# Transcription of the Genes for 5S Ribosomal RNA and Transfer RNA in Isolated Mouse Myeloma Cell Nuclei†

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ABSTRACT: Nuclei isolated from mouse myeloma cells synthesize RNA for an extended period of time at 25°. Two distinct products have been identified, 5S rRNA and 4.5S precursor to tRNA. They have been identified by polyacrylamide gel electrophoresis in native and denaturing conditions and by RNA-DNA hybridization to partially purified 5S and 4S DNA. In addition, the 4.5S RNA may be converted *in vitro* to 4S RNA. These RNAs, while a minor component of the RNAs made *in vitro*, are made in similar proportions to that found *in vivo*. Although they are transcribed from nucleoplasmic genes, they are not transcribed by the major nucleoplasmic polymerase, the α-amanitin sensitive RNA polymerase II. These species are initiated *in vitro*, as judged by the incorporation of [γ-$^{32}PO_4$]GTP.

Study of transcription and maturation of specific RNA species *in vitro* requires a system which synthesizes RNA for an extended period of time. In addition, the system must continually reinitiate and complete the synthesis of defined RNA species. Isolated cell nuclei provide such an opportunity to study both transcription and maturation of specific RNA species *in vitro*.

Limited transcription of the rRNA species in isolated nuclei has been reported by Zylber and Penman (1971) and Reeder and Roeder (1972). Both concluded that the 45S rRNA precursor is transcribed by RNA polymerase I. However, the nuclei apparently only completed the RNA chains which were initiated *in vivo*.

Further information on RNA synthesis *in vitro* has come from the study of virus-infected cells. Isolated nuclei from adenovirus-infected HeLa cells synthesize adenovirus-specific RNA (Price and Penman, 1972a,b; Wallace and Kates, 1972). The majority of the adenovirus mRNA molecules species are transcribed by RNA polymerase II. However, one low molecular weight adenovirus RNA species was not transcribed by RNA polymerase II, but possibly by RNA polymerase III (Price and Penman, 1972b).

Previously (Marzluff *et al.*, 1973), we reported that isolated myeloma cell nuclei are capable of synthesizing RNA for an extended time. Among many different species of RNA synthesized by these nuclei *in vitro* we report here the transcription of two classes of nucleoplasmic genes, 5S rRNA and the 4.5S RNA, a precursor to tRNA (Bernhardt and Darnell, 1969; Choe and Taylor, 1972). Only the correct strand of the DNA was transcribed *in vitro*. In addition, the 4.5S RNA may be converted *in vitro* to 4S RNA, identical with tRNA by gel electrophoresis.

## Materials and Methods

*Isolation of Nuclei.* Myeloma 66-2 cells were grown to a density of (3–5 × $10^5$/ml) and nuclei were isolated as previously described (Marzluff *et al.*, 1973). The conditions for RNA synthesis were the same as described previously. [$^3$H]GTP was used as the RNA precursor in all experiments.

*Analysis of RNA.* RNA was prepared and analyzed on sucrose gradients and polyacrylamide gels as previously described (Marzluff *et al.*, 1973). For analysis in the presence of formamide, polyacrylamide gels (12% acrylamide, 0.6% bisacrylamide) were prepared in 70% formamide, 0.04 M Tris-acetate (pH 7.2) and 0.002 M EDTA. The RNA was dissolved in 95% formamide and warmed to 37° for 5 min. Electrophoresis was for 18 hr at 70 V at 30°. The running buffer was 0.04 M Tris-acetate (pH 7.2), 0.002 M EDTA, and 70% formamide.

*Preparation of DNA.* 5S DNA was prepared from *Xenopus laevis* as described by Brown *et al.* (1971). After the first Ag-$Cs_2SO_4$ gradient, the 5S DNA containing fractions were centrifuged in actinomycin D-CsCl. The 5S DNA strands were separated by centrifugation in alkaline CsCl (Brown *et al.*, 1971). DNA complementary to mouse 4S tRNA was also present in the *Xenopus laevis* genome and was separated as a by-product of the 5S DNA preparation.

The *Xenopus* tRNA genes have been previously shown to be physically linked (Brown and Weber, 1968, Clarkson *et al.*, 1973). Mouse tRNA cross-reacted with the *Xenopus* DNA. In the $Ag^+$-$Cs_2SO_4$ gradients, the 4S DNA was lighter than the main band DNA (main band fractions 21–24, 4S fractions 13–17, rDNA fractions 11–15, 5S fractions 6–10) and slightly denser than rRNA. The fractions containing the ribosomal and 4S DNA were pooled and centrifuged to equilibrium in CsCl gradients containing actinomycin D (2:1 w DNA/ actinomycin D). The 4S DNA banded at a lower density in these gradients than main band DNA (main band fractions 14–17, 4S fractions 10–13, 5S fractions 5–9, and ribosomal fractions 19–23), while the rDNA banded at a greater density. It is not known how many of the different mouse tRNA species are involved in the hybridization reaction.

*RNA-DNA Hybridization.* DNA was denatured and immobilized on nitrocellulose filters (B-6, Schleicher and Schuell). Hybridization was carried out in 2 or 4 × SSC, 50% formamide at 37° for 18 hr. Filters were washed two times (30 ml/filter) under hybridization conditions and twice more in 2 × SSC for 10 min each wash. In the hybridization reaction a small amount of electrophoretically purified, labeled 5S or 4S RNA was included as an internal standard to determine the hydridization efficiency (Reeder and Roeder, 1972).

*Purification of Marker RNAs.* RNAs labeled with either [$^3$H]uridine or $^{32}PO_4$ were prepared from cells labeled for one

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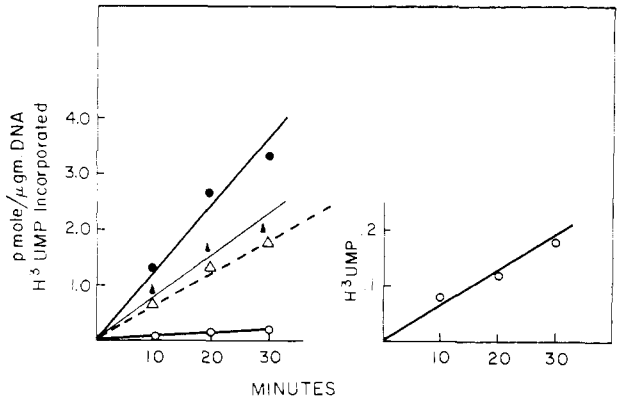


FIGURE 1: Synthesis of RNA in isolated nuclei. Nuclei were prepared from control cells and from cells which had been preincubated for 30 min with 0.04 μg/ml of actinomycin D. 2 × $10^5$ nuclei were incubated at 25°. α-Amanitin where present was at 5 μg/ml. (● — ●) control nuclei; (▲ — ▲) + α-amanitin; (Δ - -Δ) preincubated with actinomycin; (○ — ○) preincubated with actinomycin D + α-amanitin (inset).

