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Messenger and Template Activities of Chemically Modified Polynucleotides*

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ABSTRACT: Nitrous acid treatment of poly C, leading to 3–15% deamination, increased the messenger activity of the polymer in terms of proline incorporation and led to mutations in terms of serine, leucine, and phenylalanine incorporation, all effects correlated with the U content of the polymer. As templates these same samples were reduced in activity in terms of GTP incorporation, but showed some, though far from compensatory, ATP incorporation activity, both effects in approximate correlation to their U content. Hydroxylamine or methoxyamine treatment of poly C, to the point of yielding 0.5–2% of the N-4-substituted derivative of cytosine, caused marked losses in proline incorporation and no significant incorporation of other amino acids. When tested with RNA polymerase, such preparations caused less GTP incorporation, but increased ATP incorporation, resembling minimally deaminated poly C in both respects. Poly C methylated to about 5% with nitrosoguanidine or classical methylating agents was largely inactivated in the messenger test. Neither methylated poly C nor methylated poly U,G showed new amino acid incorporating specificities. As template, methylated poly C retained significant GTP incorporating activity and showed some activity in incorporating ATP, UTP, and CTP. These effects may account for the high mutagenicity of nitrosoguanidine observed under certain conditions. Poly C treated with ultraviolet light to 5% loss in absorbancy showed some loss in proline incorporation but no new amino acid incorporations. Its GTP incorporation was somewhat lowered, without definite effects on ATP, UTP, or CTP binding. Poly U irradiated with ultraviolet light up to 20% loss of absorbancy also showed no evidence for mutagenesis by either test method and comparatively little loss in its incorporating activities. It appears from these facts that cell-free amino acid incorporation supplies a reliable tool for the detection of mutagenic events if, and only if, these represent typical base replacements.

Triphosphate incorporation, in contrast, can reveal base changes which only simulate true base replacements, but gives reliable results only with poly C derivatives. This system has nevertheless shown good correspondence with the biological methods of detecting TMV mutants and may be useful in screening for potential mutagens.

The molecular basis of mutagenic events can at times be deduced from resultant protein or tRNA sequence changes but more often the chemical basis of the phenotypic alteration remains obscure. Since polyribonucleotides have been found to act as both messengers and templates in cell-free amino acid or ribonucleoside triphosphate incorporation systems, attempts have been made to use such systems for the identification of mutagenic reactions. The first reaction to be used for the modification of polynucleotides, the deaminating action of nitrous acid, has produced the expected mutagenic effects when the modified polynucleotide was used as messenger (Basilio *et al.*, 1962). The effect of deamination on the template activity of poly d(A-T) was also studied (Kotaka and Baldwin, 1964). Various other modifying reactions have since been reported to produce new coding or template activities in polynucleotides, some in line with expectation and others not. However, no comparative studies of the effects of different reactions as tested in both systems are known to us.

It is the purpose of the present paper to summarize our studies of the effects of treatment with nitrous acid, nitrosoguanidine, or classical alkylating agents, hydroxylamine or methoxyamine, and ultraviolet irradiation on both the coding and the template properties of poly C. We have attempted to obtain quantitative indications of the sensitivity of detecting mutagenic events in both systems by means of preparations of poly C that had been subjected to limited deamination. Using amino acid incorporation, we have not detected mutational events in the messenger activity of any of the modified poly C preparations excepting HNO_2-treated samples containing as little as 3% of U. When testing for new template activities in the presence of GTP, significant

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increases in ATP incorporation, indicative of C residues acting like U, were seen after HNO_2 hydroxylamine and methoxyamine treatment. After alkylation by nitrosoguanidine or dimethyl sulfate, small amounts of all 3 noncomplementary bases were incorporated. Ultraviolet irradiation caused no significant increases in incorporation. All of the modification reactions decreased the GTP incorporation to a varying extent.

Some data on the consequences of modification on the messenger and template activities of poly U,C and poly U,G are also reported, as well as the effect of ultraviolet irradiation on the incorporating activities of poly U.

Materials and Methods

The polynucleotides and labeled polynucleotides were commercial preparations. *Nitrous acid treatment* was performed in 0.25 M pH 4.0 acetate, using 1 M $NaNO_2$. Several hours at room temperature were needed to obtain 2–3% deamination of poly C[1] or 13% deamination of poly A. The polymers were recovered by G-25 Sephadex chromatography in water. Low levels of deamination were determined by analysis for [^{3}H]- or [^{14}C]uridylic or [^{3}H]inosinic acid after pancreatic or T2 RNase digestion of the polymers and electrophoretic or chromatographic separation of the products, adding nucleotides as marker. It appeared from spectrophotometric analysis of more deaminated preparations that about half of the radioactivity of the C residues was lost upon deamination of Miles poly [^{3}H]C and the values were accordingly corrected. The label in poly [^{3}H]A was not lost upon deamination.

