Kuriki, Y., and Kaji, A. (1967a), *Biochem. Biophys. Res. Commun. 26*, 95.
Kuriki, Y., and Kaji, A. (1967b), *J. Mol. Biol. 25*, 407.
Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 941.
Lindahl, T., and Fresco, J. R. (1967), *Methods Enzymol. 12A*, 601.
Mans, R. J., and Novelli, G. D. (1960), *Biochem. Biophys. Res. Commun. 3*, 540.
Momose, K., and Kaji, A. (1965), *Arch. Biochem. Biophys. 111*, 245.
Muench, K. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 539.
Rychlik, I. (1966), *Collection Czech. Chem. Commun. 31*, 2583.
Schleich, T., and Goldstein, J. (1966), *J. Mol. Biol. 15*, 136.
Shaeffer, J., Arlinghaus, R., and Schweet, R. (1968), *Arch. Biochem. Biophys. 125*, 623.
Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.

Subfractionation and Polyacrylamide Gel Analysis of Liver Ribonucleic Acid from Normal and Glucocorticoid-Treated Rats*

Ronal R. MacGregor† and Henry R. Mahler‡

ABSTRACT: A new subfractionation procedure for the separation and study of ribonucleic acid has been devised. Rat livers were fractionated into the nuclear, free polysomal, and total reticular fraction, and ribonucleic acid was extracted by means of phenol. The cytoplasmic fractions were extracted first at pH 6.0 and 0° in the presence of 0.05 M Tris-acetate containing 0.1% sodium lauroyl sarcosinate plus 1 mg/ml of bentonite; second at pH 8.3 and 0° with 0.05 M Tris-acetate, containing 2.5% sarcosinate plus 1 mg/ml of bentonite; and third with the same components, but at 40°. The ribonucleic acid from the second and third extractions showed high specific radioactivity on pulse labeling and appeared to contain species of molecular weight greater than that of the 28S ribosomal ribonucleic acid. Nuclear ribonucleic acid was extracted once at pH 6.0 and 0° in 0.05 M Tris-acetate containing 0.1% sarcosinate plus 1 mg/ml of bentonite, then with the same medium, but at 65°. The specific activity of the latter fraction was 10–30 times that of the former.

Ribonucleic acid from all fractions was analyzed as to molecular weight and labeling distributions by means of polyacrylamide gel electrophoresis. Gel profiles indicated (1) the presence of a rapidly labeled ribonucleic acid species migrating with the 18S ribosomal ribonucleic acid present in reticulum, but not in polysomes; (2) the existence of other nonribosomal ribonucleic acids in the cytoplasm of rat liver cells with molecular weights from 5×10^5 to $>10^7$ which also become rapidly labeled.

Utilizing a double-label technique, ribonucleic acid was analyzed to detect selective induction(s) of any species by triamcinolone, a synthetic highly effective glucocorticoid. No such induction was detected in studies ranging from 1 to 2.5 hr after hormone administration.

During recent years many aspects of the mode of action of glucocorticoid hormones have become established. Beginning with studies on glycogen deposition and gluconeogenesis, continued refinements and innovations in technique have permitted the demonstration that following hormone administration to an appropriate animal, tissue, or cell culture system, increases occur in the intracellular concentrations of certain enzymes concerned with protein catabolism and with glucose synthesis from amino acid precursors (Feigelson and Greengard, 1962; Kenney, 1962; Segal and Kim, 1965). These rises in enzyme concentration have been shown to be due to increases in the rate of synthesis of the enzyme involved, rather than to decreases in its rate of degradation (Kenney, 1962; Segal and Kim, 1963, 1965; Knox *et al.*, 1964; Schimke *et al.*, 1964).

Recent studies suggest that the hormone may be acting at the level of transcription. The relevant observations include (1) increases in the transcriptive ability of chromatin from hormone-treated systems (Bonner *et al.*, 1968; Chambon *et al.*, 1968; Stackhouse *et al.*, 1968); (2) increases in the translational capabilities of RNA synthesized in nuclei (or nuclear systems) from treated cells (Dukes *et al.*, 1966; Schmid *et al.*, 1967); (3) alterations in the DNA–RNA hybridization characteristics of RNA from treated animals (Drews and Brawerman, 1967); (4) sensitivity of enzyme inductions to agents which interfere with DNA-directed RNA synthesis (*i.e.*, actinomycin D and other antibiotics) (Greengard and Acs, 1962; Kvam and Parks,

* Contribution No. 1675 from the Chemical Laboratory, Indiana University, Bloomington, Indiana 47401. *Received April 4, 1969.* This research was supported by Research Grants GB 4855 and GB 7205 from the National Science Foundation, and by Training Grant T01-GM-01046 from the National Institutes of Health, U. S. Public Health Service.

† Present address: Research Unit, U. S. Veteran's Administration Hospital, Kansas City, Mo.

‡ Recipient of Public Health Service Research Career Award GM 05060 from the National Institute of General Medical Sciences, U. S. Public Health Service.

1960), and (5) increases in general RNA synthetic rates (Feigelson and Greengard, 1962; Feigelson *et al.*, 1962a,b; Kenney *et al.*, 1965).

Finally, there is strong indication that mRNA for an inducible enzyme accumulates in the absence of protein synthesis and of gross changes in RNA metabolism (Peterkofsky and Tomkins, 1968). This, the most direct evidence to date for the induction of enzyme synthesis *via* an increase in mRNA concentration, has been corroborated by similar work with chick embryo retinas (Reif-Lehrer and Amos, 1968).

Two requirements remain to be satisfied before the hypothesis that steroids exert an effect at the level of transcription can be considered established: (1) a demonstration of a selective increase in the concentration of a specific fraction of RNA prior to enzyme induction, and the isolation (or at least partial purification) of this RNA; (2) evidence that this RNA codes for one or more inducible enzymes in an *in vitro* protein synthesizing system. Work with cell cultures (Tomkins *et al.*, 1966) has provided indications that, following steroid treatment, small changes in nonribosomal RNAs are detectable by means of sucrose gradients (Gelehrter and Tomkins, 1967). Evidence implicating the possible involvement of a cytoplasmic particle similar in size to the small ribosomal or preribosomal subunit (Girard *et al.*, 1965; Henshaw *et al.*, 1965; Perry and Kelley, 1968) in this process has also been reported (Finkel *et al.*, 1966; Gelehrter and Tomkins, 1967).

The need remains for a method of subfractionating RNA populations into functionally distinct fractions, specifically the separation of rRNA (which comprises the bulk of particulate cytoplasmic RNA) from other species, and especially from mRNA. The development of polyacrylamide gel electrophoresis (Richards and Coll, 1965; Loening, 1967; Watanabe *et al.*, 1967; Peacock and Dingman, 1967, 1968; Dingman and Peacock, 1968) as an analytical tool for RNA analysis suggests the feasibility of a much more sensitive resolution of RNA species differing slightly in molecular weight. The main restriction on the fullest exploitation of this technique, however, has been the limited amounts of RNA that could be applied to each gel. Routine studies on cytoplasmic nonribosomal RNAs, therefore, would require either: (1) the use of large quantities or very high activity levels of radioactive isotopes to permit the detection of the minute quantities actually present, or (2) a method for enriching an RNA population for these minor constituents by means of the prior removal of rRNA, or *vice versa*. Such a technique would allow the study of nonribosomal RNAs following exposure to isotope for longer duration than had been possible heretofore, and might be utilized for studies concerning hormone action, tissue differentiation, and the separation of nonribosomal RNAs with varying rates of turnover.

Recently, Brawerman and Hadjivassiliou (1967) reported that RNA with several characteristics of mRNA could be isolated from cytoplasmic fractions of rat liver, and that this RNA comprised about 1% of the total cytoplasmic RNA. Although this procedure led to a large enrichment of this species, there remained the possibility that the high specific radioactivity associated with this RNA was of nuclear origin, perhaps as a result of the breakage of, or leakage from, nuclei (Perry and Kelley, 1968). Furthermore, it has been our experience that RNA isolated in the absence of detergents, as in the procedure described by the above authors, invariably suffered from some degradation.

In order, therefore, to detect possible differences between populations of nonribosomal RNAs following various treatments, we felt that an improved procedure for their fractionation had to be developed. The method described below was based on th edifferential pH-extraction procedure of Brawerman and Hadjivassiliou, modified to fit the following criteria: (1) minimal degradation of RNA during cell fractionation and RNA isolation; (2) reproducible profiles of RNA subfractions on polyacrylamide gels; (3) reduction of cross-contamination of one cellular or RNA fraction by another. Finally the possibility of locating differences between RNA populations was tested by attempting to detect an RNA fraction induced by the synthetic glucocorticoid, triamcinolone.

Materials and Methods

Materials. Bentonite, a product of Fisher Chemical Co., was purified according to Fraenkel-Conrat *et al.* (1961). [^{3}H]-Orotic acid (5–10 Ci/mmole) was purchased from Schwarz BioResearch Inc.; [6-^{14}C]orotic acid (36.5 mCi/mmole) was purchased from Schwarz BioResearch Inc. or from New England Nuclear Corp. (4.6 mCi/mmole or 39.6 mCi/mmole). Phenol was distilled immediately before use. Sodium lauroyl sarcosinate (Sarcosyl N1-97) was a gift of Geigy Industrial Chemicals and was recrystallized from ethanol–acetone (1:1). Triamcinolone, both as the acetate and as the soluble acetonide phosphate, was a generous gift of Lederle Laboratories, Pearl River, N. Y.

