Rifkin, D. B., Rifkin, M. R., and Konigsberg, W. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 586.
Sager, R., Weinstein, I. B., and Ashkenazi, Y. (1963), *Science 140*, 304.
Scher, S. (1966), *Biochem. Biophys. Res. Commun. 22*, 572.
Schweet, R. S., Lamfrom, H., and Allen, E. (1958), *Proc. Natl. Acad. Med. Sci. U. S. 44*, 1029.
So, A. G., and Davie, E. W. (1964), *Biochemistry 3*, 1165.
So, A. G., and Davie, E. W. (1965), *Biochemistry 4*, 1973.
Speyer, J. F., Lengyel, P., Basilio, C. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 684.
Szer, W., and Ochoa, S. (1964), *J. Mol. Biol. 8*, 823.
Ts'o, P., and Vinograd, J. (1961), *Biochim. Biophys. Acta 49*, 113.
Weinstein, I. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 579.
Weinstein, I. B. (1964), *Bull. N. Y. Acad. Med. 40*, 89.
Weinstein, I. B., and Schechter, A. N. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1686.
Weisblum, B., Gonano, F., Von Ehrenstein, G., and Benzer, S. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 328.

Fluorinated Pyrimidines. XXVII. Attempts to Determine Transcription Errors during the Formation of Fluorouracil-Containing Messenger Ribonucleic Acid*

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ABSTRACT: The hypothesis is presented that the inhibition of β-galactosidase production in *Escherichia coli* and the mutants of tobacco mosaic and polio viruses produced by 5-fluorouracil are a consequence of the incorporation of 5-fluorouracil into ribonucleic acid (RNA) in place of cytosine during the process of transcription or viral replication, and that the 5-fluorouracil so incorporated would be read in translation as if it were uracil instead of cytosine. This was tested using a synthetic deoxyribonucleic acid (DNA), with one strand consisting of alternating thymine and guanine sequences and the other strand consisting of alternating adenine and cytosine sequences, as the primer for purified *E. coli* RNA polymerase. In this system, the incorporation of isotope from [α-^{32}P]5-fluorouridine triphosphate and from [α-^{32}P]uridine triphosphate into the 2′-(3′)-adenosine monophosphate, obtained on alkaline hydrolysis of the polyribonucleotides, was measured. However, no difference could be detected between the two labeled precursors in this system, which were both incorporated into polyribonucleotides at a frequency of replacement of less than one uracil or 5-fluorouracil per 3000 cytosines. This low degree of incorporation can be explained by the presence of a very small amount of deoxyribonucleic acid in the purified ribonucleic acid polymerase preparation, which also had a little polyadenylate polymerase activity.

The primary mechanism whereby 5-fluorouracil[1] (Duschinsky *et al.*, 1957) inhibits the growth of bacterial, mammalian, and neoplastic cells involves the inhibition of the enzyme, thymidylate synthetase, by 5-fluoro-2′-deoxyuridine 5′-monophosphate (Cohen *et al.*, 1958; Hartmann and Heidelberger, 1961; Reyes and Heidelberger, 1965). On the other hand, the incorporation of FU into ribonucleic acid (RNA) has been reported in mouse tumors (Chaudhuri *et al.*, 1958), *Escherichia coli* (Horowitz and Chargaff, 1959), tobacco mosaic virus (TMV) (Gordon and Staehelin, 1959), polio virus (Munyon and Salzman, 1962), etc. The consequences of such incorporation have not always been clear. Polyfluorouridylic acid (poly-FU) has been

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[1] Abbreviations used: FU, 5-fluorouracil; FUDRP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; U, uracil; C, cytosine; T, thymine; G, guanine; A, adenine; ATP, adenosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; FUTP, 5-fluorouridine triphosphate; 2′,3′-AMP, adenosine 2′-(3′)-monophosphate; poly-dAT, polydeoxyadenylate–polythymidylate C (alternating sequence); [^{3}H]GTP, tritiated guanosine triphosphate; 2′,3′-UMP, uridine 2′-(3′)monophosphate; TCA, trichloroacetic acid.

