THE BIOSYNTHESIS OF CHONDROITIN-SULFATE PROTEIN COMPLEX*

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Communicated by George W. Beadle, July 15, 1965

Although considerable progress has been made in the elucidation of the biosynthesis of both proteins and complex polysaccharides, there is as yet little information concerning mechanism of formation of compounds in which polysaccharide and protein are covalently linked. Previous studies have demonstrated that the radioactivity from labeled uridine nucleotide sugars is incorporated into hyaluronic acid by particulate enzyme preparations of Group A streptococcus[1, 2] and embryonic rat skin[3] and into chondroitin sulfate (CS)[4, 5] by particulate preparations of embryonic chick cartilage. In the case of the streptococcal enzyme, net synthesis of hyaluronic acid has been achieved and no primer requirement could be established.[2]

Chondroitin sulfates, like other sulfated mucopolysaccharides, are covalently linked to protein in tissues.[6] Whether the polysaccharide molecules are linked to a single large protein unit or to a number of smaller subunits remains to be determined.[7] It is clear, however, that chondroitin sulfate A of bovine nasal septum[8] and chondroitin sulfate C of shark cartilage[9] are linked to serine residues in the protein.

In considering the problem of the biosynthesis of such complex mucoproteins, three possible mechanisms may be entertained: the sequential addition of monosaccharides or oligosaccharides to a completed protein, the sequential addition of amino acids to a completed polysaccharide, or the linkage of a completed protein to a completed polysaccharide. Recent studies on formation of glycoproteins in rat liver[10–12] indicate that these compounds are synthesized by the first of the three mechanisms.

The experiments reported here were designed to test which of these mechanisms obtains in the biosynthesis of chondroitin sulfate-protein complex.

Materials and Methods.—Carrier CS was prepared as previously described,[4] with the exceptions that whole epiphyses were digested with papain; the alkali treatment and Dowex-1 column purification were omitted. In all experiments epiphyses from 13-day-old chick embryos were used.

Carrier-free $Na_2S^{35}O_4$ was obtained from the Union Carbide Nuclear Co., Oak Ridge Laboratory. Sodium acetate-1-C^{14} (19 mc/mmole) was obtained from the Volk Radiochemical Corporation, Chicago, and L-serine-U-C^{14} (110 mc/mmole) from the New England Nuclear Corporation. Puromycin hydrochloride was obtained from General Biochemicals, Inc., and DON (6-diazo-5-oxonorleucine) was a gift from Dr. J. R. Dice of Parke, Davis & Company.

GalN-1-P was prepared by the procedure of Carlson, Swanson, and Roseman[13] and UDP-GalNAc-H^3 by the procedure of Roseman *et al.*[14] UDP-GlcUA was purchased from the California Biochemical Corp.

Preparation of cartilage suspensions: Epiphyses of femurs and tibias from 50–55 embryos were dissected free of soft tissues, chilled, and minced by being forced through a stainless steel wire screen (30 mesh) and suspended in 5 vol of Krebs-Ringer-bicarbonate which contained 3 mg of glucose per ml. The suspensions were incubated at 37°, with shaking, in an atmosphere of 95% O_2–5% CO_2 for 2 hr, and the reactions were terminated by immersion of the flasks in a boiling water bath for 2 min.

Preparation of the particulate fraction: Epiphyses from 50–55 embryos were dissected, chilled, and homogenized in a Kontes-Duall glass homogenizer with 12 ml of the following buffer: Tris, 0.05 *M* (pH 7.6); KCl, 0.07 *M;* $MgCl_2$, 0.03 *M;* EDTA, Na_2, 0.001 *M;* cysteine-HCl, 0.006 *M*. The homogenate was centrifuged at 10,000 × *g* for 10 min and the supernatant solution was then centrifuged at 105,000 × *g* for 90 min. The 105,000 × *g* pellet was suspended in 12 ml of the same buffer by gentle hand homogenization in a Potter-Elvehjem homogenizer with a Teflon pestle and centrifuged again at 105,000 × *g* for 60 min. The final pellet was suspended in 2–8 ml of the same buffer. Samples (1.0 ml) of the final suspension were incubated with sugar nucleotides, ATP, and additions in a final volume of 1.1 ml.

At the end of the incubation, tubes were heated in a boiling water bath for 2 min and the polysaccharide was isolated after digestion with papain as described for the isolation of the carrier CS. When the cell-free system was used, 3–5 mg of carrier CS was added to each tube after the digestion with papain.