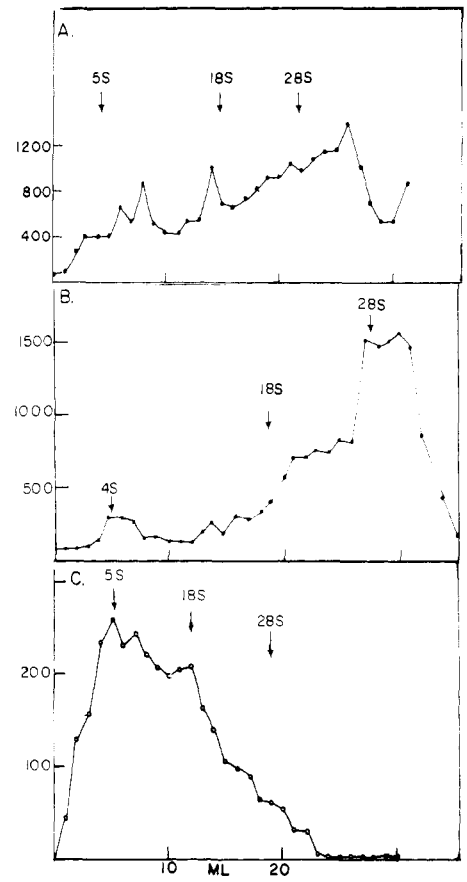


FIGURE 2: Sucrose gradient analysis of RNA synthesized *in vitro*. RNA synthesized *in vitro* for 30 min at 25° was analyzed by sucrose gradient centrifugation in the SW 25.3 rotor for 19 hr (A and C) or 21 hr (B) at 22,500 rpm at 21°; 0.025 ml of each 0.5-ml fraction was taken for determination of trichloroacetic acid precipitable counts. (A) control nuclei; (B) + α-amanitin; (C) preincubated with actinomycin D + α-amanitin.

generation in the presence of the appropriate precursors. 5S RNA was purified from purified ribosomes by sucrose gradient centrifugation followed by polyacrylamide gel electrophoresis. 4S TRNA was prepared from the post-ribosomal supernatant by sodium dodecyl sulfate–phenol extraction, followed by polyacrylamide gel electrophoresis.

Unlabeled RNA used as competitor in hybridization experiments were similarly prepared by gel electrophoresis.

*Preparation of 4.5S RNA "Maturation" Enzyme.* Cells were swollen in hypotonic buffer (0.025 M KCl, 2 mM MgOAc, 3 mM $CaCl_2$, 10 mM Tris (pH 8), and 1 mM dithiothreitol) for 5 min at 4°; 2.2 M sucrose was added to a final concentration of 0.30 M and the cells were broken by homogenization in a Dounce homogenizer (10 strokes with B pestle). Crude nuclei were prepared by centrifugation at 1000*g* for 10 min. The nuclei were suspended in 25% glycerol, 0.1% Triton X-100, 0.15 M KCl, 5 mM MgOAc, 1 mM dithiothreitol, and 10 mM Tris (pH 8) and recentrifuged. The enzyme was found quantitatively in the supernatant. Two volumes of enzyme solution were mixed with one volume of labeled 4.5S RNA and 40 μg of 28S RNA in water and the reaction was incubated for 20 min at 37°. The RNA was purified by extraction with sodium dodecyl sulfate–phenol at pH 5 and analyzed by polyacrylamide gel electrophoresis.

*Chemicals.* α-Amanitin was obtained from the Henley Co. [$^3$H]GTP (15 Ci/mmol) was obtained from Amersham Searle. [γ-$^{32}PO_4$]GTP was prepared by the method of Glynn and Chappell (1964). [α-$^{32}PO_4$]GTP was obtained from New England Nuclear.

## Results

Previously (Marzluff *et al.*, 1973), we have described an *in vitro* system which actively synthesized RNA for at least 30 min at 25°. Here we characterize two distinct low molecular weight RNA species synthesized *in vitro*. They were identical with 5S rRNA and the 4.5S precursor to tRNA and tRNA by several criteria. These species were repeatedly transcribed *in vitro*, new chains being continually reinitiated during the incubation period.

*Effect of Preincubation with Actinomycin D on Nuclear RNA Synthesis.* Nuclei were prepared from cells preincubated with 0.04 μg/ml of actinomycin D for 30 min. This treatment specifically inhibited the synthesis of 45S ribosomal precursor RNA (Perry, 1962) in HeLa cells and also in the myeloma cells. Nuclei prepared from cells preincubated in actinomycin D synthesized RNA 60% as efficiently as nuclei from control cells (Figure 1). α-Amanitin inhibited the nuclei from preincubated cells to 5–10% of the control value, although the residual synthesis followed the same kinetics at 25° (Figure 1, inset). Thus three populations of RNA synthesis could be defined by these inhibitors, 40–50% was sensitive to low concentrations of actinomycin D and presumably represented 45S ribosomal precursor RNA, an additional 40–50% was transcribed by RNA polymerase II and was sensitive to α-amanitin (Roeder *et al.*, 1970), and a third population (5–10%) was insensitive to both inhibitors. Previously, we have presented evidence that the RNA species synthesized *in vitro* and sensitive to α-amanitin may include the mRNAs (Marzluff *et al.*, 1973).

RNA synthesized under various conditions was analyzed by sucrose gradient centrifugation (Figure 2). α-Amanitin inhibited primarily the synthesis of the 8–18S RNA species and most of the RNA synthesized under these conditions was of high molecular weight (>28 S). In contrast, RNA synthesized in the presence of both inhibitors was of low molecular weight and no high molecular weight RNA was synthesized.

