[16] Such higher oxidation states of iron have been observed in peroxidase and ferrimyoglobins: Theorell, H., A. Ehrenberg, and B. Chance, *Arch. Biochem. Biophys.*, **37**, 237 (1952); George, P., and D. H. Irvine, *J. Chem. Soc.*, 3142 (1954); George, P., and D. H. Irvine, *Biochem. J.*, **60**, 596 (1955); George, P., and J. S. Griffith in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Adademic Press, 1959), vol. I, p. 347.

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*ENZYMATIC INCORPORATION OF RIBONUCLEOSIDE TRIPHOSPHATES INTO THE INTERPOLYNUCLEOTIDE LINKAGES OF RIBONUCLEIC ACID**

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Two separate reactions have been described for the enzymatic polymerization of nucleotides. In 1955, Grunberg-Manago and Ochoa[1] reported that an enzyme isolated from the microorganism *Azotobacter vinelandii* catalyzes the synthesis of highly polymerized ribonucleotides from 5′-ribonucleoside diphosphates with the release of inorganic phosphate. Because of its similarity to the action of phosphorylase on polysaccharides, i.e., its reversible phosphorolysis, the enzyme was called polynucleotide phosphorylase. Since polynucleotide phosphorylase acts on single as well as mixtures of ribonucleoside diphosphates, to form single or mixed polymers, its role as a general mechanism for the biosynthesis of specific ribonucleic acids has been debated.[2] This enzyme is widely distributed in microorganisms and its presence in higher forms has been briefly reported by Hilmoe and Heppel.[3]

DNA[4] polymerase was first described in 1956 by Kornberg, Lehman, and Simms[5] in extracts from *E. coli*. Further investigations by this group[6] clearly showed that this enzyme catalyzes a net synthesis of deoxyribonucleic acid which is dependent on the presence of all four deoxynucleoside triphosphates, as well as magnesium ions and "primer" DNA, and occurs with the elimination of inorganic pyrophosphate. A mammalian system which catalyzes a reaction similar to DNA polymerase has been reported.[7]

Terminal addition of one or more ribonucleotides to RNA molecules is another reaction observed by a number of investigators.[8–13] This reaction is specific for the ribonucleoside triphosphate and results in the release of inorganic pyrophosphate. However, no extensive polymerization occurs.

In view of the importance of specific ribonucleic acid molecules as carriers for individual amino acids in protein synthesis,[14, 15] and the widely held view that RNA may provide templates for the synthesis of specific proteins, the present work was undertaken. In the course of this investigation, a new type of reaction for the incorporation of ribonucleotides has been found. This paper will describe the enzymatic incorporation of ribonucleoside triphosphates into the interpolynucleotide linkages of RNA, by an enzyme system of mammalian origin, which

requires that all four triphosphates be present. In this respect, the reaction resembles the action of DNA polymerase rather than polynucleotide phosphorylase.

A preliminary account of some aspects of this work has been published.[16]

Materials and Methods.—Preparation of labeled cyt-P^{32}-P-P: 4 mc of P^{32} and 100 μmoles of phosphoric acid were dried overnight in an oven at 110°. To the tube, 20 mg of PCl_5 was added and thoroughly mixed. The tube was tightly stoppered and incubated for 30 min in a 45° bath. 0.20 ml of dimethylformamide was added and the tube incubated for another 30 min to insure complete formation of $POCl_3$. 22 mg of dried 2′,3′-benzylidene-O-cytidine, prepared by the method of Baddiley,[17] was added, mixed, and the vessel incubated at 45° for four hr. The reaction was stopped by the addition of 2 ml of 0.20 *N* HCl and the tube heated in a boiling water bath for 12 min. After cooling, the mixture was diluted to 200 ml, the pH adjusted to 9, passed over a column of Dowex 1 formate resin and eluted with a gradient elution system containing 0.04 *N* formic acid in the upper reservoir as previously described.[18] The P^{32}-cytidine 5′-phosphate emerged from the column after an earlier material believed to be dicytidine monophosphate. The 5′ isomer peak was identified with the aid of the specific 5′-nucleotidase of *Crotalus adamanteus* venom. No 2′ or 3′ isomer was detected. In this manner, 14 μmoles of labeled CMP were prepared.

Labeled cyt-P^{32}-P-P was prepared from labeled CMP by a crude brewers yeast extract.[19] The yeast extract was purified 2.5 fold, with respect to its cytidylate kinase activity, by ammonium sulfate fractionation. The reaction mixture consisted of 250 μmoles of $MgCl_2$, 1.5 mmoles of Tris buffer of pH 7.4, 50 μmoles of ADP, 200 μmoles of phosphopyruvic acid, 500 μgrams of pyruvic kinase, 30 μmoles of labeled CMP, and 28 mg of the yeast extract in a final volume of 50 ml. After incubation at 37° for two hr, the reaction was stopped by heating in a boiling water bath for two min and then placed immediately in ice. The denatured protein was removed by filtration and the labeled CTP isolated by chromatography on a column of Dowex 1 chloride resin as described by Lehman et al.[20] The yield of CTP was 80 per cent based on the starting CMP. The final product had a specific activity of 3.5×10^7 c.p.m. per micromole. Analysis of the labeled nucleotides synthesized in this report are given in Table 1.