Analytical methods: Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer by methods previously described.[4]

Gel filtration of polysaccharides was performed on columns of Sephadex G-75 or G-25 equilibrated with 0.1 *M* NaCl. Uronic acid was determined by the carbazole method of Dische[15] and hexosamines by the Boas modification[16] of the Elson-Morgan reaction, omitting the Dowex treatment. Protein was determined by the Folin method of Lowry *et al.*[17]

Electrophoresis of acid mucopolysaccharides was performed in 0.1 *M* pyridinium formate, pH 3.0, on cellulose acetate strips for 2 hr with a voltage gradient of 10 volts/cm. Paper electrophoresis was performed on Whatman 3 MM paper in pyridinium acetate buffer, pH 5.3 (0.07 *M* in acetate) with a voltage gradient of 40 volts/cm.

Paper chromatography on Whatman no. 1 paper was performed in the following solvents: (*A*) isobutyric acid: 1.0 *N* ammonia: 0.01 *M* EDTA (100:60:3.2 by volume); (*B*) 95% ethanol: 1.0 *M* ammonium acetate buffer pH 7.0 (7:3 by volume); (*C*) phenol: ethanol: 0.880 ammonia: water (120 gm:40:1:40 by volume).

Ion exchange chromatography of uridine nucleotide sugars was performed on columns of Dowex-1-X2 Cl^-. A gradient of LiCl in 0.003 *N* HCl was used to separate the nucleotides.

Results.—In preliminary experiments, serine-C^{14} was injected into several 12-day-old embryos through a hole in the side of the shell. Twenty-four hours later the embryos were removed, the epiphyses were dissected, and the polysaccharide was isolated as described above. After acid hydrolysis and paper electrophoresis, the radioactivity in the polysaccharide was found in ninhydrin-positive spots corresponding to serine and hexosamine. In order to minimize the incorporation of radioactivity from labeled serine into hexosamine via gluconeogenesis, labeled serine was incubated with suspensions of minced cartilage for short periods of time. When radioactive polysaccharide, isolated from such incubations, was hydrolyzed in 6 *N* HCl at 100° for 20 hr, 80–90 per cent of the radioactivity was found to coincide with the serine spot after paper electrophoresis and paper chromatography in solvents *A* and *C*. No radioactivity could be detected in the hexosamine spot.

The specific activity of the serine-labeled CS remained constant through three purifications with cetylpyridinium chloride (CPC), and on cellulose-acetate-strip

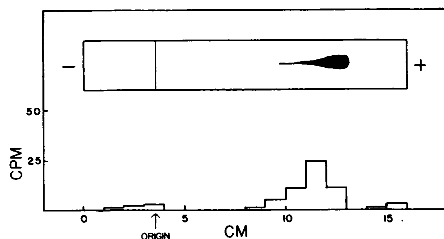

FIG. 1.—Cellulose acetate electrophoresis of labeled polysaccharide obtained by incubation of minced epiphyses with serine-C^{14}. The polysaccharide was purified three times by CPC precipitation as described in *Methods*.

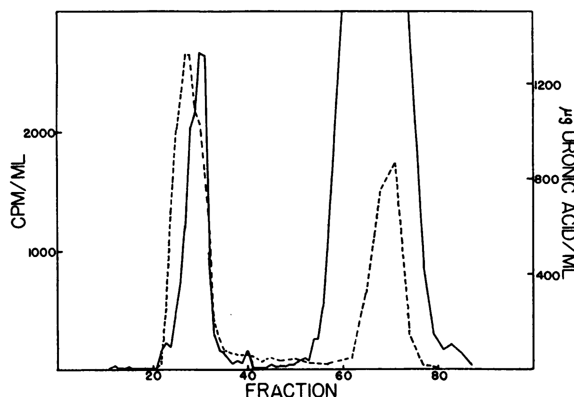

FIG. 2.—Minced epiphyses obtained from 53 embryos were suspended in 16 ml Krebs-Ringer bicarbonate buffer with 2.5 μc of serine-C^{14}. The flask was incubated at 37° for 2 hr with shaking in an atmosphere of 95% O_2–5% CO_2. The reaction was terminated by immersion of the flask in boiling H_2O for 2 min. The boiled incubation mixture was digested with papain and the insoluble material removed by centrifugation. The clear supernatant was concentrated to 4 ml and applied to a Sephadex G-75 column (beads, 1 cm diameter × 200 cm) equilibrated with 0.1 N NaCl. The flow rate was 10 ml/hr and 2-ml fractions were collected. The solid line represents cpm/ml, and the broken line represents μg uronic acid/ml.