*Polyacrylamide Gel Electrophoresis of Low Molecular Weight RNA.* The low molecular weight RNA species made *in vivo* in a short pulse with [$^3$H]uridine are shown in Figure 3. The major species synthesized in a 10-min pulse was 4.5S RNA. This RNA was apparently a precursor to the 4S tRNA which accumulated after a 30-min labeling (Figure 3b) (Bernhardt and Darnell, 1969; Choe and Taylor, 1972). 5S rRNA

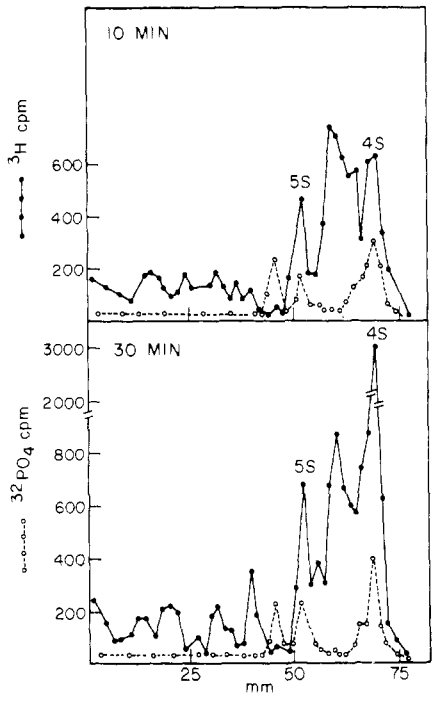


FIGURE 3: Low molecular weight RNA synthesized *in vivo*. 66-2 cells were concentrated to 1.5 × $10^6$/ml and labeled with [$^3$H]uridine (20 μCi/ml) for either 10 or 30 min. Total cell RNA was prepared by sodium dodecyl sulfate–phenol extraction at pH 5. The low molecular weight RNA (4–8 S) was prepared by sucrose gradient centrifugation and analyzed by polyacrylamide gel electrophoresis. $^{32}PO_4$-labeled 5S and 4S RNA was added to each sample. (Top) 10 min; (bottom) 30 min; (● — ●) $^3$H cpm; (○ - - ○) $^{32}PO_4$ cpm.

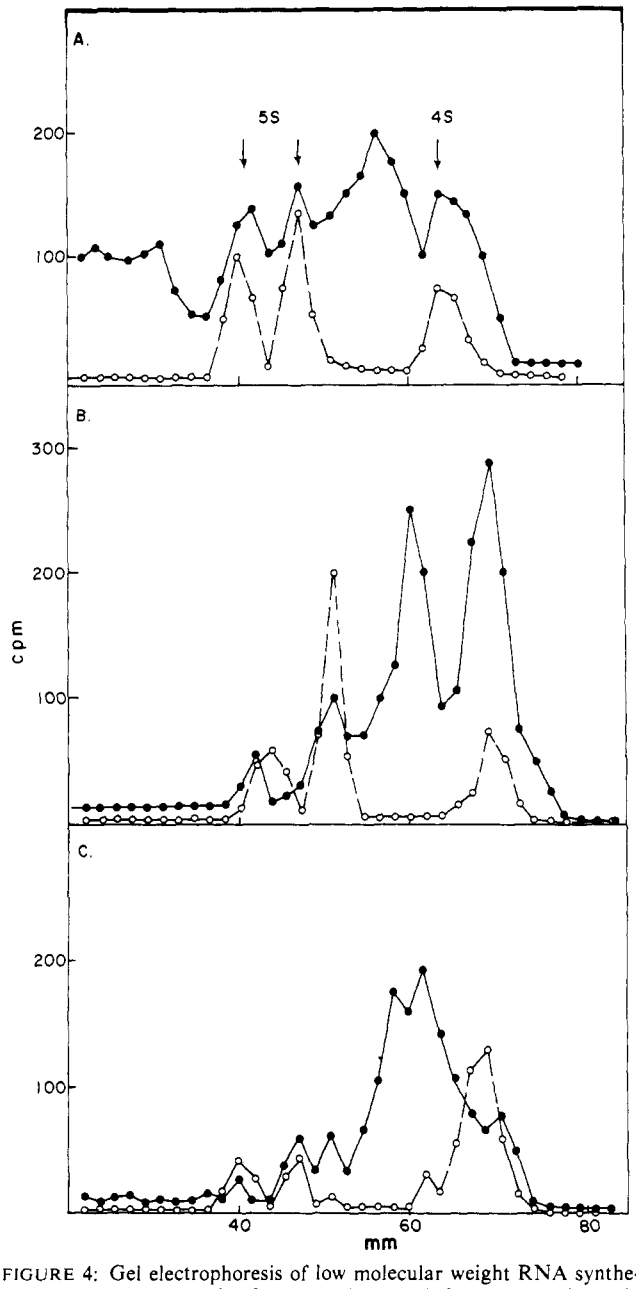


FIGURE 4: Gel electrophoresis of low molecular weight RNA synthesized *in vitro*. The 4–8S RNA (fractions 2–8) from the gradients in Figure 3 was analyzed by polyacrylamide gel electrophoresis in the presence of $^{32}PO_4$-labeled 5S and 4S RNAs. Note that 5S RNA migrates as two discrete conformers under these conditions. The slower moving conformer was found if the RNA was exposed to high concentrations of EDTA during preparation and also the proportion was increased upon heating the RNA. (● - - ●) $^3$H *in vitro* product; (○ - - ○) $^{32}PO_4$-labeled RNA markers; (A) control nuclei; (B) + α-amanitin; (C) preincubated with actinomycin D + α-amanitin.

synthesis was also apparent during this time period. After a longer labeling time, other low molecular weight nuclear RNAs became significantly labeled. Although 5S rRNA and 4S tRNA normally accumulated *in vivo* in parallel with the ribosomal 18S and 28S RNA, their synthesis was unaffected by low concentrations of actinomycin D, in contrast to its affect on the other rRNAs. In fact, excess 5S RNA accumulated in the nuclei, presumably due to inhibition of new ribosome formation.

The low molecular weight RNAs synthesized *in vitro* were analyzed by polyacrylamide gel electrophoresis. Species identical with 4S tRNA and 5S rRNAs were present (Figure 4). In addition a peak identical in mobility with the putative 4.5S precursor to tRNA was present. α-Amanitin had no effect on the synthesis of these RNAs (Figure 4b). These low molecular weight species were the major species made in nuclei prepared from cells preincubated with actinomycin D and incubated in the presence of α-amanitin (Figure 4c). The proportions of 4.5S and 4S RNAs made varied greatly from preparation to preparation presumably due to the different amounts of an enzyme(s) necessary to mature the 4.5S RNA to 4S RNA in different preparations (see below). The 5S peak was considerably smaller than the 4.5S and 4S peaks. Its total content was only 10–20% of the 4.5S plus 4S RNAs similar to the relative proportions found *in vivo* (Figure 3).

