*THE ROLE OF DNA IN RNA SYNTHESIS, IX. NUCLEOSIDE TRIPHOSPHATE TERMINI IN RNA POLYMERASE PRODUCTS**

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It has been shown previously that in RNA polymerase reactions primed with a variety of DNA preparations there is incorporation of P^{32} from $\beta\gamma$-labeled ATP into an acid-insoluble product and that triphosphate groups are present at the ends of the RNA chains formed during the reaction.[1] Two schemes for initiation of the chains can thus be envisaged. In one scheme, the initial nucleotide incorporated into RNA would retain its triphosphate end, while the growing end of the molecule would be a nucleoside. In the second scheme, the situation is reversed: The nucleoside end would be the initiation point, and the triphosphate, the growing site of the molecule. It is clear that these two schemes of initiation of RNA synthesis differ specifically in that the first would result in the initial nucleotide retaining the β and γ phosphate groups, whereas in the second scheme the last entering nucleotide would contain the β and γ phosphate group.

In the present communication, evidence will be presented that (1) initiation and subsequent elongation of RNA chains formed by RNA polymerase under the direction of a DNA template occur by a mechanism in which the first nucleotide incorporated into the RNA chain retains its triphosphate moiety, and (2) adenosine and guanosine triphosphate ends are preferentially formed.

Materials and Methods.—γ-P^{32}-GTP and UTP were prepared by photophosphorylation of the corresponding nucleoside diphosphates with $^{32}P_i$ and spinach chloroplasts by a modification of the procedure of Avron.[2] γ-P^{32}-CTP was prepared by the action of nucleoside diphosphokinase[3] on $\beta\gamma$-P^{32}-ATP and CDP in the presence of an excess of myokinase. $\beta\gamma$-P^{32}-ATP was prepared as described by Penefsky and Racker[4] in the presence of excess myokinase. These P^{32}-labeled compounds were purified by chromatography on Dowex-1-Cl^- and were free of P^{32} in the α-phosphate position. The methods of preparation of other materials, including the DNA-dependent RNA polymerase of *Escherichia coli*, have been previously described.[1, 5] Calf thymus DNA was obtained from General Biochemicals.

Enzyme assay: The presence of a triphosphate terminus in the RNA formed in an RNA polymerase reaction was measured by the incorporation of γ-P^{32} ribonucleoside triphosphate into an acid-insoluble product, and RNA synthesis was measured by the incorporation of α-P^{32} or C^{14}-labeled ribonucleoside triphosphate. Reaction mixtures (0.50 ml) contained Tris buffer, pH 8.0, 25 μmoles; 2-mercaptoethanol, 4 μmoles; $MnCl_2$, 0.5 μmole; $MgCl_2$, 2.5 μmoles; DNA, 25 mμmoles; ATP, UTP, GTP, and CTP, 10 mμmoles each, one labeled with P^{32} in the γ-phosphate group (containing $1–2 \times 10^9$ cpm/μmole) for measurement of triphosphate termini or with C^{14}-ATP or GTP (containing $2–5 \times 10^6$ cpm/μmole) for measurement of the total amount of RNA formed in the reaction. In either case, the reaction was initiated by the addition of enzyme.[6] After incubation at 37° for the desired time, the reaction mixture was chilled in ice, and 0.1 ml of a bovine plasma albumin solution (5 mg/ml) was added, followed by 0.3 ml of 7% $HClO_4$. The resulting precipitate was centrifuged for 5 min at $15,000 \times g$, and the pellet dissolved in 0.2 ml of ice-cold 0.2 *N* NaOH. This was followed by the addition of 0.1 ml of nonradioactive triphosphate (25 μmoles/ml) corresponding to the γ-P^{32}-labeled triphosphate used in the reaction mixture and 5 ml of cold 5% TCA solution containing 0.01 *M* sodium pyrophosphate. After the reaction mixture had stood in ice for 5 min, the acid-insoluble material was collected by centrifugation, and

the washing procedure was repeated two more times. The final pellet was dissolved in 1.5 ml of 0.2 N NH_4OH, transferred to an aluminum planchet, and after drying, counted in a windowless gas-flow counter. The high specific radioactivities of the γ-P^{32}-labeled substrates necessitated the washing procedure to obtain consistently low blanks. When the total amount of RNA synthesized from C^{14}-nucleoside triphosphate (specific radioactivity 10^6–10^7 cpm/μmole) was measured, the washing procedure was not necessary, and the acid-insoluble RNA product was isolated by filtration on membrane filters as described previously.[1]

