11 November 1955, Volume 122, Number 3176 SCIENCE

Enzymatic Synthesis of Nucleic Acidlike Polynucleotides

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The mechanisms of synthesis of the polynucleotide chains of nucleic acids have remained obscure despite notable advances in our understanding of the enzymatic mechanisms involved in the synthesis of the mononucleotides, the purine and pyrimidine bases, and the sugar moieties.

As briefly reported in a recent note from this laboratory (*1*), an enzyme isolated from the microorganism *Azotobacter vinelandii* catalyzes the synthesis of polynucleotides from 5′-nucleoside diphosphates with release of orthophosphate. The reaction requires magnesium ions and is reversible. The available evidence indicates that the *Azotobacter* enzyme catalyzes the reaction

$$n\ \text{X—R—P—P} \rightleftharpoons (\text{X—R—P})_n + n\ \text{P} \quad (1)$$

where R stands for ribose, P—P for pyrophosphate, P for orthophosphate, and X for one or more of the following bases: adenine, hypoxanthine, guanine, uracil, or cytosine.

Chemical and enzymatic degradation of the biosynthetic polynucleotides showed that they are made up of 5′-mononucleotide units linked to one another through 3′-phosphoribose ester bonds as in RNA (*2*). Thus, in analogy with polysaccharides, reversible phosphorolysis may be a major mechanism in the biological breakdown and synthesis of polynucleotide chains. For this reason, the name polynucleotide phosphorylase has been proposed (*1*) for the new enzyme. Evidence that this enzyme brings about the synthesis of RNA-like polynucleotides is presented in this article (*3*).

Polynucleotide Phosphorylase

The enzyme was discovered in the course of a study of biological phosphorylation mechanisms (*4*) when it was found that, in the presence of Mg^{++}, *Azotobacter* extracts catalyzed an exchange of P^{32}-labeled orthophosphate with the terminal phosphate groups of the 5′-nucleoside diphosphates of adenosine, inosine, guanosine, uridine, and cytidine. Following partial purification of the activity, there was no reaction with 5′-nucleoside mono- or triphosphates such as AMP, ATP, IMP, or ITP (*2*). The "exchange" was accompanied by the liberation of orthophosphate, as one would expect from the reaction of Eq. 1. With mixtures of 5′-nucleoside diphosphates, radioactive phosphate was incorporated in all of them.

As previously reported (*1*), the enzyme activity was purified about 40-fold through ammonium sulfate fractionation and calcium phosphate gel adsorption steps; the rate of the ADP-orthophosphate exchange was employed as an assay. The ratio of the rates of the ADP-orthophosphate exchange to orthophosphate liberation remained constant on purification, suggesting that the two activities were related to each other.

Single Polymers

On incubation of purified polynucleotide phosphorylase with nucleoside diphosphates in the presence of Mg^{++}, there is a disappearance of nucleoside diphosphate with liberation of a stoichiometric amount of orthophosphate. The reaction reaches equilibrium and comes to a standstill when about 50 to 60 percent of the nucleoside diphosphate has disappeared. The disappearing diphosphate is converted into a polynucleotide, as is borne out by the following facts. (i) The newly formed compound is strongly negatively charged, for it is retained by Dowex-1 anion exchange columns (*5*) following elution with formic acid at concentrations higher than those required to elute the most acidic mononucleotides. (ii) The product is nondialyzable against distilled water or dilute salt solutions and is quantitatively precipitated by trichloroacetic acid or alcohol in the cold. It can be isolated in this way. (iii) It is soluble in water, yielding more or less viscous solutions with a typical nucleotide ultraviolet absorption spectrum. (iv) It remains at the origin of paper chromatograms, whichever the solvent system used, and cannot be eluted with water.

Single polymers containing AMP, IMP, GMP, UMP, or CMP as the only basic unit have been obtained by incubating polynucleotide phosphorylase with the corresponding 5′-nucleoside diphosphates (*6*). If the use of heat and/or acid is avoided in the isolation of the polymers, their average molecular weight may be very high. Values of 570,000 and 800,000, respectively, were obtained by light scattering for the AMP and IMP polymers (*7*).