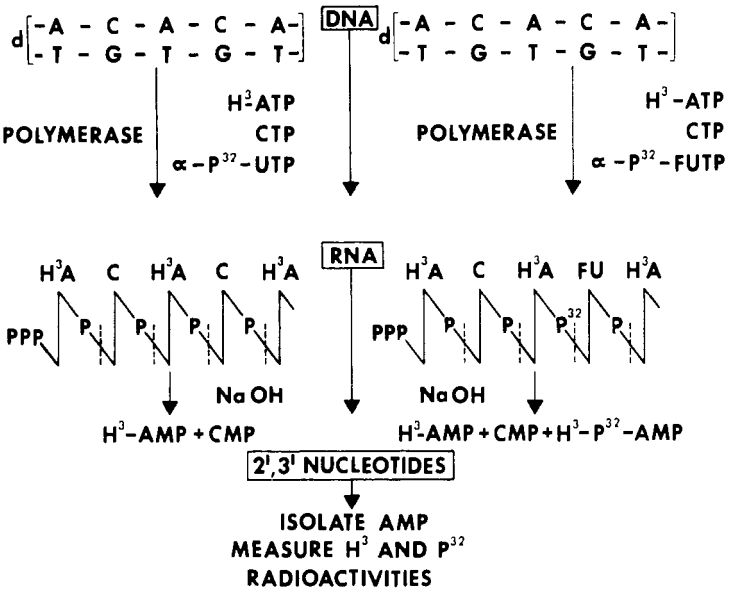

FIGURE 1: The experimental design.

shown to code only for phenylalanine, and with less ambiguity than does polyuridylic acid (poly-U) (Wahba *et al.*, 1963; Grunberg-Manago and Michelson, 1964). Tobacco mosaic virus containing up to 5% of the uracil replaced by FU, has a normal coat protein as determined by quantitative amino acid analysis (Houlabek, 1962) and by immunochemical techniques (Sutic and Djordjevic, 1964), and the same has been found for polio virus (Munyon and Salzman, 1962). Yet a five- to tenfold increase of mutants of TMV grown in the presence of FU have been found (Kramer *et al.*, 1964). When the coat protein of the pure mutants were isolated, changes in the amino acid composition were found, and the following exchanges have been determined: valine → alanine, threonine → alanine, serine → glycine (Wittman, 1964). In the case of polio virus, heat-sensitive mutants have been produced when FU was present in the medium (Cooper, 1964). It is clear, at least in one case (Wittmann, 1964), that an altered coat protein has resulted from FU incorporation, and the other failures to observe changes (Houlabek, 1962; Sutic and Djordjevic, 1964) were most likely due to the fact that with the techniques used, amino acid substitutions in the very small number of mutants that may have been present could not have been detected.

Other lines of evidence that the presence of FU leads to the production of altered proteins comes from the work on enzyme induction in *E. coli*. The immediate inhibition of the induction of β-galactosidase by the addition of FU (Horowitz *et al.*, 1960) results in the production of an enzymatically incompetent, but serologically cross-reacting protein (Bussard *et al.*, 1960), and it has been clearly demonstrated that this is a consequence of the incorporation of FU into a messenger ribonucleic acid (m-RNA) (Nakada and Magasanik, 1964). Moreover, alkaline phosphatase isolated from *E. coli* grown in the presence and absence of FU had differences in heat stability (Naono and Gros, 1960).

Since FU has a lower pK_a (8.2) than that of uracil (9.5), at physiological pH it would be more enolized than U. Therefore, it might occasionally act as if it were cytosine during transcription [deoxyribonucleic acid (DNA) dependent m-RNA synthesis], viral RNA replication, or during translation (m-RNA-dependent protein synthesis). However, the fact that only a very few mutants are produced from TMV containing up to 56% replacement of U by FU indicates that FU is generally read in translation as if it were U. This is supported by the experiments with poly-FU cited above (Wahba *et al.*, 1963; Grunberg-Manago and Michelson, 1964).