Acrylamide was purchased from Eastman Organic Chemical Co. and recrystallized from chloroform (Loening, 1967); ethylene diacrylate was a product of K & K Laboratories, Inc. Agarose was "Seakem," distributed by Bausch & Lomb, a product of Marine Colloid, Inc.

Suspension and Homogenization Media. Medium P consisted of 0.05 M Tris-acetate, 0.025 M KCl, 0.01 M $Mg(Ac)_2$, and 0.35 M sucrose (pH 7.6). Medium (P + spd[1]) consisted of medium P containing 0.001 M spermidine. Buffer E consisted of 0.04 M Tris-acetate, 0.02 M potassium acetate, and 0.002 M EDTA (pH 7.4).

Crude RNase Inhibitor. A crude fraction was isolated by a procedure modified from published data (Bont *et al.*, 1965). Four to six rats were starved overnight and killed, and their livers were gently minced immediately in 2.5 liver volumes of medium P and then homogenized with a Potter-Elvehjem homogenizer. The preceding and all further operations were performed at 0–2°.

Nuclei plus mitochondria were first pelleted by a 20-min centrifugation at 15,000*g*, followed by sedimentation of the microsomes by a 2-hr spin at 120,000*g*.

The soluble component was removed and brought to pH 5 with glacial acetic acid, followed by $(NH_4)_2SO_4$ to 40% saturation, then centrifuged at 40,000 rpm for 15 min. Pellets were discarded. To the clear supernatant was added $(NH_4)_2SO_4$ to 60% saturation followed by sedimentation as before. The final pellets were dissolved in 5 ml/10 g of liver of medium P made 5 mM in EDTA and stored until used in 0.5-ml aliquots at −20°.

Isolation of Cellular Subfractions. Four male Wistar rats, 40–43-days old, were starved for 22–24 hr and then killed by cervical dislocation. In experiments utilizing a double-isotope label, two rats received [^{3}H]orotate, and the others [6-^{14}C]orotate as the sodium salts. Rat livers were removed quickly and

TABLE I: Conditions for RNA Subfractionation.

Fraction No.	Source	Extraction Conditions
6R	Reticulum	pH 6, 0°, 0.1% sarcosinate
6P	Polysomes	pH 6, 0°, 0.1% sarcosinate
8-1R	Reticulum	pH 8.3, 0°, 2.5% sarcosinate
8-1P	Polysomes	pH 8.3, 0°, 2.5% sarcosinate
8-2R	Reticulum	pH 8.3, 40°, 2.5% sarcosinate
8-2P	Polysomes	pH 8.3, 40°, 2.5% sarcosinate
N-1	Nuclei	pH 6, 0°, 0.1% sarcosinate
N-2	Nuclei	pH 6, 65°, 0.1% sarcosinate

placed in 2.5 liver volumes of medium (P + spermidine) containing 2.5 mg/ml of bentonite (Fraenkel-Conrat *et al.*, 1961), then homogenized with a Potter-Elvehjem glass-Teflon homogenizer. Homogenates were diluted to 4:1 with medium (P + spermidine), then centrifuged for 15 min at 7500*g* to sediment debris, nuclei, and mitochondria. The supernatant fluid was poured off, layered (23 ml/tube) over two-step discontinuous gradients containing 6 ml of 2 M sucrose under 6 ml of 1.5 M sucrose (both in medium P containing 0.1 ml of the RNase inhibitor), then centrifuged for 8 hr at 42,000 rpm (140,000*g*) in the A211 rotor of the International B-60 ultracentrifuge.

The crude nuclear plus mitochondrial pellets from the centrifugation at 7500*g* were suspended by gentle homogenization in 8 ml/g of liver of 2.3 M sucrose–1 mM $Mg(Ac)_2$ and centrifuged at 30,000 rpm for 90 min in the 30 rotor of a Beckman–Spinco L-2 ultracentrifuge.

Fractionation Procedure for Enzyme Induction Studies. All injections were intraperitoneal. Treated rats were injected with triamcinolone and [^{3}H]orotate at various times before being killed. Controls received saline in place of the hormone. Male Wistar rats (40–43-days old and starved for 22–24 hr) were killed by cervical dislocation. Their livers were removed, minced gently in a Waring Blendor, and homogenized in three volumes of medium P. Nuclei were sedimented by a 10-min centrifugation at 800–1200*g* and purified by centrifugation through 2.3 M sucrose–1 mM $MgCl_2$. Aliquots of the suspension of purified nuclei were precipitated and washed twice with 5% perchloric acid, once with 80% ethanol, solubilized in 0.5 ml of Hyamine hydroxide (Packard Instrument Co.), and counted in a Packard scintillation counter.

Nuclear supernatants were centrifuged at 0° for 15 min at 15,000*g* to sediment mitochondria, and the pellets were discarded. Aliquots from mitochondrial supernatants were used for preincubation in the typtophan pyrrolase assay, and the remainder was fractionated for measurement of isotope incorporation. Total microsomal fractions were isolated from the mitochondrial supernatants by means of a 1-hr centrifugation at 50,000 rpm in the no. 50 rotor of a Spinco L-2 ultracentrifuge and assayed for incorporation into acid-insoluble material. Counts incorporated were normalized for isotope uptake. DNA was assayed by the method of Burton (1956), and RNA according to the method of Drury (1948), following extraction of nucleic acid by 5% perchloric acid at 75° (Widnell and Tata, 1964). For the study of hormone effects on RNA synthesis, nuclear incorporation was expressed as counts per minute per milligram of DNA, and cytoplasmic incorporation as counts per minute per milligram of RNA. After correction for differences in precursor uptake, both figures were converted into per cent of control values.

Tryptophan pyrrolase activity was measured essentially according to Knox *et al.* (1966), and tyrosine transaminase activity by the method of Diamondstone (1966). Activity was calculated as micromoles of product per unit time per gram of liver and expressed (for comparison of hormone effects) as per cent of the control.

Isolation and Purification of RNA Fractions. Polysomal pellets were suspended in and homogenized with two liver volumes of 0.05 M Tris-acetate containing 0.1% sodium lauroyl sarcosinate at pH 6.0 plus 1 mg/ml of bentonite. The reticulum fraction (1.5 M sucrose layer) was added with mixing to three liver volumes of the same medium and homogenized, with all solutions at 0°. Freshly distilled phenol, containing 0.1% 8-hydroxyquinoline and saturated with the sarcosinate solution minus bentonite, was added in equal volumes to the polysomal and microsomal suspensions after cooling.

Phenol extracts were obtained by homogenization with a Potter-Elvehjem homogenizer, followed by centrifugation at 15,000*g* at 0°. Aqueous phases were removed, and RNA was precipitated overnight at −20° with two volumes of absolute ethanol containing 2% potassium acetate, yielding fractions 6R (reticulum) and 6P (polysomes). The phenol plus interphase layer from each fraction was then reextracted twice as described above and the resultant aqueous phases were discarded.

The phenol plus interphase layers from the previous extractions were then homogenized at 0° with two liver volumes of 0.05 M Tris-acetate (pH 8.3), containing 2.5% sarcosinate plus 1 mg/ml of bentonite, then centrifuged as before. The RNAs obtained from aqueous phases were designated fractions 8-1R and 8-1P. The remaining phenol plus interphase layers after this first extraction at pH 8.3 were then homogenized with one liver volume of the above 2.5% sarcosinate solution, the mixture was shaken in a 40° water bath for 5 min, cooled to 0° with shaking, and centrifuged as before. The resulting RNA fractions after precipitation were called 8-2R and 8-2P.

Nuclei were suspended in and homogenized with two liver volumes of 0.05 M Tris-acetate (pH 6.0), containing 0.1% sarcosinate plus 1 mg/ml of bentonite, and then extracted at 0° with phenol as mentioned above. The resulting RNA was designated as N-1. The interphase plus phenol layers were homogenized at 0° with one liver volume of the 0.1% sarcosinate pH 6.0 buffer and subsequently shaken for 5 min at 65°, followed by cooling to 0° with shaking and centrifugation as before. The resulting RNA was precipitated and called fraction N-2. The conditions for the isolation of the various fractions are summarized in Table I.

All RNA precipitates (or convenient aliquots) were pelleted, then redissolved in a total of 5 ml of 0.05 M Tris-acetate (pH 7.6), containing 0.5% sarcosinate and 1 mg/ml of bentonite. RNA solutions were extracted three times with 5 ml of phenol–0.1% 8-hydroxyquinoline (saturated with the 0.5% sarcosinate solution) by agitating (Vortex mixer) all solutions at 0° for 5–10 min. The RNA was precipitated from the final aqueous phases by the addition of 10 ml of ethanol–potassium acetate at −20° as before, then washed and reprecipitated several times.

Final RNA suspensions were pelleted, drained, and partially dried under vacuum (in ice) for 40 min. This treatment was found to be sufficient to remove ethanol without dehydrating

TABLE II: Specific Activity (counts per minute per microgram) of RNA Fractions from Rat Liver.