Mild alkaline hydrolysis (*8*) of the IMP polymer—that is, the polynucleotide formed from IDP—yielded an approximately equimolecular mixture of 2′-IMP and 3′-IMP. These products were identified by paper chromatography with the solvent system No. 3 of Markham and Smith (*9*). As is well known, RNA yields mixtures of the 2′- and 3′-mononucleotides on mild hydrolysis with alkali (*10*). The chromatographic identification of the alkaline hydrolysis products of the IMP polymer as 2′- and 3′-IMP has been confirmed by the following experiments (Table 1). (i) About 90 per cent of the product of alkaline hydrolysis of the IMP polynucleotide is hydrolyzed by 1.0*N* hydrochloric acid in 20 minutes. It will be observed that, under these conditions, both 2′-IMP and 3′-IMP are hydrolyzed to a similar extent—that is,

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90 percent each—while 5′-IMP is hardly attacked. (ii) The alkaline hydrolysis product is not attacked by the specific 5′-nucleotidase of snake venom (*11*), which hydrolyzes 5′-IMP to the extent of 96 percent. (iii) The 3′- specific plant nucleotidase of Schuster and Kaplan (*12*)—which, as shown in Table 1, hydrolyzes 3′-IMP but not the 2′- or 5′-derivatives—hydrolyzes about half of the material. Inosine and 2′-IMP have been identified chromatographically as the products of incubation with this enzyme.

Further light on the structure of the biosynthetic polynucleotides has been shed by a study of their degradation by specific phosphodiesterases. These experiments have been partly carried out in collaboration with Leon A. Heppel of the National Institutes of Health. As shown in Fig. 1, snake venom phosphodiesterase (*13*) hydrolyzes RNA to yield 5′-mononucleotides, whereas the spleen phosphodiesterase of Heppel and Hillmoe (*14*) yields the corresponding 3′-mononucleotides (*10*). The action of each of these enzymes on various biosynthetic polymers, such as the AMP, the IMP, and some of the mixed polymers to be described in the next section, yields the expected 5′- or 3′-mononucleotides. These products were identified by chromatography and by the action of the specific mononucleotidases previously mentioned. Exhaustive digestion of the UMP polymer with crystalline pancreatic ribonuclease, a phosphodiesterase that mainly hydrolyzes the pyrimidine nucleotides of RNA as the 3′-derivatives, yields 3′-UMP while, with dilute enzyme, cyclic uridylic acid (2′-3′-uridine monophosphate) also accumulates to some extent (*15*). This is in agreement with observations on the action of pancreatic ribonuclease on RNA (*16*).

X-ray diffraction patterns of fibers (*17*) obtained from the AMP and other biosynthetic polymers closely resemble those produced by native RNA fibers (*18*).

Mixed Polymers

Mixed polymers—that is, polynucleotides containing two or more different basic units—have been prepared by incubation of mixtures of 5′-nucleoside diphosphates with polynucleotide phosphorylase. To date, two such polymers have been prepared, one from equimolecular mixtures of ADP and UDP (A-U polymer) and one from mixtures of ADP, GDP, UDP, and CDP in molecular proportion 1:0.5:1:1, respectively (A-G-U-C polymer). Degradation of the A-U polymer with purified snake venom phosphodiesterase yields 5′-AMP and 5′-UMP in a molar ratio of approximately 1:1. On digestion with pancreatic ribonuclease, it yields (*15*) 3′-UMP, as well as di-, tri-, tetra-, and pentanucleotides containing one uridylic acid unit and one or more adenylic acid units, along with a "core" consisting of adenylic acid residues.

Table 1. Products of alkaline hydrolysis of IMP polynucleotide. Polynucleotide was precipitated twice with trichloroacetic acid and hydrolyzed for 39 hours at 37°C in 0.4*N* KOH. Mixture was then treated with trichloroacetic acid; negligible precipitate was removed by centrifugation and discarded. The neutralized supernatant fluid contained 14.3 μmoles of mononucleotide based on ultraviolet absorption (λ, 260 mμ).