To further establish that the 5S, 4.5S, and 4S RNAs synthesized *in vitro* were identical with those found *in vivo*, the RNA products were isolated from the gels and reanalyzed on acrylamide gels equilibrated with 70% formamide. Separation of RNA species should be due solely to chain length and independent of conformation in 70% formamide (Suzuki *et al.*, 1972). The *in vitro* synthesized 5S RNA migrated identically to 5S rRNA in formamide and the 4.5S and 4S RNA migrated similarly to those species also (Figure 5). This result indicated that the 4.5S RNA was actually longer (10–20 nucleotides) than mature tRNA and not simply an unfolded precursor with the same molecular weight.

*Identification of RNAs by RNA–DNA Hybridization.* 5S RNA was further identified by RNA–DNA hybridization, using 5S DNA prepared from *Xenopus laevis* erythrocytes (Brown *et al.*, 1971). The mouse 5S RNA was sufficiently similar to *Xenopus* 5S DNA to use RNA–DNA hybridization to assay for 5S RNA sequences. The DNA did not bind 28S or 18S rRNA or 4S tRNA. In addition, it hybridized only to 5S RNA among the mouse low molecular weight nuclear RNAs

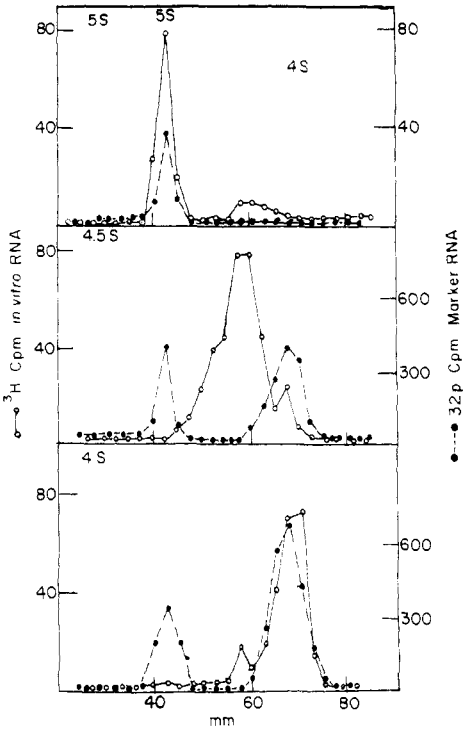


FIGURE 5: Analysis of RNA by electrophoresis in 70% formamide. RNA samples from Figure 4B were eluted from the gels, dissolved in 70% formamide, and analyzed by electrophoresis in polyacrylamide gels polymerized in 70% formamide. Electrophoresis was for 16 hr at 70 V at 27°. $^{32}$P-labeled 5S and 4S were added to the 4.5S sample and 4S sample as markers. Note that 5S RNA is a single sharp component under these conditions. (O — O) $^3$H, *in vitro* product; (● - - ●) $^{32}$P RNA markers; (top) 5 S; (middle) 4.5 S; (bottom) 4 S.

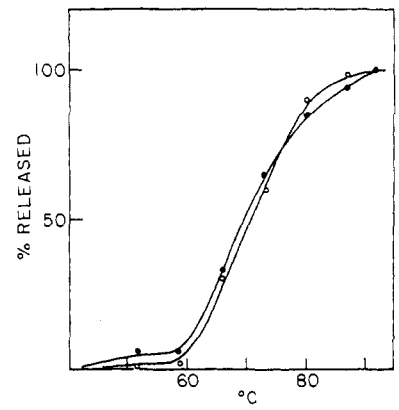


FIGURE 6: Melting of 5S RNA hybridized with 5S DNA. The filters from Table I, 1.6 were incubated in 0.1 × SSC at the indicated temperatures for 2 min and $Cl_3CCOOH$ precipitable counts released determined at each temperature. (O - O) $^{32}PO_4$-labeled 5S marker; (● - ●) $^3$H-labeled 5S synthesized *in vitro*.

(Table I, 1-3). By adding a small amount of $^{32}PO_4$-labeled 5S rRNA to the unknown sample, the amount of $^3$H-labeled 5S RNA in the sample can be estimated (Reeder and Roeder, 1972). 5S RNA sequences were found only in the 4-8S region in *in vitro* synthesized RNA (Table I, 4 and 5). Moreover, RNA from the 5S region of the gel hybridized efficiently with 5S DNA (Table I, 6). The amount of 5S RNA calculated from the hybridization data was similar to that found by polyacrylamide gel electrophoresis. The strands of 5S DNA were separated in alkaline CsCl (Brown *et al.*, 1971). RNA synthesized *in vitro* was complementary only to the + strand, the same strand which is transcribed *in vivo* (Table I, 7 and 8). The hybrid melted at the same temperature as mouse ribosomal 5S RNA hybridized with 5S DNA (Figure 6).

From these data 5S RNA was calculated to be about 0.5% of the RNA synthesized *in vitro*. In nuclei from cells preincubated with actinomycin D and incubated in the presence of α-amanitin, 5% of the RNA made was 5S RNA. *In vivo*, 0.7-1.0% of the RNA synthesized in 10 or 30 min was 5S RNA as judged by polyacrylamide gel electrophoresis.

*Xenopus laevis* DNA complementary to 4S tRNA also hybridized to mouse 4S tRNA. The partially purified 4S DNA did not hybridize with either 5S or rRNA (Table II, 1 and 3). However, 4.5S RNA made *in vitro* or *in vivo* in a 20-min pulse hybridized to the 4S DNA as or more efficiently than 4S tRNA (Table II, 3 and 4). The hybrid with 4.5S RNA melted at a higher temperature and more sharply ($T_M$ = 74°, range

TABLE I: Hybridization to *Xenopus* 5S DNA.[a]

| RNA Sample | 5S DNA | Input RNA $^3$H (cpm) | Input RNA $^{32}$P-labeled 5 S (cpm) | Bound RNA $^3$H (cpm) | Bound RNA $^{32}$P (cpm) | Competed with 5 S (3 μg) $^3$H (cpm) | Competed with 5 S (3 μg) $^{32}$P (cpm) | % 5 S in Sample Hybrid | % 5 S in Sample Gel |
|---|---|---|---|---|---|---|---|---|---|
| *in vivo* 4 S | Total | 35,000 | | 20 | | | | | |
| *in vivo* 28 S | Total | 20,000 | | 0 | | | | | |
| *in vivo* nuclear 4-8 S | Total | 30,000 | 1400 | 885 | 147 | 70 | 20 | 29 | 24 |
| *in vitro* 4-8 S[a] | Total | 35,000 | 2000 | 97 | 138 | 7 | 8 | 4 | 5.5 |
| *in vitro* 12-18 S* | Total | 15,000 | 1750 | 2 | 350 | 0 | 20 | 0.2 | 0 |
| *in vitro* 5 S* | Total | 3,000 | 4000 | 86 | 173 | | | 70 | 90 |
| *in vitro* 4-8 S** | + strand | 33,000 | 3000 | 80 | 50 | | | 14 | 10 |
| *in vitro* 4-8 S** | − strand | 33,000 | 3000 | 3 | 0 | | | | |