TABLE 1

ANALYSIS OF THE RIBONUCLEOSIDE TRIPHOSPHATES

Labeled nucleotide	Absorbance ratios*		Per cent radioactivity†		
	λ250/λ260	λ280/λ260	Monophosphate	Diphosphate	Triphosphate
CTP	0.45	2.09	0.20	1.8	98
UTP	0.75	0.35	0.15	1.3	98
ATP	0.84	0.23	0.30	5.5	94

* All values were determined at pH 2.

† Labeled compound was added to a mixture of appropriate ribonucleoside mono-, di-, and triphosphates. The mixture was subjected to paper electrophoresis in 0.025 *M* citrate buffer of pH 4.6, at 250 volts for 16 hr. The individual nucleotides were located under ultraviolet light and their radioactive content determined. The per cent radioactivity is based on the total number of counts placed on paper.

UTP^{32}, labeled in the ester phosphate only, was prepared from cyt-P^{32}-P-P by the procedure of Lohman.[21] The labeled UTP was purified by chromatography on a column of Dowex 1 chloride resin as described by Bessman and co-workers.[22] UTP was obtained in 67 per cent yield with a specific activity identical to CTP.

ATP^{32}, labeled in the ester phosphate only, was prepared from P^{32}-labeled

adenosine 5′-phosphate. The labeled mononucleotide was synthesized by a method suggested by Goldwasser,[23] using the isopropylidene derivative of adenosine as starting material. 50 μmoles of inorganic phosphate, containing 6 mc of P^{32}, was dried in a small vial at 110° overnight. 1 ml of dimethylformamide, 40 mg of dried 2′,3′-O-isopropylidene adenosine and 0.50 ml of dicyclohexylcarbodiimide were added and the stoppered vial stirred for 12 hr by magnetic mixing. The reaction was stopped by the addition of 6 ml of 0.05 *N* HCl and allowed to stir for another 12 hr. The solution was neutralized with 2 *N* NaOH and the insoluble dicyclohexylurea product removed by filtration. The filtrate was extracted with ether and after removal of dissolved ether, by a gentle stream of air, passed over a column of Dowex 1 formate resin. AMP^{32} was eluted with a gradient elution system with 1.0 *N* formic acid in the upper reservoir and 250 ml of water in the mixing chamber. Three radioactive peaks were located containing adenine. In the order eluted, these peaks were diadenosine monophosphate, adenosine monophosphate, and adenosine diphosphate as determined by the ratio of radioactivity to adenine nucleotide. The fractions containing AMP^{32} were concentrated and samples subjected to paper electrophoresis as described in Table 1. Although partially contaminated with the other two adenosine reaction products, the labeled AMP isolated after paper electrophoresis was shown to be the 5′ isomer by the action of the specific 5′-nucleotidase of snake venom. The yield of labeled AMP was 3 μmoles. It was diluted threefold with unlabeled AMP and converted to ATP^{32} using the crude yeast extract described above which also contained adenylate kinase. The conditions for the reaction were essentially the same as that described for CTP formation except no ADP was present and 1 μmole of ATP was added to initiate the reaction. Labeled ATP was isolated in a similar manner described for CTP. 6.3 μmoles of ATP were obtained with a specific activity of 3.4×10^7 c.p.m. per micromole.

Preparation of rat liver enzyme: A 20 per cent rat liver homogenate, in 0.25 molar sucrose containing 0.001 *M* $MgCl_2$, was prepared with the aid of a loose fitting stainless steel homogenizer. The homogenate was filtered through gauze and centrifuged at approximately 600 × *g* for six min at 4°. The supernatant was decanted and the loosely packed pellet washed twice more with 20 volumes of homogenizing medium. Microscopic examination of the pellet at this stage showed a high concentration of nuclei. The residue was washed once more in isotonic sucrose to remove excess $MgCl_2$, suspended in 20 volumes of 0.05 *M* Tris buffer of pH 7.4, and allowed to stand at 0° for 10 min. The lysed nuclei were centrifuged at 10,000 × *g* for 10 min. The highly colored supernatant was discarded leaving a somewhat white pellet behind. The residue obtained from 40 grams of rat liver in this manner was suspended to a final volume of 16 ml with 0.05 *M* Tris buffer of pH 7.4. 2 *M* KCl was added to the suspension dropwise, with adequate stirring, to a final concentration of 0.40 *M* KCl. Within a few min a white aggregate formed that could be separated from solution by lifting on glass rods. The isolated aggregate was washed twice in the Tris-KCl medium and finally suspended in 0.05 *M* Tris of pH 8.1 by vigorous homogenization with a glass homogenizer to give a protein concentration of 10–12 mg per ml of suspension. This preparation was used as the source of enzyme for most of the experiments reported here and shall be referred to as the aggregate-enzyme.