Treatment	Percentage of total P liberated				
	3′-IMP	2′-IMP	5′-IMP	Alkaline hydrolysate	
				Sample 1	Sample 2
1.0*N* HCl, 20 min, 100°C	90	90	6.5	89	93
5′-Nucleotidase			96	0.01	0.1
3′-Nucleotidase	80	1.6	0	54	53

The mixed polymers are more readily attacked by ribonuclease than mixtures of the corresponding single polymers (*19*). This is shown in Table 2 for the A-U polymer and the corresponding mixture. The same point is illustrated in Table 3 for the A-G-U-C polymer and the control mixture of single polymers. The AMP polymer, as expected, is not attacked by ribonuclease. Paper chromatography of the ribonuclease digestion mixtures with the solvent system of Krebs and Hems (*21*) confirmed these results. It appears as if the purine nucleotide polymers interfere with the action of ribonuclease on the pyrimidine nucleotide polymers in the mixtures. It may further be seen (Table 3) that the reactivity of the A-G-U-C polymer toward ribonuclease was similar to that of yeast RNA. This similarity was further stressed by the fact that upon exhaustive ribonuclease digestion of (i) yeast RNA and (ii) the A-G-U-C polymer, 20 and 22 percent, respectively, remained as a "core" (*22*) that was nondialyzable against distilled water, while after the same treatment of the mixture of AMP, GMP, UMP, and CMP polymers used in experiment 4 of Table 3, 43 percent remained as a nondialyzable "core."

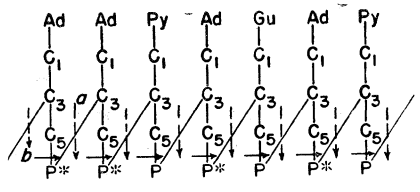

Fig. 1. Cleavage of polynucleotides by snake venom (vertical dashed arrows *a*) and spleen (horizontal arrows *b*) phosphodiesterase. *Ad,* adenine; *Gu,* guanine; *Py,* pyrimidine bases. The P^{32} label is indicated by the asterisk.

Hydrolysis of the A-G-U-C polymer with perchloric acid liberates the four bases, adenine, guanine, uracil, and cytosine. Figure 2 shows the ultraviolet photograph of an isopropanol-HCl paper chromatogram of the hydrolysate (*23*).

Further proof that 5′-3′-phosphoribose diester bonds between different mononucleotide units occur in mixed polymers was obtained from experiments in which such polymers were synthesized from mixtures of ADP labeled with P^{32} in two phosphate groups (*24*) with other nonlabeled 5′-nucleoside diphosphates (*25*). Through the action of polynucleotide phosphorylase, the labeled nucleotide is incorporated in a polynucleotide chain as adenosine-5′-(P^{32}) phosphate (Fig. 1) with release of P^{32}-orthophosphate. As may be seen on inspection of Fig. 1, if links are established between the labeled AMP and the other nucleotides, the latter will be released as *labeled* 3′-mononucleotides on hydrolysis of the polymer with spleen phosphodiesterase, while the specific radioactivity of the 3′-AMP thus obtained will be lower than that of the 5′-AMP incorporated. On the other hand, 5′-AMP of the same specific radioactivity as that incorporated will be the only labeled nucleotide released by hydrolysis with snake venom phosphodiesterase, the other 5′-nucleotides being unlabeled. Such results were indeed obtained in experiments in which the nucleotides liberated by phosphodiesterase digestion were identified by paper chromatography and autoradiography. These experiments, a detailed report of which is in preparation, were carried out with biosynthetic polymers prepared from (i) labeled ADP alone, (ii) UDP alone, (iii) labeled ADP together with UDP, and (iv) labeled ADP together with GDP, UDP, and CDP. Control mixtures of single labeled AMP polymer with either (i) nonlabeled UMP polymer or (ii) nonlabeled GMP, UMP, and CMP polymers, yielded only labeled AMP (either 3′- or 5′), the other mononucleotides being unlabeled in all cases.