Hydroxylamine and methoxyamine treatment was performed by adding to the polymer solution an equal volume of 2 M solutions of the hydrochlorides brought to pH 6.0 or 5.5, respectively, with NaOH. After 30 min to 3 days at room temperature, when less than 10% of the cytidines had been modified, the modified polymer was isolated by alcohol precipitation. The analytical data concerning the nature and extent of the two types of reactions occurring under these and other conditions are being reported elsewhere (Singer and Fraenkel-Conrat, 1971).

Alkylation with nitrosoguanidine and dimethyl sulfate was done at neutrality and analyzed as previously reported (Singer and Fraenkel-Conrat, 1969a,b). Preparations containing 3–12% of 3-methylcytidine were used, since more extensive alkylation caused too much inactivation.

Ultraviolet irradiation was done at 0° in shallow layers 17 cm from a Westinghouse G 15 T8 lamp (37.7 $\mu W/cm^2$ at lamp surface). Loss of ultraviolet absorbancy, tested at intervals, was used as a first approximation for the extent of reaction, although it is realized that due to reversibility of the reaction, and to its potential hyperchromic effect, the loss represents a minimal measure of the reaction. Parallel studies with ^{14}C-labeled TMV–RNA have given data on the proportion of U-hydrates and pyrimidine dimers formed which will be reported elsewhere (Singer and Fraenkel-Conrat, 1971).

[1] Both poly A and poly C react much more slowly with nitrous acid than does RNA. Different preparations of poly C contained from 3.8 to 9.4% of U after 24-hr reaction at room temperature. At 37°, deamination proceeded to 7.5 and 15% in 4 and 24 hr under the above conditions. These results were obtained with poly C-2-^{14}C, Miles Laboratory Inc.

Amino Acid Incorporation (*Nirenberg, 1963*). Pyruvate kinase (A grade) was obtained from Calbiochem; ATP (grade II), GTP (type II-s), and phosphoenol pyruvate from Sigma; the [^{14}C]amino acids from New England Nuclear and Schwarz; and stripped tRNA (*Escherichia coli* B) from General Biochemicals. The [^{14}C]amino acids ranged from 156 to 455 mCi per mmole.

S-30 was prepared from log phase *E. coli* A-19 according to Capecchi (1966); it was stored in small aliquots at −70°. The A_{260} was about 400 and the dry weight 24 mg/ml. If tRNA was used, which was usually the case for poly C and poly C derived samples, and occasionally with poly A and poly A,G as messenger for arginine (Anderson, 1969) or glutamic acid incorporation, 2 optical density units per sample were used.

The typical reaction mixture (0.05 ml) contained pH 7.8 Tris-HCl (0.034 M), magnesium acetate (0.012 M), NH_4Cl (0.06 M), mercaptoethanol (0.008 M), phosphoenolpyruvate (0.006 M), ATP (3.5×10^{-3} M), GTP (2.4×10^{-4} M), 2.5 μg of pyruvate kinase, $2–6 \times 10^{-5}$ M [^{14}C]amino acids (100–400 mCi/mmole), 2×10^{-4} M of each of the other 19 amino acids, and 0.05 ml of 1:4 diluted S-30 (5 optical density units), preincubated at 37° for 20 min with 2 μl of unlabeled amino acid mixture, 10 μl of buffers and salts, and 25 μl of energy source per ml. To the complete reaction mixture, mixed mechanically, was added messenger polynucleotide in several amounts (usually 2, 5, and 10 μg containing 0.04–0.2 optical density unit), except for the blanks (duplicate samples lacking only messenger), and the mixtures were incubated at 37° for 30 min.

To each tube was then added 3 ml of cold 10% trichloroacetic acid and the contents well mixed, then heated 20 min at 90°, chilled 30 min, and filtered on Whatman No. 1 paper presoaked in 1 M ammonium acetate. The tubes and filters were washed twice with 3 ml of cold 5% trichloroacetic acid. With poly A and related messengers, 5% trichloroacetic acid–0.25% sodium tungstate (pH 2) was used instead of trichloroacetic acid.