[a] RNA samples were hybridized to 5S DNA as described in Materials and Methods. $^{32}PO_4$-labeled 5 S (250,000 cpm/μg) was prepared by gel electrophoresis as was the ribosomal and tRNAs used (300,000 cpm/μg). The *in vitro* samples used were prepared from nuclei incubated in the presence of α-amanitin (*) or from nuclei prepared from cells preincubated in actinomycin D and incubated in the presence of α-amanitin (**). The *in vitro* synthesized 5S RNA was prepared by gel electrophoresis. The amount of $^3$H-labeled 5S RNA in each sample was calculated from the efficiency of hybridization of the $^{32}PO_4$-labeled 5S RNA included in the hybridization mixture. This was compared with the value obtained by analysis of the sample by gel electrophoresis.

TABLE II: Hybridization to *Xenopus* 4S DNA.[a]

| RNA Sample | Input | | Bound | | Competed with 4 S (10 μg) | |
|---|---|---|---|---|---|---|
| | $^3$H (cpm) | $^{32}$P-labeled 4 S (cpm) | $^3$H (cpm) | $^{32}$P (cpm) | $^3$H (cpm) | $^{32}$P (cpm) |
| *in vivo* 18 S and 28 S | 100,000 | | 10 | | | |
| *in vivo* 5 S | 30,000 | | 5 | | | |
| *in vivo* 4.5 S | 8,000 | 19,000 | 176 | 164 | 36 | 16 |
| *in vitro* 4.5 S | 4,000 | 5,500 | 44 | 30 | 7 | 5 |

[a] *Xenopus* 4S RNA was prepared as described in Materials and Methods. $^{32}PO_4$-labeled 4S was used (200,000 cpm/μg) for the internal standard. The rRNAs used were 300,000 cpm/μg. The *in vivo* 4.5S RNA was prepared from cells grown in [$^3$H]-uridine for 20 min. The *in vitro* 4.5S RNA was prepared from nuclei incubated in the presence of α-amanitin. Both samples were purified by gel electrophoresis. Background was 0.05–0.1% of the input counts.

69–80°) than the 4S tRNA ($T_M$ = 65° range 51–80°). This was presumably due to the lack of modified bases in 4.5S RNA which are present in tRNA and would interfere with the hybridization reaction. Moreover, the hybridization of the 4.5S RNA was competed by tRNA. These data conclusively show that the 4.5S RNA made *in vitro* shares the same nucleotide sequences as 4S tRNA. It is not known how many different mouse tRNA species hybridize with the *Xenopus* DNA probe. Considering one is dealing with 40–50 sequences in both the mouse tRNA (W. F. Marzluff, R. Benjamin, and R. C. C. Huang, manuscript in preparation) and in *Xenopus* (Clarkson *et al.*, 1973) the efficiency of hybridization (>1%) is very high and the specificity of the reaction is indicated by the effective competition at very low competitor concentrations (less than 0.25 μg/ml of each competitor sequence).

*Conversion of 4.5S RNA to 4S RNA in Vitro.* The ratio of 4.5S to 4S RNA made *in vitro* varied greatly from preparation to preparation. In many preparations little or no 4S RNA was found, while in some, substantial amounts of 4S RNA were found. This suggested that the enzyme system(s) responsible for the conversion of 4.5S RNA to 4S RNA might be only loosely associated with the nuclei. To test this, nuclei were prepared by a less rigorous procedure without the use of detergent. Washing the nuclei in 0.15 M KCl and 0.1% Triton X-100 released an activity which efficiently converted 4.5S RNA synthesized *in vitro* to 4S RNA (Figure 7). This activity was localized in nuclei prepared by this method (W. F. Marzluff and R. C. C. Huang, unpublished results). The reaction resulted in a decrease in chain length of the 4.5S RNA as analyzed by gel electrophoresis in the presence of formamide. Similar results have been found for 4.5S RNA made *in vivo* during a short pulse. This provided further evidence that the 4.5S RNA made *in vitro* was a precursor to 4S tRNA.

*Initiation of RNA Synthesis.* Synthesis of both the 5S and 4.5S and 4S RNAs proceeded coordinately with total RNA synthesis (Marzluff *et al.*, 1973) and substantial amounts of both RNA species accumulated throughout 30 min of incubation. The small size of these RNA molecules, together with their continued synthesis over an extended period of time, suggested that synthesis of complete molecules must be occurring *in vitro*. 5S mouse RNA is known to be initiated with pppGp (Hatlen *et al.*, 1969) and the kinetic evidence (Figure 3) suggested that the 4.5S RNA might also be a primary transcript. Incubation of nuclei with both [$^3$H]GTP and [$\gamma$-$^{32}PO_4$]GTP resulted in incorporation of significant amounts of $^{32}$P into low molecular weight RNA, much of which migrated with 5S and 4.5S RNA on gel electrophoresis (Figure 8). Digestion of the product with ribonuclease $T_1$ resulted in the release of pppGp, indicating that the RNA chains had been initiated *in vitro*. From the known composition of 5S RNA and the relative specific activities of the [$^{32}$P]- and [$^3$H]GTP, it was calculated that 10–20% of the 5S molecules contained pppGp. However, analysis of [$\alpha$-$^{32}PO_4$]GTP labeled RNA made *in vitro* revealed that over 80% had pGp as the 5′ terminal sequence is pGpU. In addition, much of the 5S RNA made *in vivo* over a long-term label in these cells contained pGP as its 5′ terminal (Figure 9), as also was found by Williamson and Brownlee (1969). Similarly, the 4.5S RNA made *in vitro* had some pGp at its 5′ terminal (not shown). These data strongly suggest that most of the 5S and 4.5S RNA molecules are totally synthesized *in vitro*.