Enzyme assay: After incubation, the reaction was stopped by the addition of 5 ml of cold 5 per cent TCA. The acid-insoluble precipitate was washed twice with cold 5 per cent TCA, two times with ethanol-ether (3:1) and extracted twice with 4 ml of 10 per cent NaCl, at pH 8, in a boiling water bath for 30 min. 2 mg of carrier RNA was added with each extraction. The combined extracts were precipitated with 2 volumes of cold ethanol, the precipitate redissolved in 5 ml of water and 2 ml samples dried and assayed in a windowless gas flow counter.

Isolation of ribonucleic acid: RNA, from various preparations, was isolated by the Kirby modification[24] of the procedure of Gierer and Schramm.[25] P^{32}-labeled RNA, isolated in this way, was exhaustively dialysed against 0.05 *M* KCl until no further counts could be detected in the dialysing medium.

Hydrolysis of ribonucleic acid: The conditions for alkaline hydrolysis are described in Table 4. After hydrolysis, the solution was neutralized with 18 per cent perchloric acid. After standing for 2 hr at 0°, the precipitated salt was removed by centrifugation. Separation of nucleotides was accomplished by paper electrophoresis in 0.025 *M* citrate buffer of pH 3.5, for 20 hr at 400 volts, and by chromatography on columns of Dowex 1 formate resin. The isolated nucleoside 2′- and 3′-monophosphates were assayed for radioactivity.

Enzymatic hydrolysis of labeled RNA was accomplished with the aid of *Crotalus adamanteus* venom. The reaction conditions and procedure are given in Figures 6 and 7.

Phosphorus was determined by the method of Gomori.[26] Inorganic-P^{32} was separated from organic-P^{32} by extraction into organic solvents as described by Borkenhagen and Kennedy.[27] Protein was determined by the method of Lowry.[28]

TABLE 2

NUCLEOTIDE REQUIREMENT FOR THE INCORPORATION OF LABELED NUCLEOTIDES INTO RNA

Experiment number	Labeled substrate	Nucleotides added	Labeled substrate incorporated into RNA, μμmoles
1*	CTP^{32}	Complete	50.0
		Omit ATP	10.0
		Omit GTP	6.0
		Omit UTP	6.8
		Omit ATP, UTP, GTP	4.7
		Complete except 1 μmole each of ATP, UTP, GTP	570.0
2*	UTP^{32}	Complete	32.0
		Omit ATP	5.5
		Omit GTP	4.3
		Omit CTP	3.5
		Omit ATP, CTP, GTP	2.8
		Complete except 1 μmole each of ATP, CTP, GTP	280.0
3†	ATP^{32}	Complete	62.0
		Omit GTP	12.1
		Omit UTP	16.5
		Omit CTP	13.9
		Omit GTP, UTP, CTP	12.4

* The complete system contained 5 μmoles of $MgCl_2$, 100 μmoles Tris, buffer of pH 8.05, 10 μmoles of cysteine, 0.06 μmoles of CTP^{32} or UTP^{32} (2.1×10^7 c.p.m. per micromole), 0.06 μmoles each of ATP, UTP, GTP, and CTP, except as indicated, and aggregate-enzyme containing 6 mg of protein. The final volume of the system was 1.0 ml. The vessels were incubated for 15 min at 37°.

† The reaction mixture was the same as above except that in addition, each vessel contained 60 μmoles of KCl, 20 μmoles of NaF, and 0.06 μmoles of ATP^{32} (1.4×10^7 c.p.m. per micromole) as the labeled substrate. 12 mg of twice washed nuclei served as the enzyme source.

Optical density measurements were made in a Zeiss spectrophotometer. The nucleotides used in this work were products of the Pabst Laboratories. Other materials used in this report were of commercial origin. *Crotalus adamanteus* venom was bought from Ross Allen's Reptile Institute, Silver Springs, Florida.

Experimental.—Enzymatic incorporation of nucleoside triphosphates into RNA: When rat liver homogenates were incubated with cyt-P^{32}-P-P, a significant incorporation of label into the acid-insoluble residue was observed. Further experiments indicated that the nuclear fraction was particularly active in this respect and that optimal incorporation occurred when ATP, UTP and GTP were present.