Pulse Time (min)	Fraction Number							
	6R	6P	8-1R	8-1P	8-2R	8-2P	N-1	N-2
15	1.3[a]		8.7[a]				697.0[b]	
	1.6[a]		9.5[a]				344.0[b]	
30	8.2	1.9	10.1	35.4[c]	17.1		35.8	927
60	24.0	13.0	24.0	80.0[c]	41.7		51.5	999
	21.0	13.0	29.0	79.6[c]	59.5		86.0	1240
90	33.5	24.0	42.3	91	63.2	82.2	70.0	860
120	37.4	27.5	48.3	100	59.5	87.3	61.0	503
	49.0	33.8	69.4	125	94.0	115	79.0	2020
	57.5	38.6	76.0	109		170	65.0	662
150	53.6	39.6	67.5	123	92.4	137	77.4	785

[a] RNA was isolated from whole microsomal fractions which were isolated by centrifuging the mitochondrial supernatant through 10 ml of 0.8 M sucrose in medium P (containing RNase inhibitor) for 2 hr at 140,000*g*, 0°. [b] RNA was isolated from nuclei by one extraction at 65°. [c] Total polysomal RNA was extracted by one extraction at pH 8.3, 40°, 2.5% sarcosinate. Each row represents a separate experiment as described under Materials and Methods.

the RNA. The resulting pellets were dissolved in as small a volume as possible (10–100 μg/10 μl) of a solution containing 0.5% sarcosinate, 25% glycerol, and 74.5% electrophoresis buffer (buffer E); 10-μl aliquots were removed for determination of the absorbance at 260 and 280 mμ and of the counting rate, for the calculation of specific radioactivities.

Electrophoresis. Polyacrylamide gels (2.25%) containing 0.5% agarose (washed five to eight times) were prepared by a method developed by Campagnoni (1968) adapted from the procedures employed by Watanabe *et al.* (1967) and Bishop *et al.* (1967). Gels were cast in Pyrex tubes, each 10.5 cm long and 7 mm in diameter closed at one end with a 1.5 cm snugly fitting Teflon plug. The plug was removed after polymerization, and the lower end of the tubes was sealed with dialysis tubing.

Samples of RNA (volume 3–30 μl) were layered on gels *previously* purified by preelectrophoresis for 1–2 hr at a constant current of 3.33 or 5.0 mA per gel, and were subsequently fractionated for either 3.5–4 hr at 3.33 mA/gel or 2.5 hr at 5 mA/gel.

Monitoring of RNA Bands for Optical Density and Radioactivity. Gels were analyzed utilizing the Gilford gel scanning attachment (Gilford Instruments, Yellow Springs, Ohio) to their Model 340 recording spectrophotometer.

Following ultraviolet scanning, gels were sliced in a wire-slicing apparatus designed by Campagnoni (1968) into slices of approximately 1.25 mm thickness. Slices were then placed into scintillation vials containing 2 ml of scintillation solution (5 g of 2,5-diphenyloxazole and 0.3 g of *p*-bis[2-(5-phenyloxazolyl)]benzene/l. of toluene), and 0.5 ml of NCS solubilizer (Amersham-Searle) was then added. After overnight incubation, vials were filled to a total volume of 12.5 ml with scintillation solution and counted on a Nuclear-Chicago Mark I scintillation counter.

Results

Characterization of RNA Fractions. In Figure 1 are shown absorbance and labeling profiles of RNA fractions isolated from polysomes, reticulum, and nuclei according to the procedure described above. The absorbance profiles for corresponding fractions from reticulum and polysomes were similar, but their labeling profiles differed in two respects: (1) the 8-1 fraction from polysomes showed a heterodisperse labeling profile which did not coincide with the absorbance and revealed the presence of small amounts of RNA species with very high specific radioactivities. Their apparent molecular weights ranged from about 10^6 to $>10^7$ daltons, assuming that the relation between mobility and molecular weight established by Peacock and Dingman (1968) was obeyed for electrophoresis at constant current. (2) In the pH 6 fractions of the RNA from the reticulum, in contrast to the polysomes, the area comprising the small rRNA species (referred to as 18S) was labeled more heavily than 28S RNA. Figure 2 shows the labeling kinetics of these fractions. In polysomes, the label appeared simultaneously in the two ribosomal species, in agreement with data presented by Dingman and Peacock (1968). In the reticulum, however, the region of the gel containing 18S rRNA became labeled more rapidly than did the 28S region.

Table II summarizes data concerning the rate of appearance of isotope in the various fractions subsequent to pulses of [^{3}H]-orotate. Contrary to recent findings from one laboratory (Murty and Hallinan, 1968), we found the rRNA from the reticulum (fraction 6R) labeled more rapidly (on the basis of counts per minute per milligram of RNA) than the same fraction from polysomes (fraction 6P) for periods up to 2.5 hr after injection of the label. The 8-1 and 8-2 fractions from polysomes, however, generally exhibited higher specific activities than did the corresponding fractions from the reticulum. This difference between the pH 8.3 fractions of reticulum and polysomes may have been due in part to varying amounts of contamination by rRNA in the two fractions. As shown in Figure 1, 28S rRNA proved more difficult to extract completely than was the 18S species, and therefore comprised the principal contaminant in the pH 8.3 fractions. Relative yields for the various cytoplasmic fractions are presented in Table

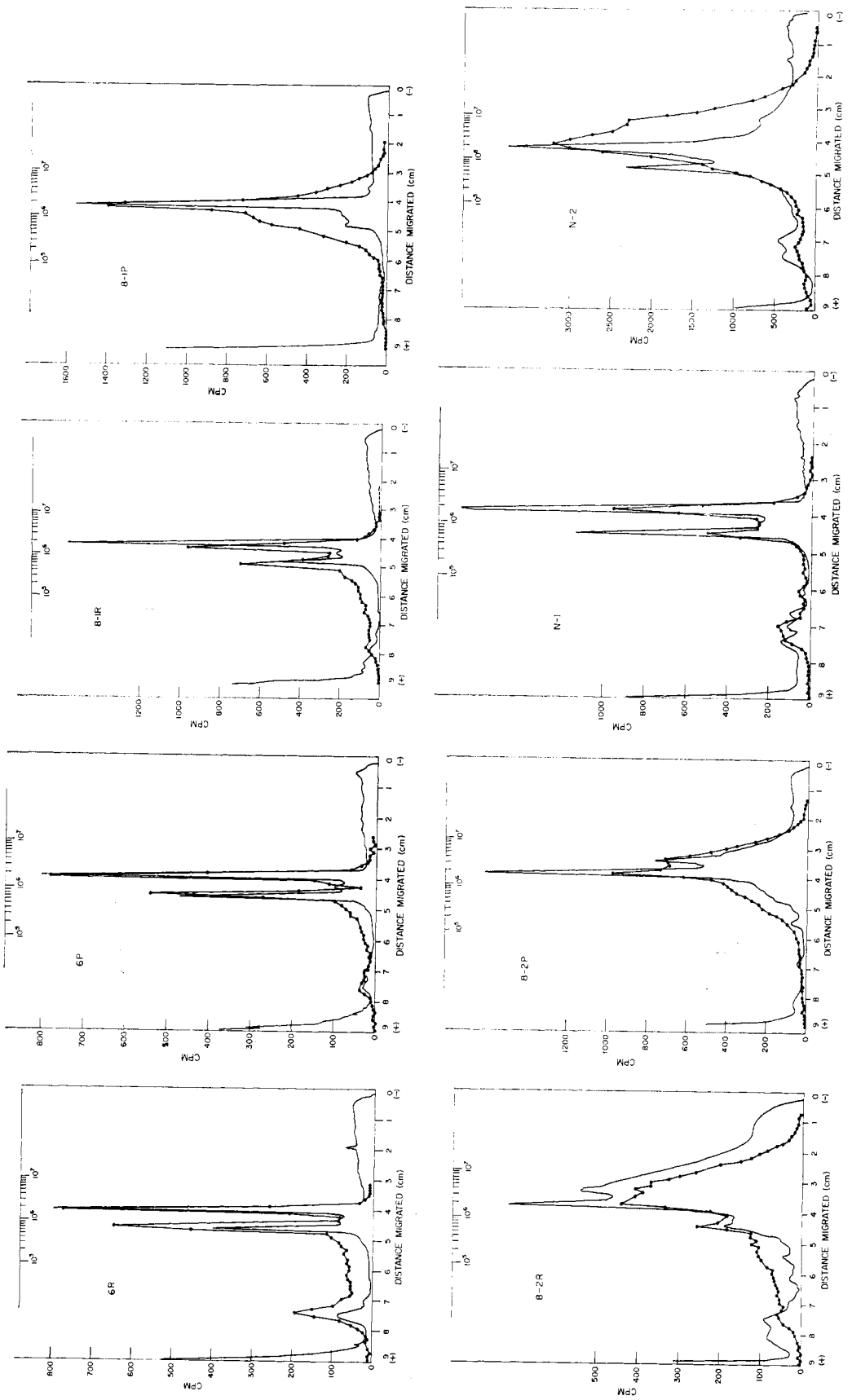

FIGURE 1: Gel electrophoresis of rat liver RNA. Four rats were starved for 24 hr. Two of them were injected with 50 μCi/100 g of [6-^{14}C]orotate, and the other two with [^{3}H]orotate (not shown) 2.5 hr prior to sacrifice. The tissue was fractionated, and RNA was isolated as described in Methods. RNA (35–50 μg) was applied to each gel, and the counts per minute are normalized to 50 μg. "R" fractions are reticular RNA, "P" fractions polysomal, and "N" fractions nuclear. (———) Ultraviolet absorbance and (●—●) counts per minute of ^{14}C.