electrophoresis, the radioactivity coincided with the acridine orange stain of the CSA (Fig. 1). Gel filtration on Sephadex G-75 (Fig. 2) indicated that the radioactivity and the uronic acid-reacting peaks appeared in the void volume, although the two peaks did not coincide exactly. This apparent separation may be due to a difference in molecular weight between newly synthesized polysaccharide (*labeled*) and the bulk of the polysaccharide already preformed in the tissue.

These results established the fact that serine was linked to polysaccharide by the minced cartilage suspension and the incorporation of radioactivity from labeled serine in such purified preparations could be taken as a measure of the formation of protein polysaccharide linkage. Previous studies have indicated that the incorporation of radioactivity from acetate-C^{14} may be used as a measure of polysaccharide synthesis.[18, 19] After polymerization, sulfate groups are added to form CS. Accordingly, $S^{35}O_4^=$ may be used as a further indicator of polysaccharide synthesis.[20]

The results in Table 1 show that the incorporation of acetate-C^{14}, serine-C^{14}, and

TABLE 1

EFFECT OF PUROMYCIN ON ISOTOPE INCORPORATION INTO POLYSACCHARIDE

	First purification sp. act.*	Second purification sp. act.*	Third purification sp. act.*	Per cent of control
Acetate-1-C^{14}	77,400	83,200	82,700	100
Acetate-1-C^{14} + puromycin	8,100	8,730	9,430	11.4
Serine-U-C^{14}	12,340	10,800	9,400	100
Serine-U-C^{14} + puromycin	368	373	352	3.0
$S^{35}O_4^=$	973,000	932,000	847,000	100
$S^{35}O_4^=$ + puromycin	83,300	78,200	100,000	8.5

* Cpm per mg of uronic acid.
Minced epiphyses (representing three embryos), suspended in 3 ml of Krebs-Ringer bicarbonate buffer, were added to each flask. Puromycin was added to a final concentration of 200 μg per ml and the flasks were incubated with shaking at 37° for 15 min in an atmosphere of 95% O_2–5% CO_2. To the appropriate mixtures, acetate-1-C^{14} (10 μc), serine-U-C^{14} (10 μc), and $S^{35}O_4^=$ (50 μc) were added and incubation was continued for 2 hr. The reactions were terminated by immersion of the flasks in boiling H_2O for 2 min and the polysaccharide was isolated as described in the text.

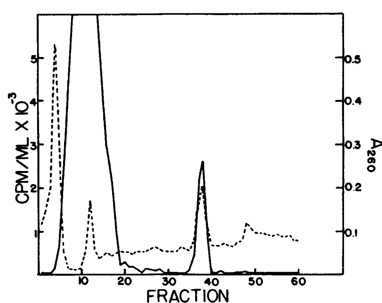

FIG. 3.—Ion exchange chromatography of the TCA extract of a cartilage suspension after incubation with acetate-C^{14}. The broken line indicates the absorbancy at 260 mμ, and the solid line the measured radioactivity of the fractions. A column of Dowex-1 × 2, Cl^- was used (1 cm diameter × 20 cm) and 15-ml fractions were collected. A nonlinear gradient was achieved by adding 1.0 liter of 0.25 *M* LiCl in 0.003 *N* HCl to a constant volume mixing vessel which contained, initially, 1.0 liter of 0.003 *N* HCl. The UDP-N-acetylhexosamine peak (tubes 35–40) was well separated from inorganic acetate (tubes 5–20).

inorganic sulfate-S^{35} into polysaccharide by the cartilage suspensions was inhibited by addition of puromycin. Under the conditions employed, serine incorporation was inhibited 97 per cent and the incorporation of acetate and sulfate, 89 and 92 per cent, respectively.

The inhibition of acetate incorporation by puromycin might result from the inhibition of synthesis of enzymes concerned with any of the steps in the synthesis of requisite nucleotides, the polymerization of the nucleotides, or of the synthesis of specific protein necessary for the biosynthesis of the protein-polysaccharide complex.