Results.—Incorporation of γ-P^{32}-labeled nucleoside triphosphates into RNA polymerase products: RNA polymerase, primed with dAT copolymer, catalyzes the incorporation of $\beta\gamma$-P^{32}-labeled ATP into an acid-insoluble polyribonucleotide product.[1] The incorporation of P^{32} is dependent on the presence of UTP, dAT copolymer, and RNA polymerase. The omission of any of these components, or the addition of RNase or DNase, results in a marked decrease in P^{32}-ATP incorporation. In contrast, similar experiments carried out with γ-P^{32}-UTP do not result in significant incorporation of P^{32}, as shown in Table 1. The low level of incorporation observed is not dependent on the simultaneous presence of ATP.[7]

TABLE 1

REQUIREMENTS FOR THE INCORPORATION OF $\beta\gamma$-P^{32}-LABELED ATP AND γ-P^{32}-UTP IN dAT-PRIMED POLY rAU SYNTHESIS

Additions	$\beta\gamma$-P^{32}-ATP incorporated ($\mu\mu$moles)	γ-P^{32}-UTP incorporated ($\mu\mu$moles)
1. Complete system	4.40	0.40
2. Omit UTP	0.37	—
3. Omit ATP	—	0.40
4. Omit enzyme	0.13	0.11
5. Omit dAT copolymer	0.29	0.28
6. Complete + DNase (1 μg)	0.30	0.24
7. Complete + RNase (1 μg)	0.30	0.24
8. Complete with C^{14}-ATP in place of $\beta\gamma$-P^{32}-ATP	7200 (poly rAU)	

The conditions of the experiment were as described under *Enzyme Assay*, except that GTP and CTP were omitted and 10 mμmoles of dAT copolymer replaced DNA. $\beta\gamma$-P^{32}-ATP incorporation and γ-P^{32}-UTP incorporation were measured in separate mixtures. In the complete system, the total amount of nucleotide incorporated was calculated from the amount of C^{14}-AMP incorporated into the poly rAU product. Mixtures were incubated for 40 min and contained 3 units of RNA polymerase.

The finding that poly rAU chains contain ATP ends and very few UTP ends prompted an examination of the relative incorporation of P^{32} from each of the four γ-P^{32}-labeled nucleoside triphosphates with different DNA templates of varying base composition. The results, presented in Table 2, show that RNA chains are

TABLE 2

INCORPORATION OF γ-P^{32}-NUCLEOSIDE TRIPHOSPHATES WITH DIFFERENT DNA PREPARATIONS

DNA primer	RNA synthesis ($\mu\mu$moles)	γ-P^{32}-Nucleotide Incorporated ($\mu\mu$moles) ATP	GTP	UTP	CTP
T2	4800	2.40	1.2	0.12	0.10
T5	4000	1.80	1.4	0.41	0.23
SP3	5480	1.25	1.0	0.39	0.12
Cl. perfringens	2800	1.60	2.1	0.28	0.25
E. coli	2660	0.43	1.4	0.13	0.10
M. lysodeikticus	2560	0.36	2.5	0.10	0.12
Calf thymus	3560	0.77	1.3	0.33	0.18
dAT copolymer	rAU = 7200	4.40	—	0.20	—
dGC homopolymer	rG = 1350 rC = 120	—	4.8	—	0.30

The conditions of the assay were as described under *Methods*. In each reaction mixture, only one of the four nucleoside triphosphates was labeled with P^{32} in the γ-phosphate group; the other three were nonradioactive. Incubation was for 40 min at 37° with 2 units of enzyme. Where indicated, 7.5 mμmoles of dGdC homopolymer and 10 mμmoles of dAT copolymer were added. Controls without enzyme and without DNA were included. In these controls incorporation was 0.2–0.3 $\mu\mu$mole of nucleotide, and the higher value was subtracted from the results listed above.