The filters were glued to aluminum planchettes, dried in an oven for 10 min, and counted in a gas-flow counter (efficiency about 8%).

Typical messengers usually caused increased incorporation with increasing amounts of messenger. In some instances, however, particularly with samples of low or doubtful activity, these curves showed a plateau or decreased with increasing amounts of messenger used. All calculations are based on the difference in incorporation between the averaged blanks and the 5-μg (0.1 optical density unit) samples. All experiments were repeated from (rarely) 2 to (usually) 3 to 6 times. If a sample caused a slight increase in incorporation (less than doubling the value obtained without messenger) in only one of several tests, or at only one of several levels of polymer used, this was scored as no incorporation. Most of the data are reported in terms of percentages of counts per minute incorporated by modified as compared to unmodified polymer, tested simultaneously (or averaging several experiments, each containing treated and untreated messenger preparations). Typical incorporations produced by untreated poly C or poly U gave 30,000 cpm which corresponds to about 150–500 μμmoles for different amino acids.

Triphosphate Incorporation (Krakow and Karstadt, 1967).

TABLE I: Effect of Various Modifications of Poly C on Its Proline Incorporating Activity.

Reaction		Incorpn of Proline[b]
Reagent[a]	Extent (%)	(%)
Hydroxylamine	0.9	32
	2	7
Methoxyamine	0.5	51
Dimethyl sulfate	5	8
Nitrosoguanidine	5	5
Ultraviolet irradiation	3	61
	5	10
Nitrous acid	3	118
	15	180

[a] For experimental details of modifying reactions see Materials and Methods. The extent of reaction is expressed in terms of approximate percentages of cytosine residues affected, averaging in most cases several similarly treated samples. [b] Average of 2–6 separate tests, performed usually with 2 or more preparations, for incorporation of proline by modified as compared to untreated control samples.

The 0.25-ml reaction mixture contained 0.01 mg of RNA polymerase and usually 0.01 mg of template polynucleotide, 20 μmoles of pH 7.8 Tris buffer, 10 μmoles of mercaptoethylamine, 1 μmole of $MnSO_4$, and 0.1 μmole of each nucleoside triphosphate (usually one ^3H labeled, containing about 6000 cpm per mμmole). After 1 hr at 37°, 0.1 ml of saturated sodium pyrophosphate, pH 7.2, was added, and the mixture cooled at 0°, treated with 3 ml of cold 5% trichloroacetic acid, and immediately filtered on Whatman GF/c Glass Fibre filters washing 5 times with 5 ml of 5% trichloroacetic acid. The filters were dried, placed in 10 ml of toluene scintillation fluid, and counted. Maximal incorporation (110,000 cpm) amounted to about 10 mμmoles.

RNA polymerase, prepared from *Azotobacter vinlandii* by a modification (Krakow *et al.*, 1968) of the original procedure of Krakow and Ochoa (1963) through the DEAE-cellulose step (σ present), was a gift from Dr. J. Krakow.

Results

This paper deals with a comparison of the effects of 4 different types of modification of poly C and to a lesser extent of other polynucleotides on the template and messenger activities of these polymers. This comparative survey by the same standard test methods has revealed certain definite facts among which is most important the finding that alkylation of cytidine is a nonspecific mutagenic event, in terms of transcription, while ultraviolet irradiation has no mutagenic effect under the same conditions. These results are contrasted with the well-known effects of nitrous acid and hydroxylamine or methoxyamine treatments which specifically transform cytidine to behave like uridine. More detailed studies are in progress on several of these modified polynucleotides which may well throw additional light on the nature of mutagenesis.

Amino Acid Incorporation. EXPERIMENTS WITH HOMOPOLYMERS. When poly C was treated with various modifying reagents, its capability to stimulate proline incorporation decreased with increasing intensity of treatment. The order of inactivating action for a given extent of base modification (*e.g.*, 5%) was alkylation and hydroxylamine more than methoxyamine and ultraviolet light (Table I). Only nitrous acid did not decrease, but rather increased, proline incorporation (Tables I, II).

Actual or effectual changes of C → U residues might be expected to lead to incorporation of serine (UCX), leucine (CUX), and phenylalanine (UUX). These amino acids were found to become incorporated upon deamination of 3–10% of the cytidine residues.[2] Neither these nor various other amino acids requiring A in their codons were consistently or significantly incorporated when hydroxylamine or methoxyamine treated, alkylated, or ultraviolet-irradiated poly C was used as messenger.