In order to test the first of these possibilities, the effect of puromycin on the incorporation of radioactivity from labeled acetate into UDP-N-acetylhexosamine was examined. The cartilage suspensions were incubated with acetate-C^{14} and puromycin for 2 hr and then the entire contents of each flask was homogenized in 5 per cent TCA. Carrier UDP-GalNAc was added and the TCA precipitate was removed by centrifugation; the supernatant solutions were neutralized with 1 *M* Na_2CO_3 and applied to Dowex columns. The elution pattern of such a column is shown in Figure 3. The radioactivity in the peak corresponding to the UDP-GalNAc was desalted by adsorption and elution from charcoal and was found to move with UDP-GalNAc during paper electrophoresis and paper chromatography in solvents *A* and *B*.

The results of this experiment are summarized in Table 2. Although puromycin inhibited the incorporation of radioactivity from labeled acetate into polysaccharide, the N-acetyl hexosamine nucleotide fraction of the puromycin-inhibited preparation actually contained a considerably higher number of counts than did the control.[21] To ensure that puromycin did not inhibit the conversion of UDP-GlcNAc

TABLE 2

EFFECT OF PUROMYCIN ON THE INCORPORATION OF ACETATE-1-C^{14} INTO POLYSACCHARIDE AND INTO UDP-N-ACETYL HEXOSAMINE

	Puromycin, μg/ml	Glucosamine, mg/ml	Total Radioactivity, cpm UDP-N-Acetyl hexosamine	CS	Specific activity CS*	Percentage CS formation
1	0	0	36,500	264,000	42,700	100
2	200	0	96,500	49,500	7,050	16
1	0	0.6	240,000	602,000	62,000	100
2	200	0.6	411,000	72,000	6,660	11

* Cpm per mg of uronic acid.

Each flask contained 10 ml of cartilage suspension (representing 28 embryos) in Krebs-Ringer bicarbonate buffer and 10 μc of acetate-1-C^{14}. Puromycin and glucosamine were added as shown in the table. The flasks were shaken at 37° for 2 hr in an atmosphere of 95% O_2–5% CO_2. A 1.0-ml sample from each flask was heated for 2 min in boiling water and polysaccharide isolated as described in the text. The remainder was homogenized in 5% TCA, and labeled UDP-N-acetyl hexosamine was assayed using column chromatography on Dowex-1 × 2 Cl.

TABLE 3

EFFECTS OF PUROMYCIN AND DON ON POLYSACCHARIDE SYNTHESIS BY PARTICULATE ENZYME

Incubation time, hr	Puromycin, μg/ml	DON, μg/ml	First purification, sp. act.*	Second purification, sp. act.*	Third purification, sp. act.*
0	0	0	60	15	56
2	0	0	970	750	748
2	10	0	985	870	735
2	100	0	1003	950	820
2	0	20	924	790	635
2	0	100	882	728	630

* Cpm per mg uronic acid.
Each flask contained 1 ml of particulate enzyme (representing six embryos) in Tris buffer (see text), 2.0 μM ATP, 0.5 μM UDP-GlcUA, and 0.1 μM UDP-GalNAc-H^3 (1.2 × 10^5 cpm). The reaction was terminated by immersion of the flasks in boiling H_2O for 2 min and the polysaccharide was isolated as described in the text.

to UDP-GalNAc, the labeled UDP-N-acetylhexosamines were subjected to mild acid hydrolysis and the N-acetylhexosamines, so obtained, were separated by paper chromatography on borate-treated paper.[4] No significant difference in the proportion of radioactive GalNAc to radioactive GlcNAc was detected in the presence or absence of puromycin. Because of the much greater technical difficulties, the effect of puromycin on the synthesis of UDP-GlcUA was not separately examined.

In order to determine whether puromycin inhibits the synthesis of CS from sugar nucleotides, two types of experiments were performed.

The effect of puromycin on the cell-free incorporation of UDP-GalNAc-H^3 into polysaccharide is shown in Table 3. No inhibition of the enzymic activity was detected at either of the two concentrations of puromycin used. In contrast, amino acid incorporation into protein, catalyzed by cell-free systems,[22] is inhibited to the extent of 95 per cent by 0.3 mM puromycin.