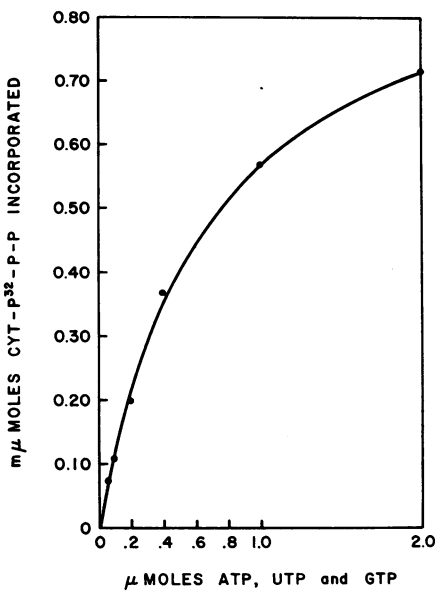


FIG. 1.—Incorporation of CTP^{32} at various concentrations of ATP, UTP and GTP. Each tube contained 5 μmoles of $MgCl_2$, 100 μmoles Tris buffer of pH 8.05, 10 μmoles of cysteine, 0.06 μmoles of CTP^{32} (9×10^6 c.p.m. per micromole), rat liver aggregate-enzyme (see methods) containing 6 mg of protein and ATP, UTP and GTP each, in the quantities shown. The final volume of the system was 1.1 ml. The tubes were incubated for 15 min at 37°.

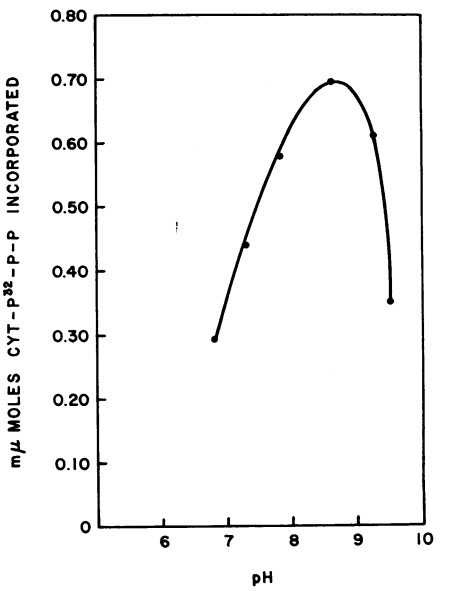


FIG. 2.—The incorporation of CTP^{32} as a function of pH. The conditions of the enzyme assay were identical with those shown for Fig. 1, except that each tube contained 100 μmoles of an equimolar mixture of phosphate and Tris. One μmole each of ATP, UTP, and GTP per reaction vessel was used. The hydrogen ion concentration of the Tris-phosphate buffer was varied to give the pH values shown, which were measured with a glass electrode after the addition of all reaction components. The tubes were incubated for 15 min at 37°.

It was found that after lysis of the nuclei, the DNA in these preparations could be aggregated by the addition of the proper amount of salt. At higher salt concentrations (1 molar), the aggregate becomes soluble and a highly viscous suspension develops. Removal of the aggregate from solution and subsequent assay for nucleotide incorporation indicated that this preparation contained the entire activity of the original nuclear fraction and still maintained the requirement for all four ribonucleotides (Table 2). This requirement is also shown for labeled ATP with whole nuclei. The incorporation of labeled substrate is increased from eight to tenfold as the concentrations of each of the unlabeled nucleotides are simultaneously increased (Fig. 1).

If ribonucleoside diphosphates are substituted for the unlabeled ribonucleoside triphosphates a considerable reduction in the incorporation is observed (Table 3). This would suggest that the incorporation of ribonucleotides into RNA occurs at the triphosphate level, and that the reaction proceeds by the elimination of inorganic pyrophosphate similar to DNA polymerase[6] and the terminal addition type reactions.[13] Such a reaction mechanism would be in agreement with the observation that inorganic pyrophosphate, but not inorganic orthophosphate, inhibits the incorporation (Table 3).

Identification of the radioactive product as RNA: The labeled product, isolated from the reaction mixture, is acid-insoluble and nondialysable. Treatment with crystalline ribonuclease, but not desoxyribonuclease, alters these properties. The product may be isolated further under conditions where DNA is not extracted.[24] Treatment with alkali, under conditions known to hydrolyze RNA, or with snake venom, results in the formation of acid-soluble labeled material. The products formed by alkaline hydrolysis can be separated by paper electrophoresis or by ion exchange chromatography and have been identified as the