When poly U was subjected to ultraviolet light until it had lost about 20% of its ultraviolet absorbancy, it retained over 70% of its phenylalanine- and slight leucine-incorporating activities, but did not acquire the ability to incorporate detectable amounts of serine or any of the many other amino acids tested (Table III).

Poly A has given much lower incorporations than the other polynucleotides, though various published methods of tungstate–trichloroacetic acid precipitation of the product were investigated. Blanks with [^{14}C]lysine were quite high, particularly if tRNA was added, even with many variations in washing and preincubation techniques. Poly A usually gave no more than a threefold stimulation, not well correlated with amount of messenger, and not, in our hands, increased by addition of various polylysines.

Experiments with Copolymers. Poly U,C was treated with methoxyamine, ultraviolet light, and dimethyl sulfate to search for differences in the ratio of phenylalanine incorporation to proline incorporation, since a change in this ratio would signify a difference in codon composition as a consequence of the modifying reaction. The increase in this ratio observed in 3 of 4 samples with methoxyamine, but not with ultraviolet irradiation (Table IV), suggests that methoxyamine causes some effective C → U changes.

The messenger activity of poly U,G was studied after alkylation to search for evidence of new coding capabilities as a consequence of up to 12% of the G becoming transformed to 7-methyl G, the only reaction known to occur under the conditions used. There was appreciable loss in typical coding activity (Phe, Leu, Gly, Val, Cys incorporation), and no increases in the incorporation of amino acids requiring A or C (Met, Asp, Ile, Arg) were detected.

Several preparations of poly AG behaved like poly A in terms of lysine incorporation and caused only very little, and doubtful incorporations of glutamic acid and arginine, respectively, even when unfractionated tRNA was added

[2] S-30 preparations obtained early in this study by Nirenberg's (1963) method gave relatively low blank incorporation (without added messenger) of serine and leucine (<1000 cpm), and showed detectable increases with 3% deaminated poly C as messenger. S-30 made by the more convenient method of Capecchi (1966) gave higher blanks (1500–4000 cpm) and lower sensitivity in the detection of U residues in slightly deaminated poly C.

TABLE II: Comparison of Messenger and Template Activities of Polynucleotides Containing Various Ratios of U to C.

	Amino Acid Incorporation[a]				Nucleotide Incorporation[a]			
	Pro	Phe	Leu	Ser	GTP	ATP	ATP[b] (+ Unlabeled GTP)	GTP[b] (+ Unlabeled ATP)
Poly C	*100*	(0.8)	(0.6)	(0.3)	*100*	0	(0.3)	114
Poly U,C (30% U)	340	22	*100*	*100*	4.0	39	54	9.5
Poly U	0	*100*	5	0	0	*100*	44	(0.4)
Poly C 3% deaminated	118	1.9	4.8	3.3	33	(0.1)	1.2	
Poly C 15% deaminated	180	12.5	31	25	26	(0.4)	2.2	

[a] The specific incorporations are taken as 100%. These were for poly C: 16,000 cpm of proline and 80,000 cpm of GTP; for poly U,C: 28,000 and 32,000 cpm of leucine and serine, respectively; and for poly U: 24,000 cpm of phenylalanine and 20,000 cpm of ATP. Background levels without messenger ranged from 700 to 1700 or 2300 cpm for different amino acids, the higher values being obtained in presence of added tRNA.[2] For GTP and ATP the values without template were 200–500 cpm. Incorporations which are in the range of duplicate incorporation data without messenger or template are given in parentheses. [b] Compared to the respective labeled control, taken as 100.

TABLE III: Effect of Ultraviolet Irradiation of Poly U on Incorporation of Amino Acids and Nucleoside Triphosphates.

	Incorporation Compared to Untreated Poly U[a]						
Polymer[a]	Phe (%)	Leu (%)	Ser, Pro, Ile, Arg, Cys, Val	ATP cpm	UTP[b] $\times 10^{-3}$	CTP[b]	GTP[b]
Untreated poly U	(16,000 cpm) = 100	(680 cpm) = 100	None	73	10.5	None	None
Ultraviolet – poly U 9% optical density loss	78	95	None	90	8.5	None	None
Ultraviolet – poly U 20% optical density loss	74	80	None	70	8.5	None	Mone

[a] Averages of two treated preparations, and control polymer tested 5–6 times for amino acid and 3 times for triphosphate incorporation. None signifies levels within the range of samples lacking messenger or template. [b] In presence of unlabeled ATP.

TABLE IV: Incorporation of Amino Acids by Poly U,C Derivatives.