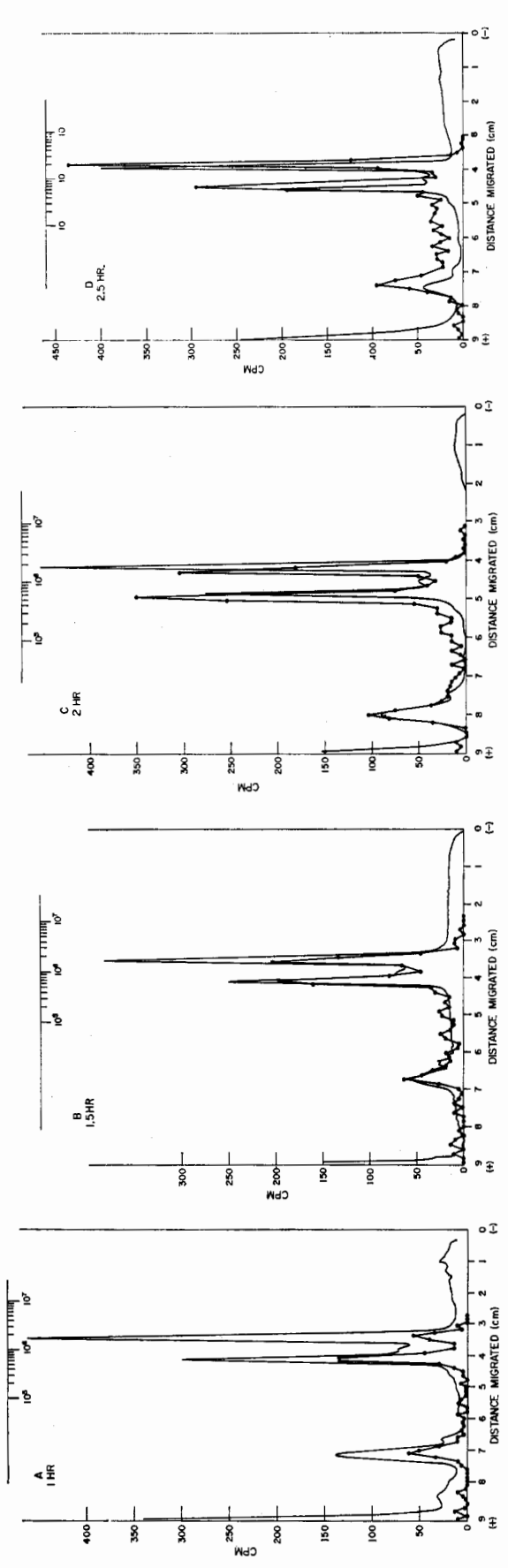

FIGURE 2: Time course showing the relative labeling rates of pH 6 RNAs isolated from polysomes and reticulum. Top: reticular RNA, bottom: polysomal RNA. Conditions were as described in Figure 1 and Methods, except that pulse times were varied as shown in the figure.

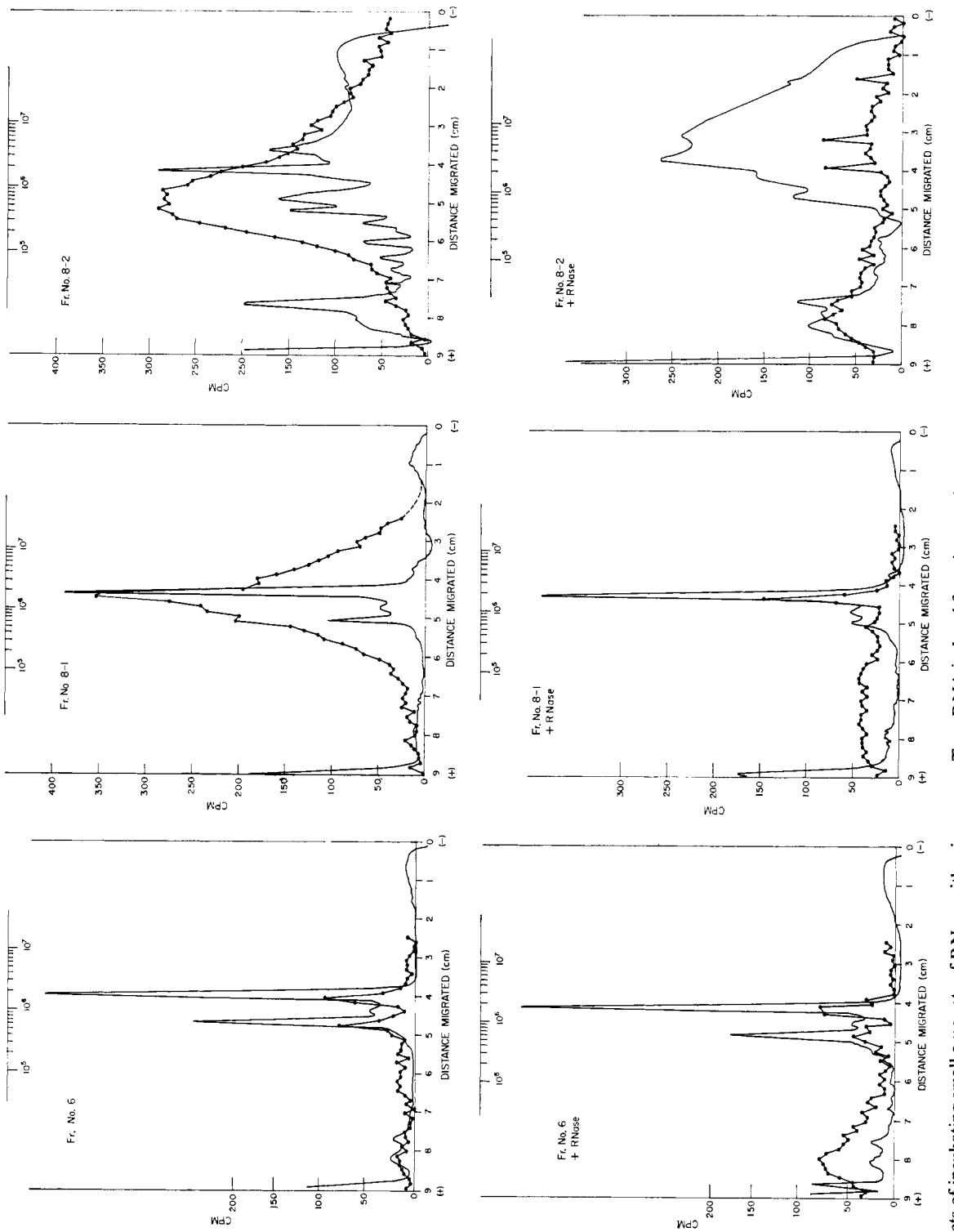

FIGURE 3: The effects of incubating small amounts of RNase with microsomes. Top: RNA isolated from intact microsomes. Bottom: RNA isolated from RNase-treated microsomes. Five rats were starved 24 hr, then each was given a 1-hr pulse of 100 μCi/100 g of [^{3}H]orotate. Livers were treated as in Methods, except that a total microsomal fraction was isolated from the mitochondrial supernatant. Microsomes were pelleted from the mitochondrial supernatant following a 1.2-hr centrifugation at 120,000g in the A211 rotor of an International B-60. Half of the microsomes was extracted immediately as described in Methods in order to isolate intact RNA subfractions. The other half was incubated for 8 min in medium P containing 0.1 μg/ml of pancreatic RNase (37°), followed by RNA isolation. Following RNA fractionation, 30–50-μg samples of RNA were applied to gels, and the results were normalized to 50 μg of RNA/gel.

TABLE III: Relative Yields of Cytoplasmic RNA Fractions (Per Cent).[a]

Fraction No.	% of Total[b]
6R	39.8 ± 6.3[c]
6P	43.0 ± 3.7
8-1R	9.5 ± 4.0
8-1P	2.3 ± 1.1
8-2R	3.5 ± 1.2
8-2P	2.0 ± 0.6

[a] Averages of five experiments. [b] Total yields varied from 14 to 45 mg of RNA for four or five livers. Variation was due to differences in liver weights and to changes in homogenization and centrifugation conditions during development of the procedure. [c] Standard deviation.