In an attempt to explain these results, the hypothesis was recently put forth (Heidelberger, 1965) that FU could occasionally base pair with guanine as if it were C during m-RNA or viral RNA synthesis, but not during translation. In other words, FU substituted in a m-RNA or viral RNA in place of U would be translated as if it were U. However, FU incorporated into RNA in place of C, would also be translated as if it were U, and mistakes in the resulting protein would be made. Since FU in poly-FU has a higher pK_a than does the monomer (Grunberg-Manago and Michelson, 1964), it would be more "U-like" in polymers. Thus, if such a shift of pK_a also occurs in natural RNA, it would appear more probable that FU causes errors in transcription and replication rather than during translation. This hypothesis would be consistent with all the above-mentioned facts. An exception, however,

appears to be the phenotypic reversions of the amber mutants in the r_{II} locus of bacteriophage T4 by FU (Champe and Benzer, 1962; Brenner *et al.*, 1965) (and possibly also the FU-repaired production of alkaline phosphatase in certain strains of *E. coli* (Rosen, 1965)), which can best be explained on the basis of relatively infrequent mistakes in translation.

The present experiments were designed to test the possible incorporation of FU into RNA in place of cytosine. This was made possible by the availability of a DNA of known alternating sequence (Wells *et al.*, 1965), which was kindly provided by Professor H. G. Khorana and Dr. R. D. Wells of this university. The synthetic DNA was used as the primer for a purified *E. coli* RNA polymerase (Chamberlin and Berg, 1962), and the design of the experiments is shown in Figure 1.

The synthetic DNA consists of one strand of alternating thymine-guanine (T-G) sequences and the other strand of alternating adenine-cytosine (A-C) sequences. In the presence of adenosine triphosphate and cytidine triphosphate, the RNA polymerase only copies the former strand to give an RNA of alternating A-C sequence. The experiments were carried out with tritiated ATP and [α-^{32}P]uridine triphosphate and fluorouridine triphosphate. The RNA so obtained was hydrolyzed by alkali and the amount of ^{32}P transferred to the 2′,3′-adenylic acid (Heidelberger *et al.*, 1956; Harbers and Heidelberger, 1959; Josse *et al.*, 1961) was determined. As can be seen from Figure 1, if FU were incorporated into RNA in place of C, then adenosine 2′,3′-monophosphate would be isolated that would be labeled with both tritium and ^{32}P. In all these experiments [α-^{32}P]UTP and [α-^{32}P]FUTP were compared under identical conditions.

Materials and Methods

Poly-d(AC:TG). This template was obtained from Professor H. G. Khorana and Dr. R. D. Wells (Wells *et al.*, 1965), and had a molecular weight of about 1×10^6. The preparation was free of polydeoxyadenylic acid (poly-dA) and polydeoxyadenylic polythymidylic acids (poly-dAT) as shown by density gradient centrifugation in alkaline cesium chloride.

RNA Polymerase. The enzyme was prepared from frozen *E. coli* cells following the procedure of Chamberlin and Berg (1962). In order to determine the proper amounts of reagents for the streptomycin and protamine sulfate precipitations, titrations were carried out by monitoring the precipitation of nucleic acids in the former case, and the RNA polymerase activity/mg of protein in the latter case. After chromatography on DEAE-cellulose, the enzyme was stored in liquid nitrogen. The final activity was 3000 units/mg of protein (10 mg of protein/ml).

Syntheses of [α-^{32}P]*UTP and* [α-^{32}P]*FUTP.* β-Cyanoethylphosphate was prepared from 0.1 mmole of radioactive phosphoric acid (obtained from Squibb and Co.), containing 180 mc/mmole, by the method of Pfitzner and Moffatt (1964). The isopropylideneuridine and isopropylidenefluorouridine (Remy *et al.*, 1961) were phosphorylated by the labeled β-cyanoethylphosphate in the presence of dicyclohexylcarbodiimide according to the procedure of Tener (1961). The tributylammonium salts of the monophosphates were reacted in dimethylformamide with carbonyldiimidazole and tributylammonium pyrophosphate as described by Hoard and Ott (1965). In the final reaction mixture the imidazolinium pyrophosphate was centrifuged and washed twice with cold dimethylformamide, and the resulting solution was evaporated to dryness with an oil pump at 30°. The residue was dissolved in 2 ml of water and put on a DEAE-cellulose column (1.5 × 35 cm) in the carbonate form. After washing with water until no more optical density was removed, the column was eluted with a linear gradient of triethylammonium carbonate solution (the mixing chamber contained 1 l. of water, and the second chamber contained 1 l. of 0.5 M triethylammonium carbonate). The chromatography was carried out at 4°. The fractions containing the triphosphates were combined and lyophilized, and the remaining triethylammonium carbonate was removed by several evaporations with absolute ethanol. The final residue was dissolved in 1.5 ml of water and passed through a 3-ml column of Dowex 50-H+, and the effluent was adjusted to pH 7.5 with NaOH and evaporated to dryness under vacuum at 25°. The residue was dissolved in water at a concentration of 2 μmoles of nucleoside triphosphate/ml. The products were shown to be pure by paper chromatography in two systems. The over-all yield was 15–30% based on the amount of [^{32}P]-phosphoric acid used, and the initial specific activities were 130 mc/mmole.