Reversibility

As already indicated, the reaction catalyzed by polynucleotide phosphorylase is reversible. In the presence of the enzyme, orthophosphate, and Mg^{++}, the biosynthetic polynucleotides undergo phosphorolysis to yield the corresponding 5′-nucleoside diphosphates. There is no reaction in the absence of orthophosphate. Quantitative data on the stoichiometry of the reaction with IDP in both directions have been presented previously (*1*). We have as yet no accurate data on the position of the equilibrium. In the direction of polynucleotide synthesis and under our usual experimental conditions (*p*H 8.1, 30°C), the reaction comes to a standstill when the ratio of the concentration of orthophosphate to that of nucleoside diphosphate is from 1.5 to 2.0. The reaction favors to some extent polynucleotide synthesis, as would be expected from the fact that the pyrophosphate bonds of nucleoside diphosphates are converted to the phosphodiester bonds of the polynucleotide.

For reasons at present not well understood, single polymers are phosphorolyzed more readily than mixed ones. As already reported (*1*) phosphorolysis of RNA isolated from *Azotobacter* has been shown through the incorporation of radioactive phosphate and chromatographic identification of radioactive ADP, GDP, UDP, and CDP. The identity of the labeled GDP and UDP thus formed was further established through specific enzymatic hydrolysis with inosine diphosphatase (*26*). It has also been found with the same technique that the *Azotobacter* enzyme can catalyze the phosphorolysis of ribonucleic acids from other sources (yeast, ox liver, *Escherichia coli*, and *Streptococcus pyogenes*); this indicates lack of specificity in this regard. However, samples of DNA from calf thymus, as well as a sample of ribonuclease-resistant "core" from yeast RNA, were not attacked (*27*).

Table 2. Brief ribonuclease digestion of biosynthetic polynucleotides. Details: 0.5 ml reaction mixture with 50 μmoles of Tris buffer, *p*H 7.35, 1.48 μg of crystalline pancreatic ribonuclease, and polymers as indicated: incubation 20 min at 30°C. Hydrolysis was determined by a modification of the method of Anfinsen *et al.* (*20*) through the disappearance of ultraviolet absorbing material (λ, 260 mμ) precipitable by uranium acetate-perchloric reagent.

Polymer	Micromoles*	Hydrolysis (%)
AMP	2.75	0
UMP	2.80	60
A-U	2.44	77
AMP + UMP†	2.26	15 (28)‡

* Expressed as mononucleotide.
† Single AMP (1.06 μmoles) and UMP (1.20 μmoles) polymers mixed and kept overnight at 30°C prior to ribonuclease digestion.
‡ Value in parentheses gives the percentage of UMP polymer hydrolyzed assuming that only this component of the mixture is attacked by ribonuclease.

Conclusion

Present information on structure, size, x-ray diffraction pattern, and behavior toward different enzymes indicates that the polynucleotides synthesized by polynucleotide phosphorylase from 5′-nucleoside diphosphates are closely related to RNA. Indeed the A-G-U-C polymer—that is, the one containing adenylic, guanylic, uridylic, and cytidylic acid residues—appears to be indistinguishable from native RNA. However, it would be desirable to ascertain whether the biosynthetic compounds exhibit biological properties such as an effect on protein synthesis before referring to them as nucleic acids.

Whether or not the reaction catalyzed by polynucleotide phosphorylase represents a general biological mechanism for the synthesis of ribonucleic acids cannot as yet be decided, although such a possibility would not appear unlikely. The enzyme is present in microorganisms other than *Azotobacter* (*28*), but we have so far been unable to obtain unequivocal evidence for its presence in yeast and animal tissues.

Since there is evidence that ribonucleic acids from different cells or tissues vary in composition (*22*), the question whether polynucleotide phosphorylases from different sources would bring about the synthesis of polynucleotides characteristic of the various sources is of obvious interest. Not unrelated to this problem, because of the specific genetic effects of deoxyribonucleic acids (*29*), is the question whether DNA-like polynucleotides can be synthesized by a similar mechanism. It is to be hoped that answers to some of these pressing questions will soon be forthcoming.