TABLE IV: Comparison of RNA Fractions from Intact and RNase-Treated Microsomes.[a]

Fraction No.	Total Yield (mg)	^{3}H Sp Act. (cpm/μg)
pH 6	21.6	15.8
pH 6, RNase	18.6	29.1
8-1	0.83	93.0
8-1, RNase	1.18	30.2
8-2	0.75	178.0
8-2, RNase	0.71	38.4

[a] Rats were administered 60-min pulses of 100 μCi/100 g of [^{3}H]orotic acid, and the mitochondrial supernatant was obtained as described in Methods. Mitochondrial supernatants were then centrifuged for 1 hr at 140,000g, 0°, to obtain a total microsomal fraction. Half of the pellets was extracted directly to obtain intact microsomal RNA fractions. The remainder was suspended in 10 ml of medium P containing 0.1 μg/ml of pancreatic RNase and incubated at 37° for 8 min followed by RNA extraction.

III, confirming earlier results of Brawerman and Hadjivassiliou (1967). The increases in specific activity and the reduction of RNA cross-contamination, however, were less than those reported by these workers.

Figure 3 and Table IV show the results of an experiment demonstrating the lability of the RNA in fractions 8-1 and 8-2, following isolation from a combined microsomal fraction. This figure also illustrates the presence of some aggregation and/or contamination by non-RNA components in the 8-2 fraction, in spite of the presence of EDTA throughout. This contaminant was resistant to RNase treatment, and possessed an OD_{260}/OD_{280} close to unity. The presence of several RNA bands migrating more slowly than the 28S ribosomal species was, however, a reproducible finding with both the reticulum and polysomal fractions, and had already been detected in early experiments utilizing acridine orange or methylene blue as stains for RNA. Furthermore, these slowly migrating RNA bands became labeled and possessed the expected OD_{260}/OD_{280} ratio close to 2.

Many bands and a shift in radioactivity to lower molecular weight regions of the gel made their appearance in the 8-2 fraction whenever the *total microsomal* fraction was pelleted together, as shown in Figure 3. This type of profile was reasonably reproducible so long as each experiment was performed in this particular manner, but did not appear when polysomes and reticulum were first separated and their component RNAs isolated. It is possible that pelleting the reticulum releases ribonucleases (Howell *et al.*, 1964; Lawford *et al.*, 1966; Loeb *et al.*, 1967), while this release is prevented if the reticulum is isolated from the 1.5 M sucrose layer.

Enzyme Induction. Preliminary studies showed that triamcinolone and its water-soluble acetonide phosphate were completely equivalent with respect to the kinetics of induction of tryptophan pyrrolase and tyrosine transaminase. The soluble form of the hormone, however, was found to be about 100 times more effective than the particulate one on a dosage basis.

No significant differences in the characteristics of the induction process were found between fed, 12-hr starved, and 24-hr starved rats. The specific activities of tryptophan pyrrolase and tyrosine transaminase began rising about 60 min after hormone administration, and continued rising for 3–4 hr, remaining at high levels until at least 8 hr after the hormone injection. No hormone effect on RNA synthesis was noted in short-term experiments (up to 3 hr), but long-term inductions produced a linear increase of 100% in the specific activity of microsomal RNA from zero time to 6-hr posthormone, followed by reduction toward normal levels at 8 hr.

Two parameters of the induction process were defined by these experiments: first, the time of initiation of enzyme induction (about 60 min after hormone administration), and second, the time at which enzyme synthesis reaches its maximum rate by 2-hr posthormone). If enzyme induction is a direct result of an increase in the concentrations of the respective mRNAs for the two enzymes, then these time points represent the time of appearance, and of maximum concentration of these mRNAs in the cytoplasm, respectively. Furthermore, an mRNA arriving in the cytoplasm by 60-min posthormone must have been synthesized in the nucleus somewhat earlier.

In order to determine at what point the induced mRNA had in fact been initially synthesized in the nucleus, we tested the sensitivity of the enzyme induction to actinomycin D as a function of time. The dosage used inhibited incorporation of [^{3}H]orotic acid into acid-insoluble material by >90%, and completely prevented enzyme induction when the inhibitor, [^{3}H]orotate, and hormone were injected simultaneously.

The induction of tryptophan pyrrolase was almost completely repressed when actinomycin was injected 30 min after the hormone; although slightly less sensitive initially, induction of tyrosine transaminase exhibited the same general pattern (Figure 4). These results are in essential agreement with those reported by Tomkins *et al.* (1966). Although these types of whole animal experiments, and experiments with actinomycin in general, are subject to rather serious criticisms, they have been widely used and are considered a useful tool for determining relationships between RNA and protein synthesis by many investigators (Tomkins *et al.*, 1966; Holten and Kenney, 1967; Grossman and Mavrides, 1967; Mishkin and Shore,

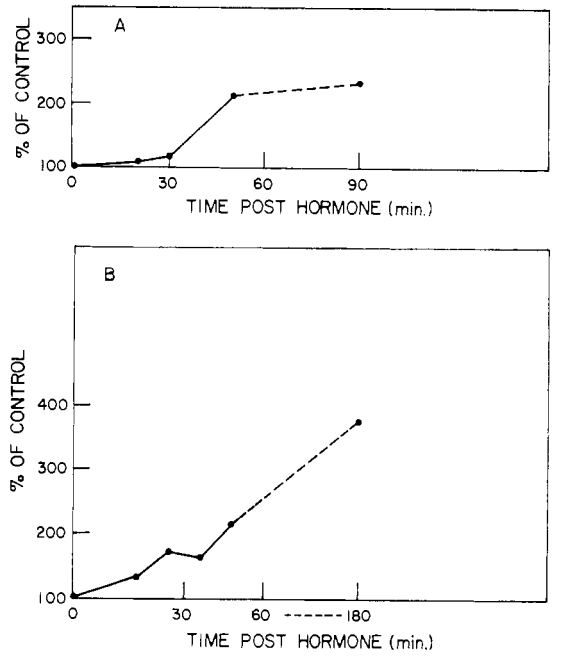

FIGURE 4: The effects of actinomycin D on enzyme induction. (A) Tryptophan pyrrolase and (B) tyrosine transaminase. Rats were starved 24 hr and injected with 100 μg/100 g of triamcinolone acetonide phosphate at zero time. At the various times shown on the abscissa (following hormone administration), experimental animals were injected with 250 μg/100 g of actinomycin D (suspended in saline). Control animals received actinomycin at zero time, *i.e.*, the same time as hormone, and all animals, experimental and controls, were killed after 3 hr. All other details are as described in Methods. Results are expressed as per cent of control and are the average of three experiments.

1967; Csány *et al.*, 1967; Peterkofsky and Tomkins, 1967, 1968).

Gel Analysis of Double-Labeled RNA. Double-labeled RNA isolated as described in Methods was fractionated on polyacrylamide gels. These experiments (hormone:control) were performed from 1 to 3 hr after hormone, and from 1 to 2.5 hr after isotope administration. The results of one such experiment utilizing a 2.5-hr hormone pulse and 2-hr pulses of [^{3}H]- and [^{14}C]orotate are shown in Figure 5. In not a single case did we observe reproducible change in isotope ratio profiles (hormone:control) in any of the cytoplasmic RNA fractions. Two possible hormone effects were, however, detected in nuclear fractions. A 10–20% increase in hormone:control ratio was observed in five of seven experiments in a region migrating slightly less rapidly than the 28S ribosomal RNA isolated from these nuclei at 0° (fraction N-1). This effect appeared to be time dependent (Figure 6), but as mentioned was not detected in two of seven experiments. The RNA in this region of the gel is probably of authentic nuclear origin, since in contrast to the cytoplasmic pH 8 fractions, N-1 exhibits higher specific radioactivity in the 28S region than in the 18S region. Furthermore, a component with somewhat similar properties had been reported for nuclear RNA isolated from HeLa cells 3 hr after isotope and 2.5 hr following actinomycin D administration (Girard *et al.*, 1964).

In addition to the effects on fraction N-1, in most experiments small but reproducible increases in the isotope ratio were noted in the region comprising RNA of $\geq$ 28S in the N-2 fraction. This fraction probably contains not only the ribosomal precursor species, but also numerous nonribosomal RNAs as well. As shown in Table II, this fraction has an extremely high specific radioactivity and is probably to be equated with the polydisperse rapidly labeled nuclear RNA found in other cell types (Yoshikawa-Fukada *et al.*, 1965; Attardi *et al.*, 1966; Soeiro *et al.*, 1966, 1968; Perry, 1967).

Discussion

The labeling kinetics summarized in Table II indicate that the 8-1 and 8-2 RNA classes are of cytoplasmic origin. Earlier experiments with liver nuclei incubated *in vitro* (MacGregor and Mahler, 1967) had shown that the nuclear membrane was stabilized by spermidine and that *in vitro* synthesized RNA was not found in the incubation medium after removal of the nuclei by centrifugation. This finding by itself does not exclude the possibility of contamination of the postmitochondrial supernatant by nuclear ribonucleoproteins; however, results of experiments with shorter pulses (15 min) of [^{3}H]-orotate suggest that contamination from this source is low (Table II). The specific activity of the 8-2 fraction of total microsomes is only 2.5% of that of total nuclear RNA, which contains 60–80% 28S plus 18S RNA. Assuming that all of the counts in the 8-2 fraction are the result of nuclear leakage or contamination, calculations based on 15-min pulses of [^{3}H]orotate indicate that a maximum of 3% of the 8-2 RNA can be of nuclear origin.