	% Incorporation of Untreated Poly U,C (see below)[a]				
Reaction	Proline	Phenylalanine	Leucine	Serine	Phe/Pro
Methoxyamine[b]	68 (6)	100 (4)	100 (2)	56 (1)	1.5
	11 (3)	7 (3)	11 (3)	10 (3)	0.6
	5 (3)	6 (3)	8 (3)	4 (3)	1.2
	2 (2)	4 (2)			2.0
Ultraviolet irradiation[b]	44 (4)	44 (4)	48 (2)	33 (2)	1.0
Untreated poly UC (cpm)	11,600 (9)	6400 (7)	8000 (9)	4600 (10)	

[a] Number of averaged tests in parentheses. [b] See Methods and Materials for experimental details.

TABLE V: Effect of Various Modifications of Poly C on Its Template Activity.

Reaction	Number of Samples[a]	GTP cpm[a]	ATP cpm[b]	UTP cpm[b]	CTP cpm[b]
Poly C, untreated[c]		80,700[c]	687 ± 177 (18)	409 ± 144 (11)	437 ± 193 (11)
Hydroxylamine	4	6,500–33,000	*1055* ± 423 (14)	390 ± 179 (10)	328 ± 133 (9)
Methoxyamine	3	27,000–43,000	*1135* ± 224 (11)[d]	554 ± 366 (8)	514 ± 283 (8)
Alkylating agents	4	3,500–19,000	*1075* ± 387 (20)	*842* ± 282 (15)	*721* ± 284 (12)[d]
Ultraviolet irradiation	8	7,200–29,000	775 + 210 (41)	379 ± 144 (33)	327 ± 114 (30)
Nitrous acid	2	30,000–40,000	*2414* ± 1203 (8)	612 ± 241 (7)	523 ± 110 (6)

[a] Increasing intensities of treatment (in terms of time, temperature, and occasionally reagent concentration) usually give roughly proportional decreases in GTP incorporation, as indicated by the range of data in the GTP column. However, the range of incorporation of the other triphosphates was similar for each reagent as these various levels of GTP incorporation, and these data were therefore pooled and the standard deviation was calculated for the entire group. The conclusions concerning significant differences (italicized) derived from this statistical treatment were, however, borne out almost infallibly by each individual experiment. [b] Incorporation of labeled triphosphate in the presence of unlabeled GTP (number of tests averaged in parentheses). Without added GTP, incorporations approached in most instances those obtained without template (200–300 cpm) which were not subtracted from the above data. [c] Decreasing amounts of poly C used as messenger (from 10 to 1.25 μg) gave roughly proportionally decreasing incorporation of GTP, but the other triphosphates were reduced by less than half. [d] Nearest neighbor analyses indicated that 85–95% of the radioactivity introduced as [α-^{32}P]ATP was in (2′)3′-guanylic acid, and the rest in adenylic acid. With [α-^{32}P]CTP, over 90% of the incorporated label was in Gp.

to compensate for the deficiency of *E. coli* extracts for Arg-tRNA (Anderson, 1969). No definite incorporation of these two amino acids was observed with poly A deaminated up to 13%. No incorporation was detected at all if only trichloroacetic acid was used as precipitant.

Nucleoside Triphosphate Incorporation. The activity of poly C to stimulate GTP incorporation was decreased upon treatment with all reagents discussed in this paper, including minimal deamination (Tables II, V). In contrast, ATP incorporation, indicative, presumably, of the presence of U or a base behaving like U in the poly C, was increased by hydroxylamine and methoxyamine treatment. Incorporation of UTP and CTP was not significantly affected by these poly C derivatives. Ultraviolet irradiation showed no significant effect on ATP incorporation into the poly G, even when using the lower pH (6.5) and temperatures (15 or 20°) advocated by Ono *et al.* (1965) for the detection of this incorporation as directed by irradiated poly C. The important new result of this study is that alkylation by means of dimethyl sulfate or nitrosoguanidine caused slight increases in the incorporation of all three triphosphates (ATP, UTP, and CTP) over untreated poly C in most preparations tested.