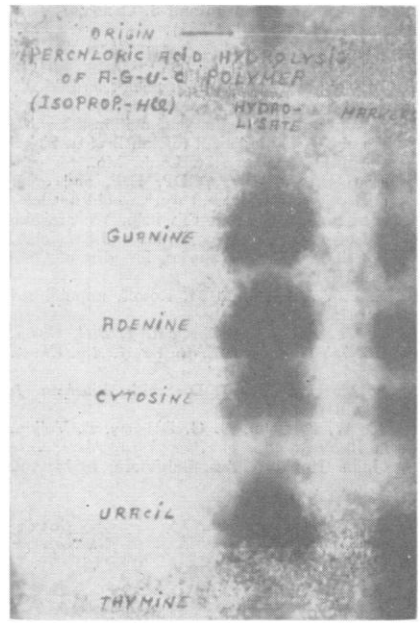

Fig. 2. Perchloric acid hydrolysis of A-G-U-C polymer.

Table 3. Prolonged ribonuclease digestion of biosynthetic polynucleotides and yeast RNA: Details: 1.6 ml reaction mixture with 20 μmoles of Tris buffer, *p*H 7.0, 29.6 μg of ribonuclease, and polymers as indicated. Incubated at 37°C. Other details as in Table 2.

Expt. No.	Polymer	Micromoles*	Hydrolysis (%)		
			3 hr	4 hr	7 hr
1	AMP	19.3	0	0	0
2	Yeast RNA	13.9	77	84	84
3	A-G-U-C	17.9	88	90	90
4	Mixture†	14.0	19 (34)‡	32 (57)	37 (65)

* Expressed as mononucleotide.
† Mixture of single polymers as follows: AMP, 4 μmoles; GMP, 2 μmoles; UMP, 4 μmoles; CMP, 4 μmoles.
‡ Values in parentheses give the percentage hydrolysis of the pyrimidine nucleotide polymers in the mixture, which make up 57 percent of the total, assuming that only these components are attacked by ribonuclease.

References and Notes

1. M. Grunberg-Manago and S. Ochoa, *J. Am. Chem. Soc.* 77, 3165 (1955).
2. Abbreviations: ribonucleic acid, RNA; 5′-diphosphates (pyrophosphates) of adenosine, inosine, guanosine, uridine, and cytidine, ADP, IDP, GDP, UDP, and CDP; 5′-monophosphates of the same nucleosides, AMP, IMP, GMP, UMP, and CMP; corresponding 3′-monophosphates, 3′-AMP, and so forth; adenosine and inosine triphosphates, ATP and ITP; deoxyribonucleic acid, DNA; tris(hydroxymethyl)aminomethane, Tris.
3. This work was aided by grants from the National Institute of Arthritis and Metabolic Diseases (grant A-529) of the National Institutes of Health, U.S. Public Health Service; the American Cancer Society (recommended by the Committee on Growth, National Research Council); the Rockefeller Foundation, and by contract N6onr 279, T.O.6 between the Office of Naval Research and New York University College of Medicine. These results were presented at the third International Congress of Biochemistry, Brussels, Belgium, 3 Aug. 1955.

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6. We wish to thank M. Staehelin and M. C. Schneider for the preparation of some of the polynucleotide phosphorylase used in this work. We are deeply indebted to Dan Broida, Sigma Chemical Company, St. Louis, Mo., for generous gifts of UDP, GDP, IDP, and other nucleotides. The present work would not have been possible without this help. We are also indebted to Alexander Frieden and Samuel A. Morell, Pabst Laboratories, for gifts of CDP and UDP.
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22. S. Zamenhof, in *A Symposium on Phosphorus Metabolism* (Johns Hopkins Press, Baltimore, 1952), vol. 2, p. 301.
23. This experiment was carried out by J. R. Fresco.
24. Prepared by the Schwarz Laboratories, Mount Vernon, N.Y.
25. These experiments were suggested by G. W. E. Plaut.
26. G. W. E. Plaut, *Federation Proc.* 14, 263 (1955). We are indebted to Plaut for a generous gift of this enzyme. It catalyzes the hydrolysis of IDP, GDP, and UDP, but is inactive on ADP and CDP.
27. Our thanks are due to A. W. Bernheimer for the samples of bacterial and ox liver RNA, to J. R. Fresco for those of yeast RNA and calf thymus DNA, and to L. A. Heppel for the sample of yeast RNA "core."
28. In extracts of *Alcaligenes faecalis* (supplied by G. B. Pinchot, Yale University). Also, D. O. Brummond (unpublished experiments) has found the enzyme in extracts of *Clostridium kluyveri*.
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Egon Brunswik, Psychologist and Philosopher of Science