The second type of experiment involved the preparation of particulate enzyme from cartilage which had been pretreated with puromycin. Inhibition of measured polymerase activity in this experiment might result from inhibition of enzyme synthesis or from inhibition of formation of a requisite primer protein. Since previous experiments had shown that sulfated hexasaccharide from CS (a gift from Dr. L. Rodén) stimulates incorporation of radioactivity by the particulate enzyme, this substance was utilized to help separate direct effects on the formation of polymerase

TABLE 4

EFFECT OF INCUBATION OF MINCED EPIPHYSES WITH PUROMYCIN ON THE ACTIVITY OF THE PARTICULATE ENZYME

Pretreatment	Incubation time, hr	Hexasaccharide, mg	First purification, sp. act.*	Second purification, sp. act.*	Third purification, sp. act.*
None	0	0	80	21	44
None	2	0	935	846	795
None	2	1.0	2400	1318	1112
Puromycin	0	0	49	21	4
Puromycin	2	0	—	515	550
Puromycin	2	1.0	1790	1018	853

* Cpm per mg of uronic acid.
Minced epiphyses incubated with 200 μg per ml of puromycin for 2 hr in Krebs-Ringer bicarbonate before preparation of particulate enzyme. Controls were also preincubated for 2 hr. Each flask contained 1 ml of particulate enzyme (representing seven embryos) in Tris buffer (see text), 2.0 μM ATP, 0.5 μM UDP-GlcUA, and 0.1 μM UDP-GalNAc-H^3 (1.2 × 10^5 cpm). The reaction was terminated by immersion of the flasks in boiling H_2O for 2 min and the polysaccharide was isolated as described in the text.

TABLE 5

THE EFFECT OF DON ON ISOTOPE INCORPORATION INTO POLYSACCHARIDE

	First purification sp. act.*	Second purification sp. act.*	Third purification sp. act.*	Per cent of control
Acetate-1-C^{14}	77,400	83,200	82,700	100
Acetate-1-C^{14} + DON	873	—	—	1.13
Acetate-1-C^{14} + DON + glucosamine	98,200	97,700	102,500	127
Acetate-1-C^{14} + DON + galactosamine	1,038	1,110	1,110	1.34
Serine-U-C^{14}	12,340	10,800	9,400	100
Serine-U-C^{14} + DON	5,520	4,500	3,950	44.7
Serine-U-C^{14} + DON + glucosamine	7,730	7,700	6,880	62.6
$S^{35}O_4^=$	973,000	932,000	847,000	100
$S^{35}O_4^=$ + DON	127,200	122,000	119,200	13.1
$S^{35}O_4^=$ + DON + glucosamine	690,000	678,000	661,000	71

* Cpm per mg of uronic acid.

Minced epiphyses (representing three embryos), suspended in 3 ml of Krebs-Ringer bicarbonate buffer, were added to each flask. DON was added to a final concentration of 15 μg/ml, and glucosamine HCl or galactosamine HCl was added to a final concentration of 1 mg/ml. After 15 min, preincubation with the inhibitor, acetate-1-C^{14} (10 μc), serine-U-C^{14} (10 μc), or $S^{35}O_4^=$ (50 μc) were added to the appropriate flasks and incubation was continued for 2 hr at 37°, with shaking, in an atmosphere of 95% O_2–5% CO_2. The reactions were terminated by immersion of the flasks in boiling water for 2 min, and the polysaccharide was isolated as described in the text.

from effects on the synthesis of primer protein. The data presented in Table 4 indicate that enzyme obtained from puromycin-treated cartilage retained considerable activity compared to the control, and like the control was markedly stimulated by the addition of hexasaccharide. The decrease in specific activity on repurification of samples from reaction mixtures to which hexasaccharides were added has been repeatedly observed and is assumed to be due to losses of low-molecular-weight oligosaccharides in the purification procedure.

The results of this experiment indicate that the effects of puromycin on polysaccharide synthesis in minced cartilage cannot be explained on the basis of inhibition of formation of the CS-synthesizing polymerase.

In order to study further the mechanism of polysaccharide synthesis, the effect of DON, an inhibitor of hexosamine synthesis,[23] was examined in the hope that this might affect synthesis of polysaccharide without influencing the formation of protein acceptor.

Table 5 shows such effects on the incorporation of acetate-C^{14}, serine-C^{14}, and sulfate-S^{35} into polysaccharide by the minced cartilage suspensions.