TABLE 3

COFACTOR REQUIREMENTS FOR THE INCORPORATION OF LABELED NUCLEOTIDES INTO RNA

Experiment number	Labeled substrate	Additions	Labeled substrate incorporated into RNA, $\mu\mu$moles
1	CTP^{32}	Complete	61.0
		Complete: ADP, UDP, GDP in place of ATP, UTP, GTP	18.0
		Complete: +5 μmoles of P-O-P	22.0
		Complete: +10 μmoles of P-O-P	3.7
		Complete: in 100 μmoles of Pi buffer pH 7.5 (no Tris)	60.0
2	UTP^{32}	Complete	32.0
		Complete: ADP, CDP, GDP in place of ATP, CTP, GTP	20.0
		Complete: +5 μmoles of P-O-P	11.0
		Complete: +10 μmoles of P-O-P	1.2
		Complete: in 100 μmoles of Pi buffer pH 7.5 (no Tris)	32.0

The contents of the complete system and the conditions of the reaction were identical to that described in Table 2, except that 10 μmoles of $MgCl_2$ were used. The ribonucleoside di- and triphosphates used were 0.06 μmoles each.

mononucleotides of cytidine, adenosine, uridine and guanosine.

Optimal pH for incorporation of CTP into RNA: When the hydrogen ion concentration of the reaction mixture was varied over a considerable range (Fig. 2), the highest incorporation took place in the range of pH 8 to 9. The activity falls off rapidly at values below pH 7.5, and above pH 9.

Requirement for divalent cations: The enzymatic incorporation of ribonucleotides into RNA requires the presence of divalent cations. At low concentrations, both magnesium and manganese can activate the system; magnesium was more effective. However, at higher concentrations both cations become inhibitory; the inhibition with manganese was more pronounced (Fig. 3). Calcium was unable to activate the enzyme system. Low concentrations of calcium (0.001 *M*), in the presence of optimal amounts of magnesium ion, were inhibitory.

The incorporation of CTP into RNA as a function of time: Under conditions of these experiments, optimum reaction time for the incorporation of CTP into

RNA occurred in ten min (Fig. 4). The diminished incorporation observed for longer periods of incubation most probably represents product breakdown. The reaction was linear for the first two min with half maximum incorporation occurring at the end of one minute.

Position of the nucleotide incorporated into RNA: When P^{32}-labeled RNA, prepared enzymatically with whole nuclei or aggregate-enzyme, was subjected to alkaline hydrolysis, each of the nucleoside 2′- and 3′-monophosphates separated by paper electrophoresis or by ion-exchange chromatography was labeled (Table 4). An electropherogram of the products separated after hydrolysis is shown in Figure 5. The slight amount of ultraviolet absorbing radioactive material migrating more slowly than 2′(3′)-CMP in Figure 5 is believed to be some unhydrolyzed dinucleoside monophosphate. Although these results suggest

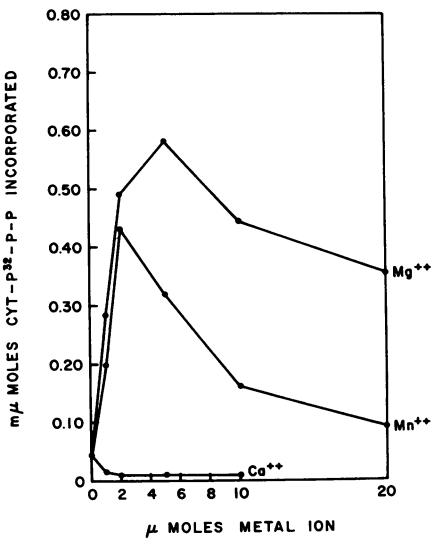


FIG. 3.—The requirement of divalent cations for the incorporation of CTP^{32}. The conditions of the enzyme assay were identical with those shown for Fig. 1, except that the time of incubation was 15 min and the added divalent cation concentration varied as indicated. One μmole each of ATP, UTP and GTP per reaction vessel was used.

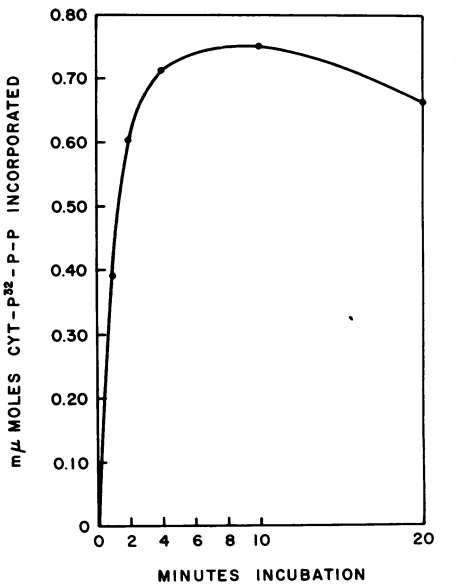


FIG. 4.—Incorporation of CTP^{32} as a function of time. The contents of each vessel were the same as indicated for Fig. 1, except that 1 μmole each of ATP, UTP and GTP were used. The final volume of the system was 1.1 ml. The reaction was incubated at 37° for the time intervals shown above.

that the incorporation of substrate is into the inner linkages of the RNA chain, the possibility of a terminal addition to different end groups of different ribonucleic acids, present in the enzyme preparation, cannot be excluded by this evidence alone.