formed predominantly with ATP and GTP ends, whereas few chains are initiated with UTP and CTP. The relative number of triphosphate ends beginning with adenosine or guanosine varied with the DNA used to direct the reaction (Table 2). With various bacterial DNA's and calf thymus DNA, GTP ends predominated, although significant ATP incorporation also was observed. With DNA preparations from phages T2, T5, and SP3 (which have an A + T/G + C ratio > 1), ATP ends occurred slightly more often than GTP ends. Denaturation of DNA had marked effects on both RNA synthesis and the incorporation of P^{32} from γ-P^{32}-labeled nucleoside triphosphates (Table 3). These effects can be summarized as

TABLE 3

EFFECT OF DENATURATION OF DNA ON THE INCORPORATION OF γ-P^{32}-LABELED NUCLEOSIDE TRIPHOSPHATES

DNA primer	RNA synthesis ($\mu\mu$moles)	γ-P^{32}-Nucleotide Incorporated ($\mu\mu$moles) ATP	GTP	UTP	CTP
T2	4800	2.3	1.2	0.13	0.10
T2 heat-denatured	1000	3.6	5.1	0.88	0.40
T2 alkali-denatured	1050	3.5	4.7	0.91	0.45
Calf thymus	5700	1.0	1.8	0.33	0.20
Calf thymus heat-denatured	2000	3.8	10.1	0.82	0.66
E. coli	2000	0.6	1.5	0.13	0.10
E. coli heat-denatured	1300	2.4	8.1	0.56	0.44

The condition of the assay was as described under *Methods*, with 20 mμmoles of each of the various DNA preparations, 2 units of enzyme, and 40 min of incubation at 37°. RNA synthesis was followed by incorporation of both C^{14}-AMP and C^{14}-GMP, and total RNA synthesis was calculated by multiplying the sum of these values by two.

follows: (1) RNA synthesis was markedly inhibited, (2) the incorporation of all four γ-P^{32} nucleoside triphosphates increased severalfold, and (3) there was an increase in the ratio of GTP to ATP termini as well as a significant though small number of RNA chains containing UTP and CTP ends.

Identification of guanosine triphosphate ends of RNA formed in the RNA polymerase reaction: As with $\beta\gamma$-P^{32}-labeled ATP,[1] the incorporation of γ-P^{32}-GTP into an acid-insoluble material had the same requirements found for RNA synthesis.

For identification of the site of GTP incorporation, an RNA product containing γ-P^{32}-GTP was prepared using denatured thymus DNA as template. Subjecting the P^{32}-labeled RNA product to the action of alkaline phosphatase or to acid hydrolysis (1 *N* HCl for 10 min at 100°) rendered the P^{32} acid-soluble and Norit nonadsorbable. The P^{32} present in the RNA product was not converted to P_i by the action of prostatic phosphomonoesterase.[8] The P^{32} product was insensitive to pancreatic DNase, since it remained acid-insoluble, whereas pancreatic RNase and alkaline hydrolysis released all the P^{32} into an acid-soluble but Norit-adsorbable form. These results indicate that the P^{32} incorporated from γ-P^{32}-GTP was in a terminal portion of the RNA structure, presumably $\overset{*}{p}$ppGpXpYpZ—, and not in an internucleotide link.

The expected products of alkaline hydrolysis of polynucleotides with the structure $\overset{*}{p}$ppGpXpYpZ— are 2′(3′)-nucleoside monophosphates and nucleoside tetraphosphates. Radioactivity from γ-P^{32}-GTP should be found only in guanosine tetraphosphate ($\overset{*}{p}$ppGp). The prediction was tested as follows: An alkaline hydrolysate (0.3 *N* KOH at 37° for 18 hr) of the labeled product was neutralized with Dowex-50 (H^+), and an aliquot of the solution containing 30,000 cpm was mixed with 2 μmoles each of GMP, GDP, GTP, ATP, and guanosine-5′-tetraphosphate.[9] The mixture was added to a column (1 × 12 cm) of Dowex-1-Cl^- (100–