The new incorporating activities of mutagenized poly C preparations were definitive only if the labeled triphosphate was used in the presence of (unlabeled) GTP and they reached in individual experiments only about threefold of the background of ATP, UTP, or CTP incorporation by the unmodified poly C. When compared to the GTP incorporation produced by the same poly C derivative, they amounted to 20–30% for the most active alkylated or hydroxylamine-treated preparations. These effects may be compared to that of minimal deamination (2–3%) which increased ATP incorporation by fourfold, while decreasing GTP to 38%, both compared to untreated poly C. All these facts were evident in individual experiments. However, the scatter of data at low incorporation levels (particularly in the controls lacking template) was great and sufficient numbers of experiments were performed, therefore, to allow statistical evaluation of the effects of each reagent (Table V). Nearest neighbor analyses showed that the α-phosphate of the ATP and CTP were genuinely introduced into the G polymer.

A few experiments were also performed with a preparation of *E. coli* RNA polymerase kindly put at our disposal by Dr. M. Chamberlin. These gave the same type of results, hydroxylamine treatment increasing only ATP incorporation and alkylation increasing ATP, UTP, and CTP incorporation. Both treatments decreased GTP incorporation.

In an attempt to use template activity for the detection of mutagenized A residues the well-known tendency for

TABLE VI: Nucleotide Incorporation with Poly A and Deaminated Poly A as Templates.

Triphosphates Used[a]	Nitrous Acid Treated Poly A: Poly A, cpm	13% Hx,[b] cpm	22% Hx,[b] cpm
UTP	15,700	8,860	9,200
ATP	209	276	365
ATP (+ unlab. UTP)	26,030	23,400	18,050
CTP	156	200	245
CTP (+ unlab. UTP)	250	333	337
GTP	121	140	133
GTP (+ unlab. UTP)	245	265	262

[a] Radioactive, unless specified; all data averages of 2–4 analyses. [b] Hx = hypoxanthine.

TABLE VII: Nucleotide Incorporation with Poly U,G and Methylated Poly U,G as Templates.

Triphosphate Used[a]	Poly U,G (67% U)		Poly U,G (50% U)		
	Untreated cpm	~5% 7-MeG cpm	Untreated cpm	~6% 7-MeG cpm	~12% 7-MeG cpm
ATP	31,500	24,100	5760	3600	2040
CTP	375	478	400	326	(242)
UTP	149	241	220	220	(206)
ATP + CTP	7,645	2,970	1250	945	(708)
ATP (+unlabeled CTP)	7,500[b]				
ATP (+unlabeled UTP)			3610	2440	1380
CTP (+unlabeled ATP)	722[c]				
UTP (+unlabeled ATP)	13,100	7,280	895	605	697
UTP (+unlabeled CTP)	178	196	203	233	(224)
UTP (+unlabeled ATP,CTP)	4,680	2,500	290	208	(350)

[a] Radioactive unless specified; all data averages of 2–4 analyses, excepting those in parentheses. [b] Ranges from 5450 to 16,000 for 10^{-3} to 10^{-7} M CTP. [c] Ranges from 722 to 253 for 10^{-4} to 10^{-6} M ATP.

reiteration of A and U oligonucleotides became evident, and this probably accounted for our failure to detect C incorporation proportional to the inosine content of partly deaminated poly A (Table VI). Possibly for the same reasons, U but not C was incorporated by two preparations of poly U,G (Table VII).

Discussion

The results of the first phase of this research, the investigation of the effects of chemical modification on the coding properties of poly C and other polynucleotides, were rather disappointing. None of the reactions studied excepting deamination produced detectable new amino acid incorporation specificities, indicative of mutagenic events. As far as poly UG is concerned, this is confirmatory of the results of Wilhelm and Ludlum (1966). Concerning ultraviolet irradiated poly C and poly U, however, the present data do not confirm earlier results by Wacker *et al.* (1964), nor do they support the recent conclusion by Ottensmeyer and Whitmore (1968) that U hydrates code (and bind) like C, and U dimers like UG.