I first met Egon Brunswik in Vienna in the winter of 1933–34. He was chief assistant in Karl Bühler's Institute of Psychology at the University of Vienna. He became Privatdozent the following fall. In an exciting series of experiments, he and his students were then demonstrating that, in perceiving, the organism may be "achieving" either the "thing-characters" —size, shape, brightness, and so forth— (of objects) or the immediate "perspective characters," of these same objects or more often some compromise between thing characters and perspective characters. And he had just finished his theoretical and empirical discussion of these findings in the book *Wahrnehmung und Gegenstandswelt* (1934), with the subtitle "Psychologie vom Gegenstand her." As a result of our meeting Brunswik spent a year as a Rockefeller fellow in the department of psychology at the University of California at Berkeley in 1935–36 and in the fall of 1937 permanently joined the Berkeley department.

Brunswik has made four major contributions to psychology: (i) his work on perception as such; (ii) his applications of the history and philosophy of science to psychology; (iii) his demonstrations that not only perception but also thinking and valuing are fruitfully conceived as only in some degree probabilistically valid achievements; and (iv) his insistence on the need for truly "representative design" in the sampling of the environment—for, as he points out, the environment usually presents to the organism cues of only low "ecological validity."

Brunswik's untimely death on 7 July 1955, at the age of 52, came just as his doctrines of functionalistic achievement, representative design and ecological validity had begun to arouse widespread attention both in this country and abroad. It is fortunate that his last work, now in press, a revision and expansion of his monograph on representative design, will serve as an effective and definitive statement of these positions. His was an extraordinarily informed, rich, and subtle mind. And he was never willing to oversimplify or restrict the actual complexities of the relationships with which he was concerned. This always makes the reading of whatever he wrote a difficult task but an exciting and stimulating challenge.

Although, intellectually, somewhat rigorous and aloof, in his human relationships he was simple, friendly, and generous. Unfortunately, he had suffered for many years from very high blood pressure. And he had had to give up the long walks, the mountain climbing, and most of the social activities of his youth.

Born in Budapest of a Hungarian father and an Austrian mother, he was sent as a small boy to the famous Gymnasium of the Theresianische Akademie in Vienna to be prepared for a position as a government official in the old Austro-Hungarian Empire. This education was interrupted by World War I. After the war he was sent for some months to Sweden to overcome the effects of the malnutrition that resulted from the war years. Completing the gymnasium in 1921, he first studied for 2 years to be an engineer at the Technische Hochschule; he then changed to philosophy and psychology at the University of Vienna. Here be came not only under Karl Bühler and the latter's interest in cognitive processes but also under Moritz Schlick and the entire "Vienna Circle" who were then developing the tenets of logical positivism. In this country Brunswik became an active member of the movement toward the unity of science. In the summer of 1953 he organized and participated in the Berkeley Conference for the Unity of Science at the University of California. This, in which he performed brilliantly, took a heavy toll from his health.

Although his own empirical work was largely restricted to perception and learning, Brunswik was extremely sympathetic to the wider sociological and psychoanalytical studies of his wife, Else Frenkel-Brunswik. He was indeed hospitable to all systematic ideas, even when he was unable immediately to incorporate them into his own thinking. His was not only a generous personality but also a generous mind.

His friends, colleagues, and students, and our science as a whole, will miss profoundly his eager, insightful personality and the further vigorous, devoted development of his own original and important basic presuppositions concerning the problems and methodology of psychology.

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