200 mesh, 2% cross-linked), and the nucleotides were eluted as follows: (*a*) 150 ml of 0.01 *M* HCl + 0.05 *M* LiCl (GMP); (*b*) 150 ml of 0.01 *M* HCl + 0.1 *M* LiCl (GDP followed by ATP); (*c*) 150 ml of 0.01 *M* HCl + 0.2 *M* LiCl (GTP); (*d*) 150 ml of 0.05 *M* HCl + 0.2 *M* LiCl, which eluted guanosine 5′-tetraphosphate. The elution profile and identification of each of the nucleotides were determined by measuring the optical densities of the effluents at 260 and 280 mμ. More than 90 per cent of the P^{32} added to the column was eluted as a sharp symmetrical peak in the last solvent with guanosine 5′-tetraphosphate, whereas 8 per cent of the added radioactivity was eluted in the GTP region. No P^{32} was detected in the other regions of the chromatogram. These results are consistent with the presence of *pppGp. To characterize further the P^{32}-labeled product in the alkaline hydrolysate, another aliquot of the alkaline hydrolysate (containing 30,000 cpm) was incubated with 3.5 units[10] of prostatic phosphomonoesterase at pH 5.0 at 37° for 30 min. The mixture was then chromatographed on Dowex-1-Cl^- under the conditions described above. Approximately 75 per cent of the added radioactivity now chromatographed with GTP and the remainder with guanosine 5′-tetraphosphate. These results are consistent with the structure *pppGpXpYpZ----, i.e., γ-P^{32}-GTP is incorporated as such at the end of RNA chains.

Kinetics of nucleoside triphosphate incorporation: The rates of RNA synthesis and $\beta\gamma$-P^{32}-ATP incorporation were compared (Table 4). Whereas T2 DNA-directed

TABLE 4

KINETICS OF RNA SYNTHESIS VERSUS $\beta\gamma$-P^{32}-ATP INCORPORATION

Time of incubation (min)	RNA synthesis (μμmoles)	$\beta\gamma$-P^{32}-ATP incorporated (μμmoles)	Ratio
1	300	1.2	250
2	600	1.6	375
5	1500	2.4	630
10	2700	2.8	960
20	4500	3.2	1410
40	6100	3.7	1695
60	8400	3.7	2270

The assay was performed as described under *Methods*, except that 5 μmoles of $MgCl_2$ replaced $MnCl_2$, 2 units of RNA polymerase and 20 mμmoles of each of the nucleoside triphosphate were added, and the incubation was carried out at 25°. This procedure permitted slow growth of the RNA chains with T2 DNA as primer.

RNA synthesis continued during the entire experiment, 65 per cent of the total amount of $\beta\gamma$-P^{32}-ATP was incorporated by 5 min. The ratio of $\beta\gamma$-P^{32}-ATP ends to total nucleotide incorporation decreased progressively with time from a ratio of 1 ATP terminus per 250 RNA nucleotides during the first minute to 1 ATP terminus per 2270 nucleotides after 60 min.

A comparison of the kinetics of P^{32} incorporation from γ-P^{32}-GTP with native and with heat-denatured T2 DNA as template is summarized in Table 5. With native T2 DNA as template the incorporation of P^{32} was virtually complete in 20 min, although RNA synthesis continued throughout the incubation period. The ratio of GTP ends to RNA formed decreased progressively with time from a ratio of 1 GTP terminus per 1300 nucleotides in the first 5 min to 1 GTP terminus per 5100 nucleotides after 90 min. Under the same conditions, experiments with $\beta\gamma$-P^{32}-ATP indicated the presence of 1 ATP terminus per 600 nucleotides during the first 5 min of incubation and 1 ATP terminus per 3100 nucleotides after 90 min. In contrast, with denatured DNA as template, the incorporation of γ-P^{32}-GTP was