The phosphodiesterase of snake venom has been shown to attack nucleic acid, in a stepwise fashion from the 3′-hydroxyl end of the chain, to liberate nucleoside 5′-monophosphates.[29] The free nucleoside 5 -monophosphates may be attacked next by the phosphomonoesterase of snake venom to form nucleosides and inorganic phosphate. If terminally labeled P^{32}-RNA is digested with crude snake venom, one might expect a disproportionate release of inorganic-P^{32} and inorganic phosphate if the kinetics of the over-all reaction were essentially those of the diesterase activity. That such is the case may be demonstrated by the reaction of

snake venom with terminally labeled P^{32}-RNA[13], prepared by the "pH 5 enzyme" and RNA isolated from the soluble fraction of rat liver homogenates (SRNA). Under these conditions, more than 90 per cent of the radioactivity was released while less than 10 per cent of the RNA chain had been degraded (Fig. 6). However, venom digestion of RNA labeled with CTP^{32} or UTP^{32} by the aggregate-enzyme (NRNA), results in the release of inorganic-P^{32} and inorganic phosphate at nearly identical rates (Fig. 7*a* and 7*b*). The slight discrepancy in rates observed in Figure 7*b* indicates that the distribution of labeled uridylate in the RNA chain is not statistically uniform. Higher concentrations of venom were used in the NRNA experiments so that digestion of 80 to 90 per cent of the material could be achieved in a reasonable length of time.

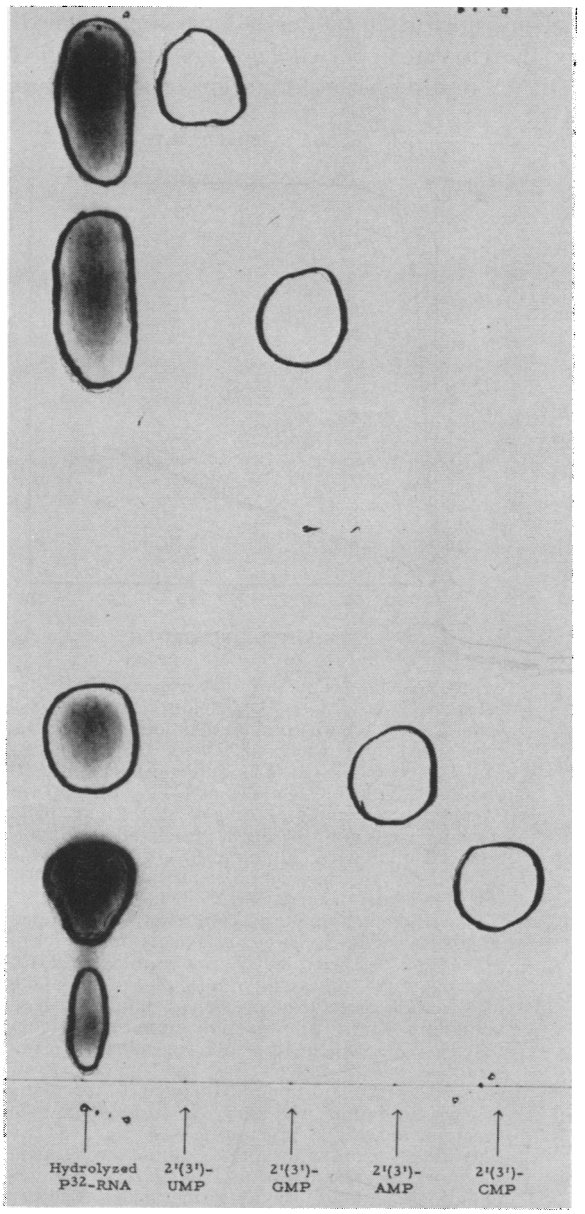


FIG. 5.—Paper electrophoresis of P^{32}-RNA, labeled with CTP^{32}, after alkaline hydrolysis. The conditions for alkaline digestion and paper electrophoresis are indicated in Table 4. The ultraviolet absorbing areas are encircled. The exposed dark areas indicate the presence of radioactivity.

Discussion.—A number of bacterial and mammalian cell-free systems have been described which incorporate labeled ribonucleotides into RNA.[1, 8–13, 30] Most of these, with the exception of polynucleotide phosphorylase, have been shown to catalyze the terminal addition of nucleotides to RNA chains. Goldwasser[31] and Edmonds and Abrams[11] have presented evidence which suggests that mammalian preparations can also catalyze incorporation of ribonucleotides into the framework of RNA. However, none of the above systems demonstrate a specific requirement for all four ribonucleotides.