The electrophoretic profiles of both the 8-1 and 8-2 fractions suggest that RNAs of relatively high molecular weight may be present in the cytoplasm of rat liver cells and that this RNA is resistant to prior extraction at lower pH and detergent concentration. The molecular weight of the major nonribosomal peak in the 8-2P fraction was estimated to be 3.8 $\times$ 10^6 daltons, while in several experiments additional peaks were detected with approximate molecular weights equal to 3.0 $\times$ 10^6 and 5.9 $\times$ 10^6 daltons. The molecular weights of the major high molecular weight species from nuclear fraction N-2 were estimated as 2.4 $\times$ 10^6, 3.3 $\times$ 10^6, and 5.8 $\times$ 10^6 daltons.

It is perhaps difficult to make meaningful comparisons between polysomal and reticular RNAs since the latter are, of course, derived from membrane-bound rather than free ribonucleoprotein particles. Although the radioactivity profiles of the two 8-1 fractions are different, these differences could have been due to the membrane retaining RNA during the extraction rather than to intrinsic differences in the two RNAs. The membranes appeared to be solubilized at 40°, however, and most of the RNA should have been solubilized. The final residues remaining after this treatment have not been extracted further, since it was felt that the chances of obtaining intact RNA were slim. The kinetics of incorporation also argues in favor of meaningful distinctions between the two cytoplasmic compartments.

In this context, the existing controversy concerning the kinetics of labeling of cytoplasmic 18S and 28S rRNA may be partly resolved by the finding that the labeling patterns of polysomal and reticular RNA are different. The same results have been observed in rat brain RNA isolated from these two fractions, both on sucrose gradients and with polyacrylamide gels (Campagn ni, 1968). Thus, the finding by Dingman and Peacock (1968), that the two ribosomal species become labeled

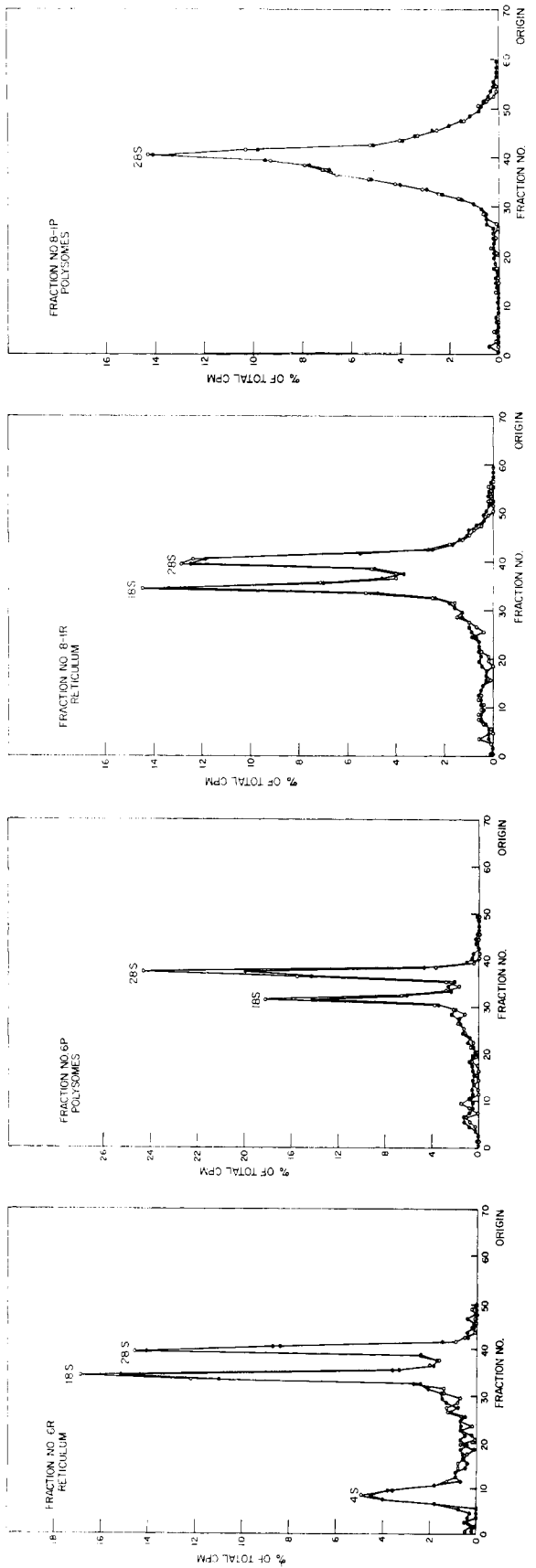

FIGURE 5: Effects of triamcinolone acetonide phosphate on liver RNA subfractions. Five rats were injected with saline 2.5 hr prior to sacrifice and with 100 μCi/100 g of [^{3}H]orotate 30 min later. The other three were injected with 100 μg/100 g of hormone 2.5 hr prior to sacrifice and with 50 μCi/100 g of [6-^{14}C]orotate 30 min later. Livers were mixed immediately and minced together. Conditions for cellular and RNA subfractionations are as described in Methods. Radioactivity on the gels was converted into per cent of total cpm for each isotope. Total cpm for each gel was as follows for ^{3}H (counts per minute of ^{3}H/counts per minute of ^{14}C = 1.4): 6R = 1659, 6P = 1201, 8-1R = 3166, 8-1P = 5560, 8-2R = 5154, 8-2P = 6577, N-1 = 3174, N-2 = 27,230. (O—O) ^{3}H controls and (●—●) [^{14}C]hormone.

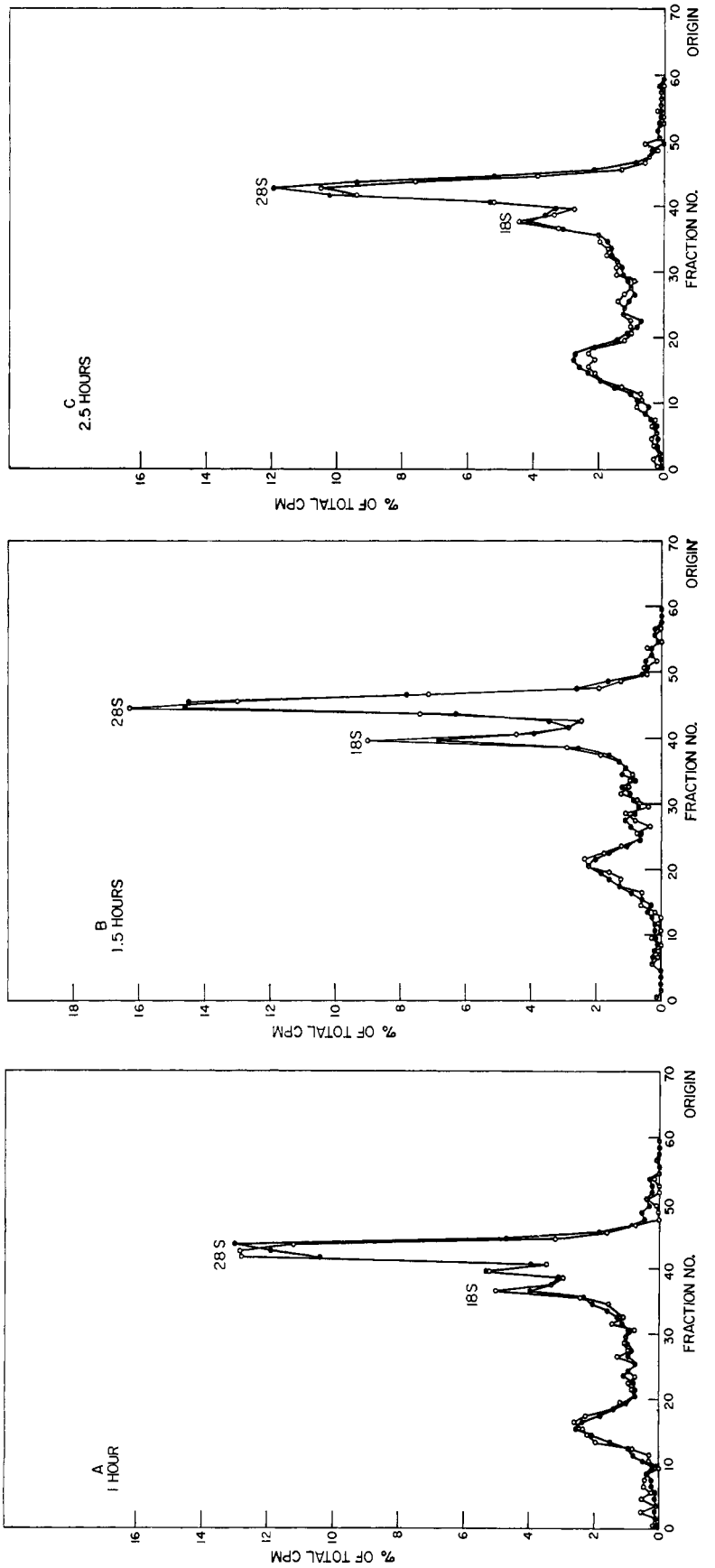

FIGURE 6: Effects of triamcinolone acetonide phosphate on the RNA of fraction N-1 at various periods following isotope and hormone administration. Four rats in each experiment were starved as before and treated as described for Figure 5, except the duration of hormone and isotope pulses was as shown in the figure.

at the same rate may reflect the situation obtaining in free polysomes, since in their experiments most of the reticulum-bound polysomes would have been sedimented with the mitochondrial fraction, which was spun out at 20,000*g* (Loeb *et al.*, 1967). On the other hand, reports favoring a prior labeling of 18S over 28S RNA have been based on the total ribosomal population (Girard *et al.*, 1965; Henshaw *et al.*, 1965; Penman, 1966; Perry, 1967), which, in liver at least, consists primarily of polysomes attached to membranes (Howell *et al.*, 1964; Blobel and Potter, 1967). Therefore, unless polysomal and reticular ribosomes are constructed by different pathways, an additional species must be present in the reticular 18S rRNA fraction that is similar to rRNA in many of its properties but becomes labeled much more rapidly[1] (Perry, 1967; Hadjiolov, 1967). These suppositions are borne out by the experiments reported above (Figure 2).