severalfold faster and continued during the entire period of RNA synthesis. Similar results were obtained with $\beta\gamma$-P^{32}-ATP. In this case, with denatured DNA as template, the ratio of ATP ends to RNA formed was lower than that found with GTP (1/160 after 5 min of incubation and 1/540 after 60 min), since with denatured DNA more γ-P^{32}-GTP than $\beta\gamma$-P^{32}-ATP was incorporated. However, with either of these two triphosphates, the ratio of triphosphate ends formed to RNA synthesized was considerably smaller than that obtained with the corresponding native DNA as template. The ratios obtained in these experiments can be used as a measure of the length of the RNA product formed. Wood and Berg[11] found that RNA products formed with denatured DNA as template were smaller than those obtained with native DNA. The results summarized in Table 5 are in agreement with their findings.

TABLE 5

KINETICS OF RNA SYNTHESIS AND γ-P^{32}-GTP INCORPORATION WITH NATIVE AND DENATURED T2 DNA AS TEMPLATES

Experiment no.	Time of incubation (min)	RNA synthesized ($\mu\mu$moles)	γ-P^{32}-GTP incorporated ($\mu\mu$moles)	Ratio
I	5	1500	1.1	1360
	10	2700	1.4	1800
	20	5000	1.7	2900
	40	6200	1.9	3200
	60	8300	1.9	4150
	90	10400	1.9	5200
II	5	150	3.2	47
	10	300	4.8	62
	20	600	6.6	90
	40	1140	7.7	143
	60	1500	8.1	180
	90	2100	10.1	210

The conditions of the experiment were as described under *Methods*, except that 20 mμmoles each of γ-P^{32}-GTP, UTP, CTP, and ATP were added. Twenty-four mμmoles of native T2 DNA (approximate size = 35*S*) were added in experiment I, and an equimolar amount of heat-denatured T2 DNA was added in experiment II. Incubation was at 37°.

Direction of growth of RNA chains: In order to determine whether incorporation of the triphosphate terminus in RNA occurs according to the scheme in which the first nucleotide incorporated retains the triphosphate end, or by a mechanism in which the triphosphate moiety is at the growing point of the RNA molecule, the following experiment was performed. γ-P^{32}-GTP was incorporated into RNA for 5 min, and then a large excess of cold GTP was added to reduce the specific activity of the labeled nucleotide. The fate of P^{32} already incorporated at the ends of the RNA chains was then followed during subsequent RNA synthesis. If the initial nucleotide incorporated was present as the triphosphate end, subsequent synthesis should have no effect on the P^{32} already incorporated. In contrast, if the last entering nucleotide existed as the triphosphate terminus, subsequent synthesis should release the previously incorporated P^{32}. The fate of γ-P^{32}-GTP ends under the conditions described above is summarized in Figure 1. As shown, the addition of unlabeled GTP halted γ-P^{32} uptake immediately, and continuing RNA synthesis did not diminish the amount of P^{32} already incorporated.

Proof that the labeled chains actually increased in length after the addition of unlabeled substrates was obtained by the following experiment. RNA synthesis was carried out in the presence of γ-P^{32}-GTP and $\beta\gamma$-P^{32}-ATP. After the reaction

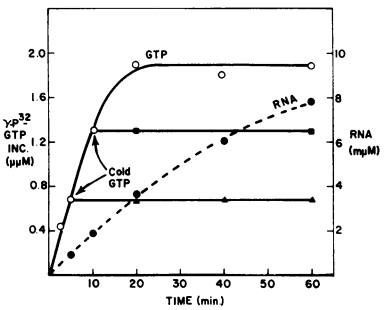

FIG. 1.—Effect of dilution on γ-P^{32}-GTP incorporation. Reaction mixtures were as described under *Methods*, with the exception that 20 mμmoles of each of the four nucleoside triphosphates were added, γ-P^{32}-GTP (1.1×10^9 cpm/μmole) was used, and incubation was carried out at 37°. In two separate reaction mixtures, one after 5 min and the other after 10 min, a 30-fold excess of unlabeled GTP was added. RNA synthesis was measured in separate reaction mixtures in which C^{14}-GTP was used as the only radioactive substrate, with all other additions as above. ▲, Incorporation of γ-P^{32}-GTP in the reaction mixture diluted with unlabeled GTP after 5 min; ■, incorporation in the reaction mixture diluted after 10 min.