Recently, Edmonds and Abrams[32] found that extracts from calf thymus nuclei

catalyze the formation of single adenylate polymers from ATP. It is apparent that incorporation studies with C^{14}-labeled nucleotides alone cannot in itself determine whether natural or atypical polynucleotide polymers have been formed. The synthesis of homopolymers by the enzyme reported here may be excluded since alkaline hydrolysis of the P^{32}-RNA results in the formation of all four nucleoside 2′- and 3′-monophosphates, each containing significant radioactivity.

The present experiments show that the omission of any one of the four ribonucleoside triphosphates resulted in a marked reduction of label incorporated when CTP, UTP, and ATP were the labeled substrates. In this respect, and also because the ribonucleoside triphosphate rather than the diphosphate appears to be required, the system is similar to the action of DNA polymerase and different from polynucleotide phosphorylase. The hydrolysis experiments with snake venom clearly demonstrate that the labeled RNA formed by the aggregate-enzyme is quite different from terminally labeled RNA. Indeed, the similar rates of release of inorganic phosphate and inorganic-P^{32} can only be interpreted to mean that the labeled substrate had been incorporated throughout the entire polynucleotide chain. This information, coupled with the four ribonucleotide requirement, suggests that polynucleotide synthesis had taken place. The same type of activity shown here for extracts from rat liver nuclei can be shown to occur with nuclei from calf thymus and ascites cells. The reaction is not stimulated by the addition of nuclear RNA, however, addition of soluble RNA causes terminal addition products besides those described above. It is also interesting that preincubation of the aggregate-enzyme with extremely small quantities of desoxyribonuclease completely inactivates the system. Similar treatment with ribonuclease is not as effective. Elucidation of the exact requirements for this enzyme must await further purification.

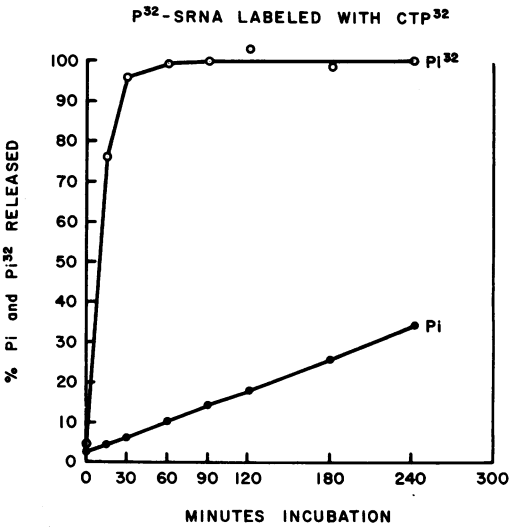


FIG. 6.—The enzymatic hydrolysis of P^{32}-labeled SRNA with snake venom. Terminal-labeled P^{32}-SRNA was prepared as follows: 4 tubes were used and each contained 10 μmoles of $MgCl_2$, 100 μmoles of Tris buffer of pH 8.05, 20 μmoles of NaF, 0.06 μmoles of CTP^{32} (3.4×10^7 c.p.m./per micromole), 1.2 mg of SRNA and "pH 5 enzyme" containing 6-7 mg of protein, in a final volume of 1 ml. After 20 min at 37°, the SRNA was isolated by the phenol procedure (see methods) with 3 mg more of carrier SRNA being added.

The snake venom incubation reaction contained 20 μmoles of $MgCl_2$, 200 μmoles of glycine buffer of pH 8.4, P^{32}-labeled SRNA containing 144,000 total c.p.m. and 13 μmoles of total phosphate, and 8 mg of crude *Crotalus adamenteus* venom, in a final volume of 4 ml. The reaction mixture was incubated at 37°. At the time intervals shown, 0.50 ml samples were removed and the reaction was stopped by the addition of 5 per cent TCA. 2 mg of carrier albumin was added to insure complete precipitation. The precipitate was removed by centrifugation and appropriate aliquots were taken for inorganic phosphate and inorganic-P^{32} determinations as described under methods. The per cent Pi and Pi^{32} released is based on the total phosphate and radioactivity content of the RNA used in the reaction.