At high salt concentrations (>0.25 M KCl) RNA polymerases cannot initiate RNA synthesis but RNA chains continue to be elongated. At high salt concentrations several changes in RNA synthesis occur in the isolated nuclei. First the temperature dependence is reversed (Figure 10) and now reflects the temperature dependence of the isolated polymerases (unpublished results). There is a net decrease in RNA synthesis relative to that at 0.15 M KCl. The RNA product is still largely high molecular weight. However, no 5S or 4.5S RNA species

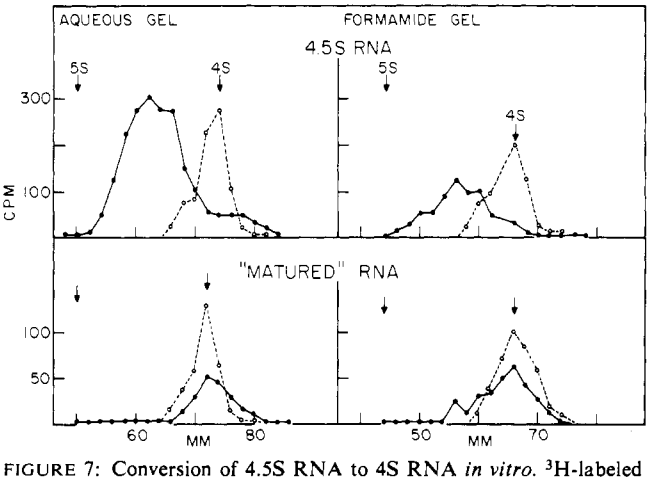


FIGURE 7: Conversion of 4.5S RNA to 4S RNA *in vitro*. $^3$H-labeled 4.5S RNA synthesized *in vitro* was eluted from the gels and incubated with an enzyme preparation from nuclei of 66-2 cells (20 min, 37°) as described in Materials and Methods. The incubated RNA (yield 85%) was analyzed by gel electrophoresis both in aqueous and formamide gels. Control samples were incubated without enzyme. Four times as much sample was applied to the top left gel. (Top) 4.5S RNA; left, aqueous gel; right, formamide gel; (bottom) incubated 4.5S RNA; left, aqueous gel; right, formamide gel; (● — ●) $^3$H *in vitro;* (O - - O) $^{32}$P-labeled 4S marker.

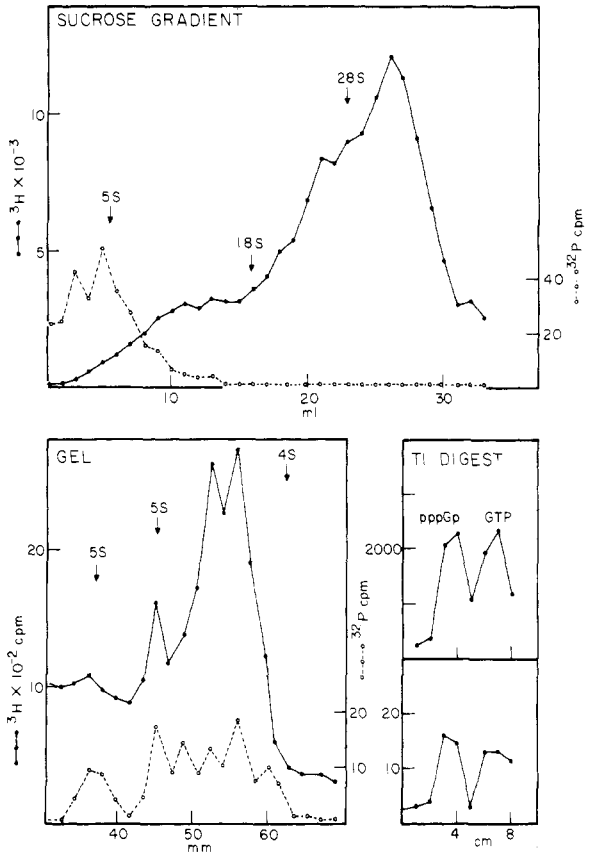


FIGURE 8: Incorporation of [$\gamma$-$^{32}PO_4$]GTP into RNA in isolated nuclei. Nuclei were incubated with 300 $\mu$Ci of [$^3$H]GTP and 300 $\mu$Ci of [$^{32}PO_4$-$\gamma$]GTP for 30 min at 25°. RNA was prepared and analyzed by sucrose gradient centrifugation (top). 20% of each fraction was ctd. 40% of the low molecular weight RNA was analyzed by polyacrylamide gel electrophoresis (bottom left). (● — ●) $^3$H; (○ - - ○) $^{32}$P. A second aliquot (20%) was digested with ribonuclease $T_1$ for 30 min at 37° and the products were analyzed by thin-layer chromatography on PEI-cellulose developed with 1.5 M $KH_2PO_4$ at pH 3.4 (Kashel *et al.*, 1971). A sample of RNA transcribed from DNA with *E. coli* RNA polymerase using the same preparation of [$\gamma$]GTP was also digested. Bottom right: top, RNA made *E. coli* RNA polymerase; bottom, RNA synthesized *in vitro*. The small amount of [$^{32}PO_4$]GTP in each sample was due to incomplete separation of the substrate from RNA.

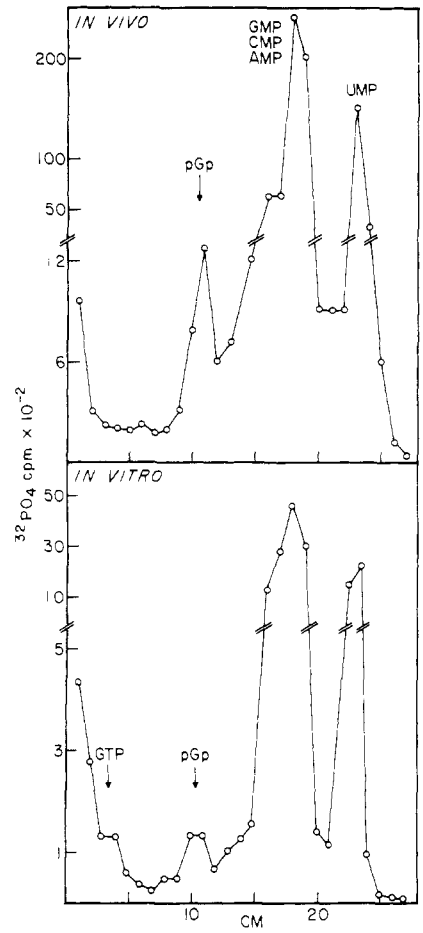


FIGURE 9: Analysis of 5S RNA. 5S RNA prepared from cells labeled *in vivo* with $^{32}PO_4$ or synthesized *in vitro* with [$\alpha$-$^{32}PO_4$]GTP was prepared by gel electrophoresis and hydrolyzed with 0.3 M KOH overnight at 37°. The products were analyzed by electrophoresis on DEAE-cellulose paper at pH 3.5 in 7 M urea. The paper was cut into 1-cm strips and counted. (Top) *in vivo* synthesized 5S RNA; (bottom) *in vitro* synthesized 5S RNA.

were found (Figure 11). In addition, no 5S RNA sequences were detected by hybridization with 5S DNA, indicating that failure to observe 5S RNA was not due to faulty termination in high salt. This result is consistent with the conclusion that the synthesis normally observed resulted from continued initiation of new chains of these two species of RNAs.

