNA.
4. Chromatographic analysis indicated that the samples of isolated RNA contained traces of carbohydrate before extraction with methyoxyethanol but were free from carbohydrate contamination after the extraction.
5. A sedimentation analysis was performed which indicated that the isolate contained three RNA fractions: a 27S fraction, an 18S component and an 8S component. It was demonstrated that the RNA isolated in the present experimental series was undegraded.
6. Competent ectoderm excised from stage 9+ Xenopus laevis and cultured in RNA-conditioned medium demonstrated no tissue-specific induction but did demonstrate enhanced development when compared to the control series.
7. Similarly, when stage 9+ Xenopus were grown in RNA-enriched medium the experimental animals were slightly advanced in development over the control animals. Again, no tissue-specific induction was observed.

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Appendix I

Extraction of calf spleen RNA (Kirby, 1956, 1962)

1. Isolation of spleen RNA.

The spleen tissue was homogenized in 0.015 M. naptha-lene-1,5-disulfonate (at a pH of 6.8 to 7.0) and water saturated phenol (2.5 ml. of 0.015 M. napthalene disulfonate and 2.5 ml. of 88-90% phenol / gram wet weight of tissue) for 2 minutes in a Waring blender at room temperature. The homogenate was filtered through a single layer of gauze in a Buckner funnel. This mixture was stirred for 30 minutes and centrifuged for 1 hour at 0°C at 2,000 r.p.m. The precipitate obtained was washed with 100 ml. of 0.015 M. napthalene disulfonate and centrifuged for 15 minutes at 5,000 r.p.m. This washing procedure was repeated at least twice. The cloudy supernatant layers from the original centrifugate and the washings were carefully suctioned off to insure against contamination from the intermediate layer (see figure 1.). The supernatant layers were pooled together and were washed with 0.5 to 1 volumes of phenol and shaken gently for 10 minutes before centrifuging at 5,000 r.p.m. for 15 minutes. The aqueous layers were removed and made up to 2% with respect to potassium acetate ($K^+CH_3COO^-$). The RNA was precipitated from the aqueous layer with 2 volumes of cold 95% ethanol and centrifuged at 2,000 r.p.m. for 20 minutes. The aqueous layer was discarded and the precipitate washed in an ethanol-distilled water solution (3:1). The precipitate was finally dissolved in 0.1 M. NaCl salt solution (pH at 7.0 to 7.2). Phenol was removed by three consecutive extractions with equal volumes of ether.

Ether was expelled by passing nitrogen gas through the aqueous layer.

2. Purification of spleen RNA.

Equal volumes of 2.5 M. K_2HPO_4, redistilled 2-methoxyethanol* and 0.05 vol. of 33.3% H_3PO_4 (upper phase, lower phase 5:1) were added to the aqueous preparation of spleen RNA. The lower layer was separated and washed with 10-20 ml. of the top layer from a mixture of 2-methoxyethanol-distilled water-2.5 M. K_2HPO_4-33.3% H_3PO_4 (1:1:1:0.05) by volume. The combined top layers contained all the RNA. The clear supernatant layers were added together and these were dialysed against 2 liters of distilled water (changed twice) for 6 to 12 hours in the cold at 4-6°C. The dialysate was centrifuged, made up to 2% with respect to potassium acetate and precipitated by 2 volumes of 95% ethanol. The precipitate was washed in ethanol and the precipitated spleen RNA stored in 95% ethanol at -20°C.

*methoxyethanol = b.p. 122-124°C.

Appendix II.

1. The Biuret method for protein. (Schneider, 1957).

1.5 g. of cupric sulfate ($CuSO_4 \cdot 5H_2O$) and 6.0 g. of sodium potassium tartrate ($Na\ KC_4H_4O_6 \cdot 4H_2O$) were dissolved in 500 ml. of distilled water. 300 ml. of 10% NaOH was added to this with constant swirling and the mixture was then diluted to 1 liter. This served as biuret reagent.