had been allowed to proceed for 4 min, a large excess of unlabeled GTP and ATP was added. One sample was removed at this time, and a second after an additional 8 min of incubation. The sedimentation of the labeled RNA is illustrated in Figure 2, which shows that the sedimentation rate increased from about 6*S* to about 20*S* after the addition of unlabeled substrates. An increase in size of RNA products with time was also noted by Bremer and Konrad.[12]

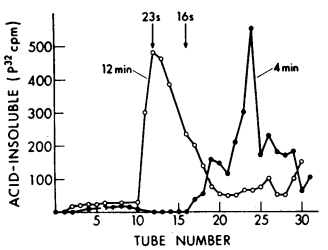

FIG. 2.—Zone sedimentation analysis of RNA. Two 0.5-ml reaction mixtures containing Tris buffer, pH 8.0, 25 μmoles; 2-mercaptoethanol, 4 μmoles; $MgCl_2$, 5 μmoles; ADP, 5 mμmoles; T5 DNA, 25 mμmoles; UTP and CTP, 25 mμmoles each; $\beta\gamma$-P^{32}-ATP and γ-P^{32}-GTP, specific activity 1×10^9 cpm/μmole, 25 mμmoles each; and 3 units of enzyme were incubated for 4 min at 25°. After that time, 1.5 μmoles each of nonradioactive ATP and GTP were added to each tube. One reaction was stopped immediately by the addition of sodium dodecyl sulfate and EDTA (0.5% and 0.01 *M* final concentration, respectively). The other tube was treated in the same manner after 12 min at 25°. Both samples were diluted to 1 ml with 0.5% sodium dodecyl sulfate, layered on 30 ml of a 15–30% sucrose solution gradient containing 0.05 *M* Tris buffer, pH 8.0, 0.1 *M* NaCl, and 0.2% sodium dodecyl sulfate, and centrifuged for 15 hr at 25,000 rpm in an SW 25.1 Spinco rotor at approximately 25°. Ribosomal RNA was used as an optical density marker. Fractions (1 ml) were collected through a hole punched in the bottom of the tube. The fractions were scanned through a Gilford recording spectrophotometer to locate the position of 23*S* and 16*S* ribosomal markers and assayed for P^{32}.

These results show that nucleoside triphosphates are incorporated at the point of initiation and not at the growing end of RNA chains, and, therefore, that chain growth occurs by the addition of nucleotides to the 3′-hydroxyl end.

Discussion.—The above results clearly show that RNA chains are initiated with ribonucleoside triphosphates, principally or exclusively ATP and GTP. Upon denaturation of DNA by heat or alkali, the incorporation of nucleoside triphosphates into terminal positions increases. Thus, RNA polymerase finds more and different initiation sites for RNA synthesis on denatured DNA than on double-stranded DNA. This conclusion is also supported by the observation that single-stranded and denatured DNA saturate RNA polymerase more effectively than native DNA.[13, 14]

The finding that the pyrimidine sites of DNA, especially when native, are preferentially utilized as initiation points for RNA synthesis was totally unexpected. In fact, the pyrimidine nucleoside triphosphate ends found in small numbers in the RNA may reflect the presence of small amounts of denatured DNA in all the primers used. The reason for this specificity is unknown, but it probably results from the

manner in which the enzyme interacts with DNA. It is probably not due to the selective binding of ATP and GTP versus UTP and CTP to the enzyme, since there is no difference in affinity constant of these nucleotides for RNA polymerase.[5] The selective copying of the pyrimidine-rich strand of the DNA of *Bacillus subtilis* phages SP8 and 2C and *Bacillus megatherium* phage α *in vivo*[15, 16] and *in vitro*[17, 18] may be related to the preferential initiation of RNA chains with purine nucleotides. The selection of one DNA strand over another may thus be governed by runs of pyrimidine bases in native DNA. The loss of asymmetric copying of DNA in RNA synthesis upon denaturation of the DNA[17] may be related to our finding that denaturation uncovers new sites in the DNA at which RNA chains can be started.