## Discussion

Several lines of evidence establish the faithfulness of the *in vitro* transcription process in the myeloma nuclei.

(1) As assayed by gel electrophoresis the low molecular weight RNAs made *in vitro* are similar to those synthesized during a short pulse *in vivo*.

(2) 5S RNA is transcribed only from the correct DNA strand. In addition, most if not all of the 5S sequences made *in vitro* are identical with 5S RNA on gel electrophoresis as determined by RNA–DNA hybridization (Table I, 4–6), indicating that the molecule is terminated correctly.

(3) Both the 4.5S and 5S RNAs represent a substantial portion of the RNA made *in vitro*, similar to amounts made *in vivo*. Transcription of these small species should take only a short time relative to the much larger rRNAs and, in addition, only a very small number of polymerases might be transcribing a given gene at one time, while many polymerases can transcribe a single rRNA gene at one time (Miller and Beatty, 1969). Hence a system which only completed 5S and 4.5S RNA chains would synthesize an extremely limited number of molecules and this synthesis would be completed quickly, probably within 1 min.

Artificially allowing only chain completion to occur by raising the KCl concentration to a point where RNA polymerase can no longer initiate RNA synthesis prevented any detectable 5S synthesis (Figure 11).

(4) The 5′-terminal phosphate of 5S RNA was labeled both with [$\gamma$-$^{32}PO_4$]GTP and [$\alpha$-$^{32}PO_4$]GTP. *In vivo* in this cell line most of the 5′ terminal was pGp indicating there was a phosphatase activity which removes most of the $\beta$ and $\gamma$ phosphate from the 5′ terminal. Some labeling (with [$\gamma$-$^{32}PO_4$]GTP) of the 5′ terminal of 4.5S RNA was detected also, implying that this species was also a primary transcription product.

(5) Extensive amounts of RNA are synthesized by the nuclei. In 30 min about 5 ng of RNA is synthesized per $\mu$g of DNA. 0.5% of this is 5S RNA and about 4% is tRNA. This corresponds to 1000–4000 molecules of 5S/haploid genome and 10,000–20,000 molecules of 4S/haploid genome. Only 400 copies of the 5S genes and 4500 copies of tRNA are found in the mouse haploid genome (W. F. Marzluff, R. Benjamin, and

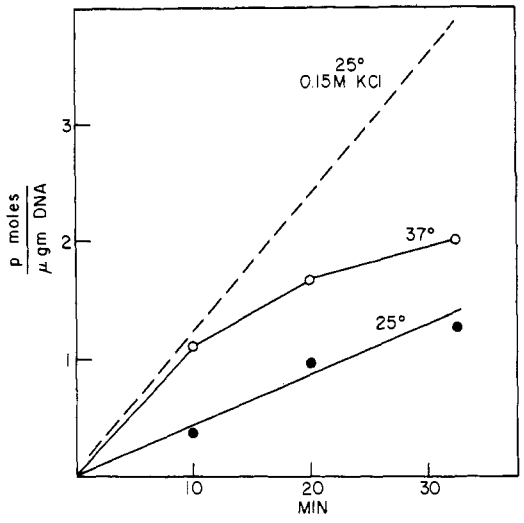


FIGURE 10: Synthesis of RNA in isolated nuclei in 0.5 M KCl. 2 × $10^5$ nuclei were incubated in 0.5 M KCl at either 25 or 37° for 30 min. (O — O) 37°; (● — ●) 25°; (- - - -) synthesis at 25° in 0.15 M KCl.

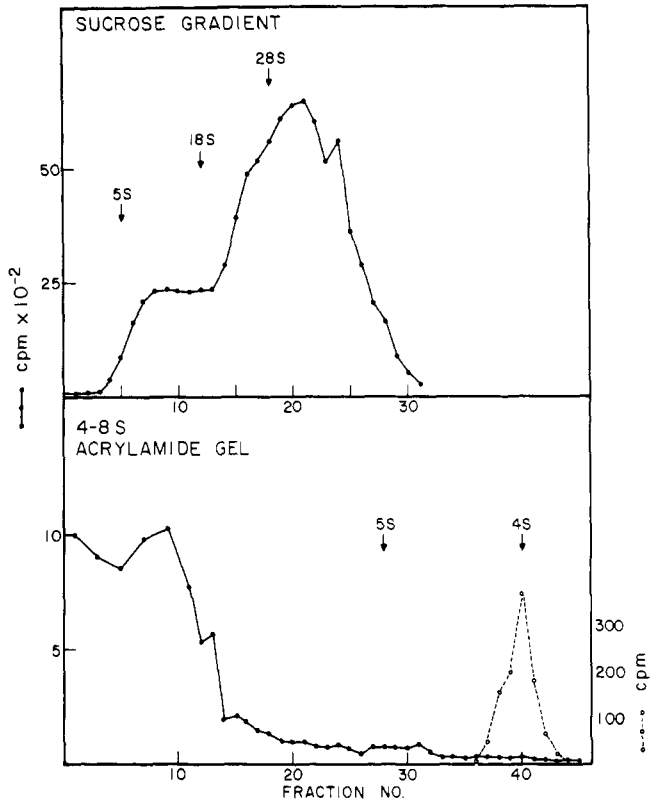


FIGURE 11: Analysis of RNA synthesized in isolated nuclei in 0.5 M KCl. RNA synthesized in isolated nuclei in 0.5 M KCl was analyzed by sucrose gradient centrifugation (top). The low molecular weight RNA was analyzed by polyacrylamide gel electrophoresis (bottom). (● — ●) $^3H$; (O - - O) $^{32}PO_4$-labeled 4S marker.