While analytical data on the extent of reaction are available for most of the modifications studied, the actual nature of the mutagenic event is unknown in most instances. Besides deamination, hydroxylamine and methoxyamine are the only reagents which transform C to a base, N^4-hydroxy- (or methoxy-) cytosine, shown to be U-like in its preferred (9:1) tautomeric state (Kochetkov and Budowsky, 1969; Lawley, 1967; Brown and Hewlins, 1968; Brown *et al.*, 1968; Janion and Shugar, 1968), and recent data from this laboratory reported by Singer and Fraenkel-Conrat (1971) indicate that only this monosubstituted product is present in poly C and RNA treated with methoxyamine at pH 5.5, while both this and the doubly substituted product formerly believed to represent the mutated species occur in hydroxylamine treated polynucleotides. The extent of the hydroxylation of the cytosine in the type of sample used for incorporation studies was between 2 and 5%, and yet in contrast to deamination which shows marked effects at this level, the hydroxylamine type of modification does not introduce new coding specificities into poly C but only results in decreased proline incorporation. These data suggest that this and other modified bases which only simulate but do not actually represent a typical RNA base do not function effectively, for reasons unknown, under our experimental conditions of *in vitro* translation. This factor, rather than an insufficient number of mutagenic events, probably accounts for the failure to detect the modified bases by amino acid incorporation in modified poly C. A suggestion, however, that nonrecognition is not complete comes from the data on the ratio of incorporation of phenylalanine to proline with methoxyamine treated poly U,C (Table VI). Also, the data of Basilio *et al.* (1962), suggest that inosine is able to behave like guanosine in amino acid incorporation tests, although we were unable to confirm this. On the other hand our data confirm the finding that methylated poly U,G fails to show any new coding activities (Wilhelm and Ludlum, 1966). We also have confirmed Ludlum's finding that limited alkylation of polyribonucleotides (as used in these studies) does not lead to their degradation (Singer and Fraenkel-Conrat, 1969a; Wilhelm and Ludlum, 1966; Ludlum and Wilhelm, 1968).

The incorporation of triphosphates has proven a much more responsive method of detecting mutagenic reaction than was amino acid incorporation. Of the reagents studied, only those which are mutagenic when acting on TMV-RNA (Singer and Fraenkel-Conrat, 1969c,d) increase the tendency of poly C to cause other triphosphates to become incorporated in the poly G made by *A. vinlandii* RNA polymerase (Table V). Besides the modified poly C preparations discussed in this paper, this has proven the case also for poly C brominated to contain varying amounts of 5-bromocytosine, which causes marked and progressive ATP incorporation (Means and Fraenkel-Conrat, 1970; Fraenkel-Conrat and Singer, 1971).

In qualitative terms, it appears that hydroxylamine and methoxyamine, like nitrous acid, lead to significant increases of only the ATP incorporating activity of the modified polymer. This is in line with expectation, since as stated above the preferred tautomer of *N*-4-hydroxy- (and presumably also methoxy-) cytosine has U-like binding properties. Our results concerning the ATP incorporation by hydroxylamine and methoxyamine treated poly C are in accord with earlier reports of Phillips *et al.* (1965) and Wilson and Caicuts (1966).

Alkylation, by classical alkylating agents and nitrosoguanidine, is well known to involve the G residues with great preference, and the resultant 7-methyl G has been presumed to code occasionally like A (Lawley, 1966; Singer and Fraenkel-Conrat, 1969d). An attempt to search for A-like behavior in a G containing polymer by triphosphate incorporation gave inconclusive results due to the irregular template behavior of poly U,G (see Table VII).

It has been observed that conditions which render nitrosoguanidine a very good mutagen favor its reaction with cytosine (Singer and Fraenkel-Conrat, 1967; Singer and Fraenkel-Conrat, 1969b,c), a reaction which under typical alkylation conditions is quite minor in RNA or DNA. Thus it was suggested that the substitution of the 3 position of C by a methyl group might represent a mutagenic event. This seems to be borne out by the present studies, since methylated poly C showed appreciable ambiguity, leading to increased and similar incorporations of UTP, ATP, and CTP, each tested in the presence of unlabeled GTP. These activities amounted to 5–10% of that of the residual GTP incorporating activity of certain preparations. The capability of methylated poly C to incorporate UTP has been reported by Ludlum and Wilhelm (1968), but these authors failed to find significant increases in ATP and CTP incorporation compared with unmodified poly C.

Ultraviolet irradiation transforms U into a 5,6-hydrate and a cyclobutane type dimer, while of C only dimeric derivatives (without or with loss of the amino groups of C) are believed to be stable (McLaren and Shugar, 1964; Pearson *et al.*, 1966; Setlow, 1966). In the case of poly U, hydrates seem to predominate. *In vitro* ultraviolet irradiation is not appreciably mutagenic (Singer and Fraenkel-Conrat, 1969c,d) and this is borne out by the present finding that this treatment (to 5% loss in absorbancy of poly C) did not produce consistent increases in incorporation of ATP, UTP, or CTP under our standard conditions, nor at the lower pH and particularly the temperature advocated by Ono *et al.* (1965) for the purpose of minimizing the reversal of the hydration reaction. Even extensive irradiation of poly U (to 20% loss in absorbancy) did not affect the incorporation of any triphosphate, compared to that caused by untreated poly U. These data also do not support Ottensmeyer and Whitmore's (1968) conclusion that U hydrates code like C and U dimers like U,G.