This compound inhibited acetate incorporation to the extent of 99 per cent and the incorporation of sulfate and serine by 86 and 59 per cent, respectively. When glucosamine was added together with DON, the inhibition of acetate incorporation disappeared, and the inhibition of sulfate and serine incorporation was considerably reduced. In separate experiments the effect of glucosamine in the absence of DON was studied. There was slight stimulation (10–20%) of the incorporation of radioactive acetate and sulfate but no stimulation of the incorporation of radioactive serine. The relief of inhibition by free glucosamine demonstrates that this compound can be utilized to synthesize CS and that the primary site of action of the DON is the formation of hexosamine. Galactosamine had no such effect.

The data in Table 3 indicate that DON has no effect on the cell-free polymerizing system. In separate experiments, utilizing minced epiphyses, DON was determined to have a markedly greater effect on serine incorporation into polysaccharide than on serine incorporation into TCA-precipitable protein.

Discussion.—Recent studies by Rodén and his co-workers in this laboratory indi-

cate that the attachment of repeating disaccharide units of chondroitin sulfate to protein is considerably more complex than anticipated.[24–26] It has been demonstrated that such chains are linked via glucuronic acid to a linkage region containing two galactose residues, a xylose residue and serine. The actual carbohydrate-protein linkage is a glycosidic bond between xylose and the hydroxyl group of serine. The nature of the protein core remains imperfectly understood.

The data presented in this paper suggest that the protein must be synthesized, presumably by conventional means on ribosomes, with subsequent addition of carbohydrates. If this be so, mechanisms must be present for the addition of xylose to the serine hydroxyl group and subsequent addition of galactose residues before the uronic acid-N-acetylgalactosamine chains can be elongated.

The presence of the polymerizing enzyme in the particulate fraction may be due to the necessary juxtaposition of the carbohydrate-synthesizing enzyme to the site of protein on the ribosomes. Recent studies regarding complex cell wall polysaccharides in bacteria suggest that the lipids of the cell membrane may play an important role.[27] Whether phospholipids of the endoplasmic reticulum may play an analogous role in formation of protein-polysaccharides in connective tissues is not yet determined.

On the basis of radioautography, Revel and Hay[28] have suggested the protein is synthesized on the endoplasmic reticulum and carbohydrate is added in the Golgi apparatus. The data presented in this paper do not permit either the support or refutation of this possibility since the particulate enzyme used undoubtedly contains elements derived from the Golgi apparatus. However, the results indicate that protein synthesis may be dissociated from polysaccharide synthesis. Regardless of site, it seems reasonable that following completion of protein on the ribosome, the requisite sugars are added to complete the protein-polysaccharide complex.

Summary.—A preparation of minced embryonic chicken cartilage catalyzes the *in vitro* incorporation of serine-C^{14}, acetate-C^{14}, and inorganic sulfate-S^{35} into chondroitin sulfuric acid. Puromycin inhibits the incorporation of all three compounds into polysaccharide: serine $>$ sulfate $>$ acetate. 6-Diazo-5-oxonorleucine also inhibits the incorporation of all three compounds into polysaccharide: acetate $>$ sulfate $>$ serine. The inhibition by 6-diazo-5-oxonorleucine is reduced by addition of glucosamine. Puromycin has no inhibitory effect on nucleotide hexosamine biosynthesis, and neither puromycin nor 6-diazo-5-oxonorleucine has a marked effect on the incorporation of hexosamine into polysaccharide, catalyzed by a cell-free system.

These observations are consistent with a biosynthetic mechanism which involves addition of carbohydrate units to preformed protein to yield a protein-polysaccharide complex.

* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases (AM-05996-04), USPHS, The National Foundation, and the Chicago Heart Association.

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*THE SYNTHESIS OF A SELF-PROPAGATING AND INFECTIOUS NUCLEIC ACID WITH A PURIFIED ENZYME**

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Communicated July 23, 1965

The unambiguous analysis of a replicating mechanism demands evidence that the reaction being studied is, in fact, generating replicas. If, in particular, the concern is with the synthesis of a viral nucleic acid, data on base composition and nearest neighbors are not sufficient. Ultimately, proof must be offered that the polynucleotide product contains the information necessary for the production of the corresponding virus particle in a suitable test system.

These conditions impose severe restraints on the type of experiments acceptable as providing information which is irrefutably relevant to the nature of the replicating mechanism. Clearly, the enzyme system employed must be free of interfering and confounding activities so that the reaction can be studied in a simple mixture