R. C. C. Huang, manuscript in preparation). Hence *in vitro* several-fold net synthesis is observed even if all the genes are active. Due to the very short length of these genes they should accommodate only one polymerase molecule at a time (Miller and Beatty, 1969). Hence during the incubation each gene must be repeatedly transcribed with reinitiation of transcription occurring.

The data presented here establish that the 4.5S RNA is a precursor to tRNA, as suggested by others (Bernhardt and Darnell, 1969; Choe and Taylor, 1972). The 4.5S RNA made both *in vivo* and *in vitro* hybridized to DNA complementary to tRNA. The 4.5S RNA hybridized more efficiently than 4S tRNA, presumably due to a lack of modified bases present in tRNA, which would tend to interfere with the hybridization reaction.

In addition, some information has been obtained on the post-transcriptional maturation process leading to tRNA. Smellie and Burdon (1970) have shown that an homogenate of L cells can convert the 4.5S RNA to 4S RNA as analyzed by chromatography on G-100. An enzyme from *Escherichia coli* can also convert 4.5S RNA from the silk worm to the size of tRNA (Chen and Siddiqui, 1973). Nuclei prepared in Triton had variable ability to make 4S RNA. However, from nuclei prepared without Triton an activity could be extracted which converted 4.5S RNA to 4S RNA. Gel electrophoresis in the presence of formamide established that this was due to a cleavage of 10–15 nucleotides from the 4.5S RNA. The enzyme is localized in nuclei prepared in this way and does not affect other low molecular weight nuclear RNA species. A more detailed report on this process will be reported elsewhere (W. F. Marzluff and R. C. C. Huang, unpublished results). One step in tRNA maturation is accomplished by an enzyme(s) loosely associated with the nuclei, possibly with the nuclear membrane.

Although the 5S RNA and tRNAs are transcribed from nucleoplasmic genes, they are not transcribed by the major nucleoplasmic RNA polymerase, RNA polymerase II. In fact, in the presence of $\alpha$-amanitin and after inhibition of 45S rRNA synthesis by low concentrations of actinomycin D, the major products *in vitro* were 5S and 4.5S RNA (Figures 2 and 4). They may either be transcribed by extra-nucleolar RNA polymerase I or another polymerase, RNA polymerase III. Roeder (1974) has recently suggested that polymerase III may be responsible for 5S and 4S RNA synthesis, as *Xenopus* oocytes, active in 5S and 4S RNA synthesis contain large amounts of polymerse III. Price and Penman (1972b) have reported that the synthesis of a low molecular weight adenovirus RNA species in isolated HeL cell nuclei possesses many of the properties reported here and suggested that this RNA was transcribed by RNA polymerase III.

This system should be useful in detecting factors which affect specific RNA transcription and in determining directly what activities are responsible for transcribing specific genes. In addition, it should be useful for studying the transcription and maturation of other RNA species, particularly specific mRNAs.

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# Mechanism of Action of Coumarins. Significance of Vitamin K Epoxide†

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ABSTRACT: The 2,3-epoxide of phylloquinone is a normal metabolite of the vitamin, and it has been demonstrated that coumarin anticoagulants inhibit an enzyme system that converts this metabolite to the vitamin. It has been postulated that this epoxide is a competitive inhibitor of vitamin K at the metabolic site where it is involved in prothrombin synthesis, and that coumarins act as anticoagulants because they increase the tissue levels of this epoxide. This study demonstrates that there is little correlation between the effectiveness of four different coumarins as anticoagulants, and their effect on phylloquinone epoxide metabolism. It also demonstrates that administration of phylloquine epoxide to vitamin K deficient, hypoprothrombinemic rats causes a significant initiation of prothrombin synthesis within 15 min. During this 15-min period, the ratio of the epoxide to the vitamin in the liver is considerably in excess of that which has previously been postulated to be inhibitory. These data would appear to rule out the hypothesis that vitamin K epoxide is an inhibitor of vitamin K action and that warfarin functions as an anticoagulant by increasing the tissue ratio of vitamin K epoxide to vitamin K.

Dicoumarol was identified by Link (Campbell and Link, 1941) as the active hemorrhagic agent in spoiled sweet clover, and since that time a large number of coumarins, including 3-(α-acetonylbenzyl)-4-hydroxycoumarin (warfarin), have been used as rodenticides and therapeutic anticoagulants. The mechanism by which the coumarin anticoagulants antagonize the action of vitamin K and depress prothrombin synthesis has not been unambiguously defined (Suttie, 1975) although it has often been assumed that they compete with vitamin K for a receptor protein or proteins at the site where vitamin K exerts its biological activity. It has also been suggested (Olson, 1970) that the coumarins interact with an alternate site on such a receptor protein, or (Lowenthal and Birnbaum, 1969) that these anticoagulants block the transport of the vitamin to its physiologically active site. More recently, Matschiner *et al.* (1970) have identified the 2,3-epoxide of phylloquinone as a natural metabolite of vitamin K and have shown (Bell and Matschiner, 1970) that warfarin administration increases the tissue levels of this metabolite. It has been postulated (Bell and Matschiner, 1972; Bell *et al.*, 1972) that warfarin exerts its anticoagulant effect through an inhibition of the enzyme which converts the epoxide to the vitamin. The epoxide of vitamin K is assumed to be the true inhibitor of the action of the vitamin, and the increase in the liver ratio of the epoxide to the vitamin is postulated as the cause of the inhibition of vitamin K dependent clotting factor synthesis. Although it has been shown that warfarin treatment will inhibit the reductase (Matschiner *et al.*, 1974) and increase the liver ratio of vitamin K epoxide to vitamin K[1] definitive proof that this increase is responsible for the antagonism of vitamin K action is lacking. All of the data that have been used to support the correlation of high oxide:$K_1$ ratios with antagonism of the vitamin have been obtained under conditions where the animals have also received warfarin. The studies reported here indicate that vitamin K epoxide is not an inhibitor of vitamin K action, and suggest that the effect of warfarin on prothrombin production is not due to the increased tissue ratio of vitamin K epoxide to vitamin K which it causes.

## Materials and Methods

Male, 190–210-g Holtzman strain rats were used throughout the study. Vitamin K deficiency was produced by feeding a vitamin K deficient diet (Mameesh and Johnson, 1959) for 8 days in cages that prevented coprophagy. The coumarin derivatives used were injected intraperitoneally in saline as the sodium salt. Plasma prothrombin concentrations were measured by the two-stage method of Ware and Seegers as modified by

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[1] The tissue ratio of phylloquinone 2,3-epoxide to phylloquinone will be referred to as the oxide:$K_1$ ratio.