The reverse of this pattern may govern the RNA isolated from nuclei at 0°. The bulk RNA in this fraction is presumably derived from nucleoprotein particles of the rough endoplasmic reticulum immediately adjacent or attached to the perinuclear envelope, particles which have been shown to have the composition of rRNA (Whittle *et al.*, 1968). However, the labeling pattern is opposite to that in the cytoplasm, with 28S RNA containing three to four times the amount of radioactivity of the 18S species, provided corrections are made for contamination by various heterodisperse-labeled species. If one discounts the possibility of a selective loss during isolation of small ribosomal subunits from the ribosomes of the nuclear pool or the outer nuclear envelope, these results suggest the presence of a component of nuclear origin with a high specific radioactivity, and with a molecular weight slightly larger than that of 28S ribosomal RNA (*i.e.*, 1.8×10^6 daltons).

The utility of the procedure described above depends upon degradation, aggregation, and contamination having been minimized or eliminated. Although degradation is an ever-present danger, the data show little evidence of extensive damage to RNA. Very little RNA with molecular weights less than 10^5 daltons appeared to be labeled, and what there was appeared to be in either tRNA or 5S RNA. Figure 3 demonstrates that, under conditions when RNA degradation is induced by RNase, not only do the radioactivity profiles shift toward low molecular weight species, but the extraction properties of the RNA species change as well. The 85% increase in the specific radioactivity of the pH 6 fraction following RNase treatment of microsomes is obviously due to extraction of RNA fragments not normally isolated under these conditions. The increase in this fraction is a direct result of the three- to fivefold reduction in the specific radioactivities of the pH 8.3 fractions. Approximately 3×10^5 cpm was transferred to the pH 6 fraction, as compared with a loss of 1.6×10^5 cpm from the pH 8.3 fractions. The difference is probably due to differences in recoveries from the two fractions. rRNA, as expected, is more resistant to RNase. Finally, authentic homogeneous RNA species, such as the rRNAs of *Escherichia coli*, when taken through the fractionation procedure, even the steps at high pH, gave no evidence of induced degradation or increased heterogeneity.

[1] There may also be some contribution by the membrane-associated, rapidly labeled RNA recently identified by Attardi and Attardi (1968) as being of mitochondrial origin.

Aggregation must be considered a serious possibility for inducing artifacts, particularly in the 8-2 fractions, which appear to contain a non-RNA contaminant in the high molecular weight region of the gels. This contaminant may contain RNA aggregates, be complexed in some manner with RNA, or simply migrate independently of RNA. It has been established that the contaminant is contained in the RNA sample rather than occurring as a gel artifact. The OD_{260}/OD_{230} ratios in this region (close to unity) indicate that it contains species different from RNA. If it were a ribosomal aggregate similar to that described by Wagner *et al.* (1967), it would possess a ratio of about 2.0 and would presumably be concentrated in the pH 6 fraction, which it is not. It is likely that glycogen is present in fairly high concentrations despite 24-hr starvation, but its behavior on gels is not known. The best method for separating glycogen from the RNA might be centrifugation of the purified RNAs in the presence of sarcosinate, but we have found that the glycogen pellets obtained following such treatment contain substantial amounts of radioactivity.

The RNA fractions studied are fairly stable after washing and reprecipitation, as shown by incubation of such purified fractions at 37° for 15 min in buffer E containing 0.25 M sucrose. Under these conditions, only fraction 8-1P was affected by the treatment, about 20% of the radioactivity shifting to lower molecular weight regions of the gel (about 1 to 5×10^5 daltons). No difference in ultraviolet absorbance profiles could be detected, with the possible exception of a slight spreading of all bands. A reasonable amount of further manipulation of nonribosomal RNAs should, therefore, be possible following isolation and purification.

It is quite possible that additional improvements in the extraction procedure might increase the purity of nonribosomal RNAs as much as five- or tenfold. Only preliminary studies of the parameters that control the fractionation have been undertaken. No investigation of other buffer systems, pH changes, temperature changes, detergent and/or detergent concentration changes, or improvements in cellular isolation and fractionation procedures have yet been attempted. The possibility of isolating polysomes from a 2.0–2.3 M sucrose interface rather than from pellets might result in reduced degradation (and elimination of possible contamination by intact nuclei). The use of various chelating agents might also promote improved separations of RNA fractions.

The major problems and limitations of the procedure are: (1) the continuing need for the prevention of RNA degradation, during both cellular and RNA fractionation; (2) the large amounts of tissue (and isotopes) necessary in order to obtain workable quantities of nonribosomal RNAs; (3) a need for further reduction of cross-contamination by ribosomal and nonribosomal species (background radioactivity levels (*i.e.*, the region between 5S and 18S RNA) on the gels indicate the possibility of extraction of nonribosomal RNAs in the pH 6 fractions, and elimination of this source of loss may lead to substantially higher yield of mRNAs); and (4) the elimination of non-RNA contaminants such as protein, DNA and glycogen.

The results of the double-labeling experiments which use pooled livers differentially labeled with the two isotopes suggest that the reproducibility of the method between runs and groups of animals is excellent. The coincidence of the profiles itself indicates either that the synthesis of all species of RNA was induced to the same extent by triamcinolone or that

if induction is specific for some RNA species, the latter are present in amounts below the level of detection. There is some evidence in favor of the former alternative based on results with whole animals (Greenman *et al.*, 1965; Wicks *et al.*, 1965; Feigelson and Feigelson, 1966; MacGregor and Mahler, 1967), and in favor of the latter based on findings with the cell culture system (Gelehrter and Tomkins, 1967). If the relevant mRNA accounts for $\leq 1\%$ of the rapidly labeled 8-1P plus 8-2P fractions and its increase in specific activity induced by hormone administration is threefold or less it might easily escape detection.

References

Attardi, G., and Attardi, B. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 261.

Attardi, G., Parnas, H., Hwang, M.-I. H., and Attardi, B. (1966), *J. Mol. Biol. 20*, 145l.

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.

Blobel, G., and Potter, V. R. (1967), *J. Mol. Biol. 26*, 279.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C., Marushige, K., and Tuan, D. Y. H. (1968), *Science 159*, 47.

Bont, W. S., Rezelman, G., and Bloemendal, H. (1965), *Biochem. J. 95*, 15C.

Brawerman, G., and Hadjivassiliou, A. (1967), *Biochemistry 6*, 1934.

Burton, K. (1956), *Biochem. J. 62*, 315.

Campagnoni, A. T. (1968), Ph.D. Dissertation, Indiana University, Bloomington, Ind.

Chambon, P., Karon, H., Ramuz, M., and Mandel, P. (1968), *Biochim. Biophys. Acta 157*, 520.

Csány, V., Greengard, O., and Knox, W. E. (1967), *J. Biol. Chem. 242*, 2688.

Diamondstone, T. I. (1966), *Anal. Biochem. 16*, 395.

Dingman, C. W., and Peacock, A. C. (1968), *Biochemistry 7*, 659.

Drews, J., and Brawerman, G. (1967), *J. Biol. Chem. 242*, 801.

Drury, H. F. (1948), *Arch. Biochem. Biophys. 19*, 455.

Dukes, P. P., Sekeris, C. E., and Schmid, W. (1966), *Biochim. Biophys. Acta 123*, 126.

Feigelson, M., and Feigelson, P. (1966), *J. Biol. Chem. 241*, 5819.

Feigelson, P., Feigelson, M., and Greengard, O. (1962a), *Recent Progr. Hormone Res. 18*, 491.

Feigelson, P., and Greengard, O. (1962), *J. Biol. Chem. 237*, 3714.

Feigelson, M., Gross, P. R., and Feigelson, P. (1962b), *Biochim. Biophys. Acta 55*, 495.

Finkel, R., Henshaw, E., and Hiatt, H. H. (1966), *Molec. Pharm. 2*, 222.

Fraenkel-Conrat, H., Singer, B., and Tsugita, R. (1961), *Virology 14*, 54.

Gelehrter, T. D., and Tomkins, G. M. (1967), *J. Mol. Biol. 29*, 59.

Girard, M., Latham, H., Penman, S., and Darnell, J. E. (1965), *J. Mol. Biol. 11*, 187.