Determination of Radioactivity. During the isolation of the RNA polymerase, the activity of the enzyme fractions was assayed by measuring the incorporation of tritiated guanosine triphosphate into acid-insoluble form. After incubation (see below), 0.1 ml of the assay mixture was deposited on Whatman 3MM filter disks, 2.3-cm diameter. The filter disks were washed three times for 15 min by gentle shaking in beakers of cold 5% TCA, followed by washing with ethanol–ether (1:1). The dried filter disks were then counted in toluene diphenyloxazole in the Packard Tri-Carb liquid scintillation spectrometer.

It was observed that with the same amount of enzyme and under identical conditions, more acid-insoluble radioactivity was apparently found if tritium-labeled nucleotides were used than was obtained when ^{32}P nucleotides had been present in the incubation mixture. This is illustrated by the experiment shown in Table I. Here, aliquots of the ^{3}H- and α-^{32}P-labeled GTP's were spotted on Whatman 3MM filter disks, and after drying were counted directly in the liquid scintillation counter to determine the original specific activity. These nucleotides were then used concurrently as precursors in the RNA polymerase assay (see below), and the acid-insoluble radioactivity was determined on Whatman 3MM filter disks following TCA precipitation and washing with ethanol–ether as described above. The greater ratio of ^{3}H:^{32}P in the acid-insoluble sam-

TABLE I: Comparison of the Determination of Acid-Soluble and Acid-Insoluble Radioactivity Using the Filter Disk Method.[a]

	Total Cpm	mμmoles on Filter Disk	$^3H/^{32}P$	
			Cpm	mμmoles
Acid-soluble nucleotides (1 mμmole)				
[^{3}H]GTP	300	1 (real)	0.045	1
[^{32}P] GTP	6700	1 (real)		
Acid-insoluble polynucleotides incorp from				
[^{3}H]GTP	3060	10.2 (apparent)	0.344	7.7
[^{32}P]GTP	8900	1.3 (apparent)		

[a] The radioactivity of [^{3}H]- and [α'-^{33}P]GTP is compared with the products of their incubation in the RNA polymerase system. See text for details.

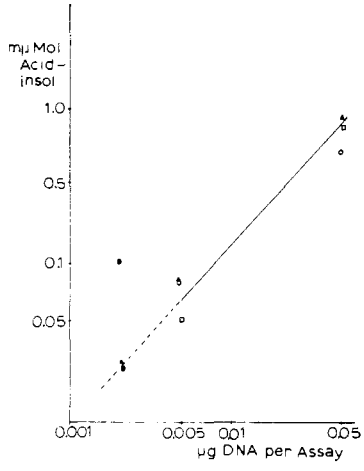

FIGURE 2: The incorporation of [^{14}C]ATP, [^{14}C]GTP, and [^{14}C]UTP into acid-insoluble polynucleotides in 30 min by *E. coli* RNA polymerase. The open points are with the addition of the indicated quantities of *E. coli* DNA as primer. The closed points represent the incorporation of the nucleotides in the absence of added DNA. After the incubation, 100 μl of the reaction mixture was spotted on Whatman 3MM filter disks and counted as described in Materials and Methods. Each point represents the mean of three experiments. 0 = [^{14}C]ATP; □ = [^{14}C]UTP; Δ = [^{14}C]GTP. In each case 0.5 μmole of the labeled nucleotide, and equivalent amounts of the other three nonradioactive ribonucleoside triphosphates were added.