TABLE 4

ALKALINE HYDROLYSIS OF P^{32}-RNA

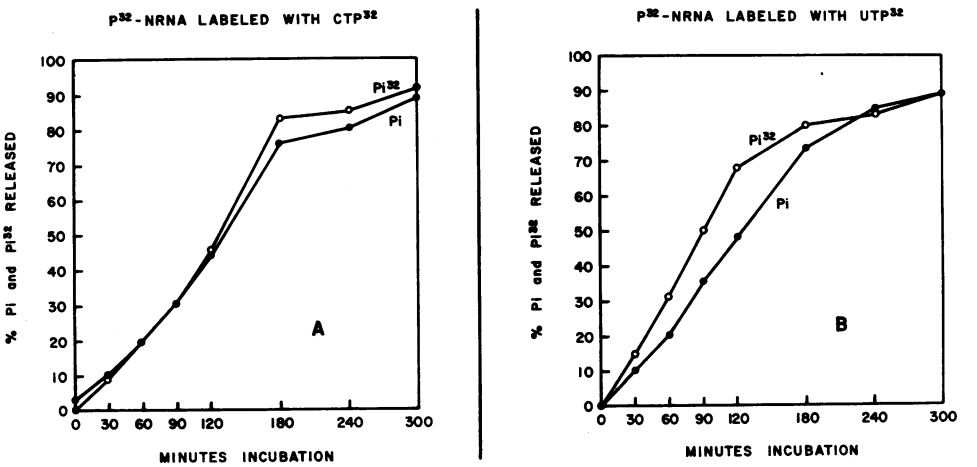
Labeled substrate used	Total RNA counts	Counts in nucleotides isolated after alkaline hydrolysis				Per cent recovery of total counts
		2'(3')CMP	2'(3')AMP	2'(3')GMP	2'(3')UMP	
CTP^{32}*	83,200	20,200	8,350	18,850	22,900	84.5
UTP^{32}†	40,000	15,400	4,200	5,800	13,000	96.0
ATP^{32}‡	31,900	6,090	6,500	8,000	8,100	90.0

Labeled RNA was prepared by the incubation of CTP^{32}, UTP^{32} and ATP^{32} with the nuclei preparations as shown in Fig. 2. The P^{32}-RNA was isolated by the phenol procedure.

* Hydrolysis carried out in 0.20 *N* KOH for 3 hrs at 80°. The mononucleotides were separated by paper electrophoresis in 0.025 *M* citrate buffer of pH 3.5, at 400 volts for 20 hrs.

† Hydrolysis carried out in 0.30 *N* KOH for 18 hrs at 37°. The separation of mononucleotides was by paper electrophoresis.

‡ Hydrolysis same as for CTP^{32}-labeled RNA. The mononucleotides were separated by gradient elution with formic acid from a column of Dowex 1 formate resin (10 per cent cross-linked).



FIGS. 7*a* and 7*b*.—The enzymatic hydrolysis of P^{32}-labeled NRNA with snake venom. P^{32}-labeled NRNA was prepared by the incubation of either CTP^{32} or UTP^{32} with the aggregate-enzyme as described previously. The reaction system was identical to that shown in Fig. 2, and 4 tubes were incubated with each label. After 15 min at 37°, the NRNA was isolated as described in Fig. 6, except that unlabeled NRNA was added as carrier.

The conditions for the snake venom reaction and procedure were identical with that shown in Fig. 6, except that 16 mg of crude *Crotalus adamanteus* venom was used. The P^{32}-NRNA used in the reaction system for Fig. 7*a* contained a total of 16,000 c.p.m. and 14.66 μmoles of total phosphate. The P^{32}-NRNA used in the reaction system for Fig. 7*b* contained a total of 19,400 c.p.m. and 14.2 μmoles of total phosphate. Per cent Pi and Pi^{32} released was calculated as described for Fig. 6.

Summary.—An enzyme system isolated from rat liver nuclei was found to catalyze the incorporation of labeled ribonucleoside triphosphates into ribonucleic acid. The enzymatic activity was dependent on the presence of all four ribonucleoside triphosphates.

Evidence is presented which shows that the incorporation of labeled ribonucleoside triphosphate occurs throughout the entire polynucleotide chain.

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[4] The abbreviations used in this report are: DNA, deoxyribonucleic acid; RNA, ribonucleic

acid; CTP or cyt-P-P-P, ATP, UTP, and GTP for the tri- and CDP, ADP, UDP, and GDP for the di- and CMP, AMP, UMP, and GMP for the monophosphates of cytidine, adenosine, uridine, and guanosine; Tris, tris-(hydroxymethyl)-aminomethane; TCA, trichloroacetic acid; c.p.m., counts per minute; Pi, inorganic orthophosphate; P-O-P, inorganic pyrophosphate.

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FORMATION OF THE PEPTIDE CHAIN OF HEMOGLOBIN

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The cytoplasmic ribonucleoprotein particles, or ribosomes, are now considered to be the major site of protein synthesis in a variety of tissues.[1] Kinetic studies with intact reticulocytes have indicated that these particles are the site of hemoglobin synthesis.[2, 3] Little is known, however, about the intra-ribosomal events which lead eventually to the final peptide chain. Two general mechanisms may be distinguished: simultaneous linkage of amino acids, and stepwise synthesis involving intermediate peptides. Intermediate peptides might be formed sequentially