Girard, M., Penman, S., and Darnell, J. E. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 205.

Greengard, O., and Acs, G. (1962), *Biochim. Biophys. Acta 61*, 652.

Greenman, D. L., Wicks, W. D., and Kenney, F. T. (1965), *J. Biol. Chem. 240*, 4420.

Grossman, A., and Mavrides, C. (1967), *J. Biol. Chem. 242*, 1398.

Hadjiolov, A. A. (1967), *Progr. Nucleic Acid. Res. Mol. Biol. 7*, 196,

Henshaw, E. C., Revel, M., and Hiatt, H. H. (1965), *J. Mol. Biol. 14*, 241.

Holten, D., and Kenney, F. T. (1967), *J. Biol. Chem. 242*, 4372.

Howell, R. R., Loeb, J. N., and Tomkins, G. M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1241.

Kenney, F. T. (1962), *J. Biol. Chem. 237*, 3495.

Kenney, F. T., Greenman, D. L., Wicks, W. D., and Albritton, W. L. (1965), *Advan. Enzyme Reg. 3*, 1.

Knox, W. E., Ogata, M., Hasegawa, N., and Tokuyama, K. (1964), *VIth Intern, Congr. Biochem.*, Abstract IX-45.

Knox, W. E., Piras, M. M., and Tokuyama, K. (1966), *J. Biol. Chem. 241*, 297.

Kvam, D. C., and Parks, R. E. (1960), *J. Biol. Chem. 235*, 2893.

Lawford, G. R., Langford, P., and Schacter, H. (1966), *J. Biol. Chem. 241*, 1835.

Loeb, J. N., Howell, R. R., and Tomkins, G. M. (1967), *J. Biol. Chem. 242*, 2069.

Loening, U. E. (1967), *Biochem. J. 102*, 251.

MacGregor, R. R., and Mahler, H. R. (1967), *Arch. Biochem. Biophys. 120*, 136.

Mishkin, E. P., and Shore, M. L. (1967), *Biochim. Biophys. Acta 138*, 169.

Murty, C. N., and Hallinan, T. (1968), *Biochim. Biophys. Acta 157*, 414.

Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.

Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7*, 688.

Penman, S. (1966), *J. Mol. Biol. 17*, 117.

Perry, R. P. (1967), *Progr. Nucleic Acid Res. Mol. Biol. 6*, 219.

Perry, R. P., and Kelley, D. E. (1968), *J. Mol. Biol. 35*, 37.

Peterkofsky, B., and Tomkins, G. (1967), *J. Mol. Biol. 30*, 49.

Peterkofsky, B., and Tomkins, G. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 222.

Reif-Lehrer, L., and Amos, H. (1968), *Biochem. J. 106*, 425.

Richards, E. G., and Coll, J. A. (1965), *Anal. Biochem. 12*, 452.

Schimke, R. T., Sweeney, E. W., and Berlin, C. M. (1964), *Biochem. Biophys. Res. Commun. 15*, 214.

Schmid, W., Gallwitz, D., and Sekeris, C. E. (1967), *Biochim. Biophys. Acta 134*, 80.

Segal, H. L., and Kim, Y. S. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 912.

Segal, H. L., and Kim, Y. S. (1965), *J. Cellular Comp. Physiol. 66, Supp. I*, 11.

Soeiro, R., Birnboim, H. C., and Darnell, J. E. (1966), *J. Mol. Biol. 19*, 362.

Soeiro, R., Vaughan, M. H., Warner, J. R., and Darnell, J. R., Jr. (1968), *J. Cell Biol. 39*, 112.

Stackhouse, H. L., Chetsanga, C. J., and Tan, C. H. (1968), *Biochim. Biophys. Acta 155*, 159.

Tomkins, G. M., Thompson, E. B., Hayashi, S., Gelehrter, T., Granner, D., and Peterkofsky, B. (1966), *Cold Spring Harbor Symp. Quan. Biol. 31*, 349.
Wagner, E. K., Katz, L., and Penman, S. (1967), *Biochem. Biophys. Res. Commun. 28*, 152.
Watanabe, Y., Prevec, L., and Graham, A. F. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1040.
Whittle, E. D., Bushnell, D. E., and Potter, V. R. (1968), *Biochim. Biophys. Acta 161*, 41.
Wicks, W. D., Greenman, D. L., and Kenney, F. T. (1965), *J. Biol. Chem. 240*, 4414.
Widnell, C. C., and Tata, J. R. (1964), *Biochem. J. 92*, 313.
Yoshikawa-Fukada, M., Fukada, T., and Kawade, Y. (1965), *Biochim. Biophys. Acta 103*, 383.

Poly-5-methyldeoxycytidylic Acid and Some Alkylamino Analogs*

Barbara Zmudzka, F. J. Bollum, and D. Shugar

ABSTRACT: 5-Methyldeoxycytidine triphosphate and several alkylamino analog triphosphates have been polymerized with the aid of terminal deoxynucleotidyl transferase to give poly-5-methyldeoxycytidylic acid and the N^4-methyl and N^4-ethyl polymer analogs of 5-methyldeoxycytidylic acid. All three of these were polymerized by the enzyme at the same rate as polydeoxycytidylic acid, and the final products have about the same sedimentation constants, $s_{20,w}$ = 4.5 S. Poly-5-methyldeoxycytidylic acid forms an acid twin-stranded helix similar to that formed by polydeoxycytidylic acid, the 5-methyl substituent being without any appreciable effect on the stability. The alkylamino polymer analogs do not form such structures. All three new polymers are considerably more resistant than polydeoxycytidylic acid to phosphodiesterase I. Poly-5-methyldeoxycytidylic readily complexes with polydeoxyinosinic acid, and the T_m for the twin-stranded helix is 17° higher than that for the corresponding polydeoxyinosinic acid:polydeoxycytidylic acid, thus confirming the significant stabilizing effect of a pyrimidine 5-methyl substituent, even in the absence of a 2′-hydroxyl. An extensive analysis of the complexes between poly-5-methyldeoxycytidylic acid and polydeoxyinosinic acid, as a function of the ionic strength of the medium, demonstrated the existence of four equilibrium reactions between the homopolymers and the twin- and triple-stranded helices formed by them. The alkylamino polymer analogs did not complex with polydeoxyinosinic acid, probably as a result of hindered rotation of the alkylamino groups by the 5-methyl substituent. The nature of the various complexes is discussed with emphasis on the so-called acid form of poly-5-methyldeoxycytidylic acid (and polydeoxycytidylic acid) in relation to the acid form of poly-5-methylribocytidylic acid (and polyribocytidylic acid).

The introduction of a 5-methyl substituent into the base residues of poly rU[1] results in an appreciable enhancement of helix stability, as demonstrated by the higher T_m value of poly rT compared with poly rU (Shugar and Szer, 1962). This effect is also observed in the complexes of the foregoing with poly A (Szer *et al.*, 1963; Barszcz and Shugar, 1968). While the acid and neutral forms of poly rC are not appreciably modified by 5 methylation, for reasons which have been presented elsewhere (Szer and Shugar, 1966), the twin-stranded helix of poly 5MerC with poly rI does exhibit a markedly higher T_m value than the corresponding complex with poly rC. A similar enhancement of helix stability by a pyrimidine 5-methyl substituent has been found in the twin-stranded complexes of poly X with poly rU and poly rT (Fikus and Shugar, 1969).

It thus was of interest to examine the influence of a pyrimidine 5-methyl substituent on the properties of poly dC and its complexes with poly dI, in part because of the absence of any secondary structure in poly dT (Riley *et al.*, 1966) as compared with poly rT (Shugar and Szer, 1962), and in poly dU as compared with poly rU (Zmudzka *et al.*, 1968). The properties of methylated polynucleotides are also of interest because of the widespread presence of methylated base residues as minor or major components in natural nucleic acids.

* From the Department of Radiobiology, Institute of Oncology, Warsaw, Poland, the Department of Biochemistry, University of Kentucky, Lexington, Kentucky, and the Institute of Biochemistry and Biophysics, Academy of Sciences, Warsaw, Poland. *Received February 25, 1969.* This investigation was supported by the Wellcome Trust, the Agricultural Research Service, U. S. Department of Agriculture (UR-E21-(30)-32), the World Health Organization, and (to F. J. B.) the National Cancer Institute, National Institutes of Health (CA 08487).

[1] For purposes of clarity the prefixes r and d, for ribosyl and deoxyribosyl compounds, respectively, are retained throughout this text where a distinction between the two is necessary. Most of the other abbreviations followed the Revised Tentative Rules of the IUPAC–IUB combined Commission on Biochemical Nomenclature (*Biochemistry 5*, 1445 (1966)). The following nonstandard abbreviations are also employed: poly 5MedC, poly-5-methyldeoxycytidylic acid; poly N^4,5-diMedC, poly-N^4,5-dimethyldeoxycytidylic acid; poly N^4Et,5MedC, poly-N^4-ethyl-5-methyldeoxycytidylic acid; poly rT, poly-5-methylribouridylic acid; poly 5MerC, poly-5-methylribocytidylic acid; poly X, polyriboxanthylic acid 3 → 2, represents a transition from a triple- to a twin-strainded helix, with similar connotations for other types of transitions.