4.0 ml. of biuret reagent was added to 1 ml. of solution containing RNA and the mixture was allowed to stand at room temperature for 30 minutes. A control consisted of 4.0 ml. of biuret reagent and 1.0 ml. of water. The absorption was read between 540 mμ and 560 mμ.

2. Orcinol test for RNA. (Schneider, 1957; Chargaff and Davidson, 1955).

1.0 g. of purified orcinol was dissolved immediately before use in 100 ml. of concentrated HCl containing 5 g. of $FeCl_3$. 0.2 ml. of RNA solution was diluted to 1.5 ml. and heated with 1.5 ml. of orcinol reagent for 20 minutes in boiling water. The intensity of the green colour at a wavelength of 660 mμ was read on the spectrophotometer.

3. Dische's diphenylamine test for DNA. (Schneider, 1957; Chargaff and Davidson, 1955).

1.0 g. of purified diphenylamine was dissolved in 100 ml. of reagent glacial acetic acid and 2.75 ml. of reagent sulfuric acid. 10 ml. of the solution containing RNA was mixed with 2 ml. of diphenylamine reagent and heated for 10 minutes in boiling water. The intensity of the blue colour at 595 mμ was read on the spectrophotometer.

4. Dreywood's anthrone test for carbohydrates. (Morris, 1948).

1.0 g. of anthrone was dissolved in 500 ml. of 95%

sulfuric acid (prepared by addition of 500 ml. of concentrated reagent sulfuric acid to 25 ml. of distilled water, and allowing the mixture to cool). 1 ml. of the RNA solution was mixed with 2 ml. of the Anthrone reagent and the mixture was permitted to stand at room temperature for 10 minutes. The intensity of the blue colour at 650 mμ was read on the spectrophotometer.

Appendix III

Chromatographic analyses.

The solvent used was n-butanol saturated with water which also contained 2.5 g. of phthalic acid in 100 ml. 1 volume of phthalic acid solution was shaken with 4 volumes of butanol and the butanol layer was separated to be used in the chromatography jar.

After the run, the Whatman #1 paper was sprayed with analine (2% in ethyl ether), allowed to dry at room temperature and then heated to 100°C in an oven to develop the spots.

Appendix IV

Ultracentrifugal analyses.

Samples of RNA to be analyses were dissolved in 0.1 M. NaCl solution. The temperature throughout the analyses was 22.4°C.

The sedimentation coefficients* were read off the ultracentrifuge plates (see figures 7, 8, and 9) and plotted on logarithmic graph paper according to Markham (1960, 1962). The protractor (designed by Markham) was placed on the graph paper with its abscissa scale along a horizontal graph line, with the line corresponding to the rotor speed at the intersection of the experimental curve with the latter. The experimental curve then intersects the ordinate scale of the protractor at the correct S value.

*The sedimentation coefficient of S of ribonucleic acid is the rate at which it would sediment through the suspending medium in a field of 1 dyne (or 1/981 of 1.0 gram) in centimeters per second, and is generally referred to water at 20°C. ($S_{20,w}$). A more useful term or unit is the Svedberg or S value which is 10^{-13}cm./sec./dyne. (Markham, 1962). Svedberg = S.

Appendix V

Preparation of modified Niu-Twitty medium (Douglas and Finnegan, unpublished).

Control medium; a defined medium was prepared as follows:

NaCl	3.400g.
KCl	0.050g.
$CaNO_3 \cdot 4H_2O$	0.080g.
$MgSO_4 \cdot 7H_2O$	0.100g.
1 N. HCl	4 ml.
glass distilled water	996 ml.
Tris* buffer	0.560g.

The 1,000 ml. solution was boiled for not less than 2 minutes and then cooled to room temperature. 2.5 mg. of streptomycin sulfate was added to the medium which was then stored for as short a period as possible at 7°C.

*Tris = hydroxy methyl amino methane or 2-amino-2-hydroxy-methyl-1,3-propanediol).