In vivo, RNA synthesis (i.e., gene expression) must begin at particular sites on DNA. Since DNA of *E. coli* (and others) is uninterrupted,[19] there must be a high degree of specificity for initiation of RNA chains within the DNA duplex. The results presented above suggest that these sites in DNA may be pyrimidine bases. In accord with this idea is the finding that sRNA molecules contain considerable amounts of guanine and adenine at the 5′-phosphate end.[20, 21] How RNA polymerase can specifically recognize initiation points on DNA, and what factor controls the accessibility of the enzyme to such sites for RNA synthesis, are problems intimately involved in the mechanism of gene control.

Summary.—In the DNA-dependent RNA polymerase reaction, the RNA chains formed contain ribonucleoside triphosphates at their starting points and grow by the subsequent addition of ribonucleotides to the 3′-hydroxyl group of the ribonucleoside end. Purine nucleoside triphosphates are preferentially found at the triphosphate end. When denatured DNA is used as a template, there are an increase in the number and a change in the kind of starting points.

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[7] In other experiments the incorporation of γ-P^{32}-UTP was <0.2 $\mu\mu$moles. Evidence that this low incorporation was not due to the presence of an inhibitor in the P^{32}-UTP preparation was obtained by the finding that γ-P^{32}-UTP supported both C^{14}-ATP and $\beta\gamma$-P^{32}-ATP incorporation with dAT copolymer as primer. The other γ-P^{32}-nucleoside triphosphates also supported RNA synthesis, and the omission of a single triphosphate resulted in a marked decrease in both RNA synthesis and chain initiation.

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*REDUCTIVE DISSOCIATION OF CHICKEN γG IMMUNOGLOBULIN IN NEUTRAL SOLVENTS WITHOUT A DISPERSING AGENT**

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The γG immunoglobulins (γG) of different animal species consist of two types of polypeptide chains: the light (L) chains (K or L chains) with a molecular weight of 20,000, and the heavy (H) chains (γ chains) with a molecular weight of about 55,000.[1-5] The chains are held together by disulfide linkages; however, reduction of the disulfide bonds and alkylation of the released −SH groups has not been reported to lead to chain separation unless followed by treatment with urea,[2] detergent,[6, 7] or weak acid.[5] Thus, either reduced human or rabbit γG in neutral buffer retains most of its antibody-combining activity and has approximately the same sedimentation coefficient as unreduced γG.[2, 8] In contrast, chicken γG antibody loses considerable activity following reduction with 2-mercaptoethanol (ME).[9] The results presented here show that, unlike rabbit γG, fractionation of reduced alkylated chicken γG in neutral or alkaline buffers yields free H and L chains.

Materials and Methods.—Preparation of γG: The globulins were precipitated with Na_2SO_4[9] from pooled sera of adult White Leghorn hens, and the γG was isolated by gel filtration on Sephadex G-200 (Pharmacia, Upsala, Sweden) in borate-buffered saline, pH 8.2, $\tau/2 = 0.16$ (borate). The $S_{20,w}$ values of γG in these preparations ranged from 6.9 to 7.1*S*. Traces of β-globulin, detected by immunoelectrophoresis (IE), was eliminated in some preparations by chromatography through DEAE-cellulose in 0.1 *M* sodium phosphate buffer, pH 6.4.[9] Sodium sulfate-precipitated rabbit γG, purified by DEAE-cellulose chromatography,[10] had a sedimentation coefficient of 6.4*S* and consisted only of γG by immunologic criteria.

Protein concentrations were determined either from measurements of absorbance at 280 mμ or by the Folin reaction as described previously.[11]

Reduction and fractionation: One to 3% solutions of γG preparations in 0.55 *M* Tris-HCl buffer, pH 8.2, were reduced with various concentrations of ME at room temperature for 1 hr. In some experiments, dithiothreitol[12] (DTT) (Calbiochem) was used as the reducing agent. A 1 *M* solution of recrystallized iodoacetamide in the same buffer was added after reduction to a molar concentration 1.5 times that of the reducing agent, and the pH was maintained at 8.0 by