In quantitative terms, the stimulation of ATP incorporation upon slight deamination of poly C (3–7%) is about three times as great as that caused by hydroxylamine and methoxyamine treatments which result in similar levels of the N-4 substituted products. Alkylation of about 5% of the C gives similar mutation frequencies (Table V). Ultraviolet induced hydration and/or dimerization of at least 5% of the C residues causes 60–90% loss in GTP incorporation by poly C (without mutagenesis), but the much greater loss of the absorbancy of poly U upon irradiation hardly affects its template activity, as it did also not greatly affect its coding potential.

While the above considerations suggest that triphosphate incorporation may represent a valid measure of mutagenic reactions in poly C, some of the quantitative aspects of these data are rather unsatisfactory. First there is the well-known lack of correlation between total template nucleotides and total triphosphate incorporation (Table II). Thus, replacement of some C by U upon nitrous acid treatment of the template does not lead to a quantitatively equivalent replacement of GTP by ATP incorporation, but rather causes a sharp drop in total incorporation capacity. This is in marked contrast to the messenger activities of this same series of poly C preparations containing increasing amounts of U since they showed progressive increases in total amino acid incorporation with increasing content in U (Table II). One might hypothesize that poly C's conformation under the reaction conditions makes it a comparatively poor messenger, but a very good template, and that introduction of U residues interferes with these conformational effects. Yet one would not expect a drop to one-third of the original activity upon deamination of a few per cent of the C residues.

Also indicative of the unpredictability of the system, as used here, is the fact that poly U,C and poly U,G incorporate considerably more ATP in the absence than in the presence of GTP or CTP, respectively (Table V). It appears probable that these are consequences of reiteration which has been found to complicate the interpretation of data obtained with oligomers and polymers of A and U. Also secondary template action of newly made complementary strands is evident with A and U containing polymers (see Table III). Most of the data and conclusions reached in this paper, however, were obtained with poly C derivatives and no indications were obtained that either of these factors played a significant role with this polymer.

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Quantitative Measurement of Isoprenoid Nucleosides in Transfer Ribonucleic Acid*

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ABSTRACT: A method is described for the quantitative estimation of submicrogram quantities of isoprenoid nucleosides from hydrolysates of tRNA. It has the advantage of being more sensitive than ultraviolet absorbance assays and more rapid, specific, and precise than bioassays. Analysis involves degradation of tRNA to its nucleosides by base hydrolysis and enzymatic digestion; separation of the isoprenoid nucleosides from the bulk of the hydrolysis products by column partition chromatography; and resolution of their trimethylsilyl derivatives by gas chromatography. The derivatives are all well separated, with retention times of 5–20 min, when applied to a 4-ft glass column of 10% DC-11 on Gas Chrom Q at 255°.

The procedure was used to determine N^6-(Δ^2-isopentenyl)-adenosine, 2-methythio-N^6-(Δ^2-isopentenyl)adenosine, and N^6-(*cis*-hydroxy-3-methylbut-2-enyl)adenosine in tRNA from *Escherichia coli*, peas, and yeast. It should be especially useful for investigation of systems yielding milligram quantities of tRNA and for the examination of tRNA subspecies for isoprenoid nucleosides.

The demonstration of a relationship between the minor nucleotide content and the biological activity of certain tRNA species (Capra and Peterkofsky, 1968; Gefter and Russell, 1969) has prompted considerable speculation concerning the role of minor nucleosides in control of protein synthesis. Of particular interest are those nucleosides shown to have cytokinin activity (Hall *et al.*, 1967a; Burrows *et al.*, 1968): IPA,[1] *cis*-ZR (the cis isomer of zeatin riboside), and msIPA. In the past, assays for these substances have relied either upon rather insensitive ultraviolet absorbance measurements following chromatographic isolation or upon time-consuming bioassays of rather low precision. Hall and his coworkers (1967a) were able to detect as little as 10 μg of IPA or *cis*-ZR in hydrolysates of plant tRNA by measuring the absorbance of spots eluted from chromatograms. However this probably represents the lower limit

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[1] Abbreviations used are: IPA, N^6-(Δ^2-isopentenyl)adenosine; ms-IPA, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine; *cis*-ZR, N^6-(*cis*-hydroxy-3-methylbut-2-enyl)adenosine; Me_3Si, trimethylsilyl.