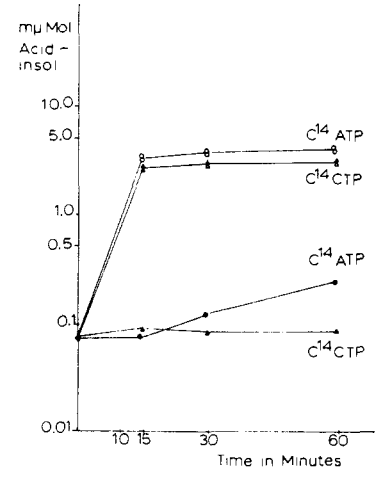

FIGURE 3: Time course of the incorporation of [^{14}C]-ATP and [^{14}C]UTP into acid-insoluble polynucleotides by *E. coli* RNA polymerase. The open points show the incorporation obtained with the addition of 5 μg of *E. coli* DNA added as primer. The closed points show the incorporation of the nucleotides in the absence of DNA primer. In each assay, 0.5 μmole of the labeled nucleotide, and equivalent amounts of the other three nonradioactive ribonucleoside triphosphates were added.

ples, suggests that the polynucleotides are attached to the surface of the cellulose fibers and are therefore counted with a higher efficiency than the mononucleotides, which penetrate more deeply into the fibers of the filter disk, and in the case of tritium are counted with a very low efficiency, thus accounting for the large shift in the $^3H:^{32}P$ ratio observed in this experiment. These results are in agreement with those of Furlong *et al.* (1965), who found a similar effect with tritium-labeled DNA, and point out the necessity of care in the comparison of acid-soluble and acid-insoluble radioactivities using tritium and the filter disk method.

RNA Polymerase Assay. The reaction mixture contained in a volume of 0.125 ml, 5.0 μmoles of Tris buffer, pH 7.4, 0.5 μmole of $MgCl_2$, 1.5 μmoles of mercaptoethanol, 0.125 μmole of $MnCl_2$, 0.04 μmole of [^{3}H]ATP, 0.02 μmole of CTP, 0.04 μmole of [α-^{32}P]UTP (or FUTP), and poly-d (TG:AC) as template (0.01–0.025 OD unit). The reaction was started by

TABLE II: Attempts to Determine Transcription Errors during the Formation of Fluorouracil-Containing RNA in the RNA Polymerase System Primed by Synthetic DNA.[a]

Expt	Labeled Precursors	Sp Act. (cpm/mμmole) [^{3}H]ATP	Sp Act. (cpm/mμmole) ^{32}P Nucleotide	Isolated AMP (cpm) ^{3}H	Isolated AMP (cpm) ^{32}P	mμmoles of [^{3}H]AMP Isolated	mμmoles of ^{32}P Transferred to AMP	% mμmoles of ^{32}P Transferred to mμmoles of [^{3}H]AMP
1	[^{3}H]ATP, CTP, [^{32}P]UTP	1,750	125,000	1,340	56	0.77	4.5×10^{-4}	0.06
	[^{3}H]ATP, CTP, [^{32}P]FUTP	1,750	125,000	4,560	114	2.6	9.1×10^{-4}	0.04
2	[^{3}H]ATP, CTP, [^{32}P]UTP	1,750	13,100	2,010	51	1.2	4.0×10^{-3}	0.34
	[^{3}H]ATP, CTP, [^{32}P]FUTP	1,750	13,100	2,880	47	1.6	3.6×10^{-3}	0.22
3	[^{3}H]ATP, CTP, [^{32}P]UTP	1,750	6,540	2,570	63	1.5	9.6×10^{-3}	0.66
	[^{3}H]ATP, CTP, [^{32}P]FUTP	1,750	6,540	5,080	124	2.9	1.9×10^{-2}	0.65
4	[^{3}H]ATP, CTP, [^{32}P]UTP	2,100	4,500	2,510	1.3[b]	1.2	2.9×10^{-4}	0.024
	[^{3}H]ATP, CTP, [^{32}P]FUTP	2,100	4,500	3,560	6.8[b]	1.7	1.5×10^{-3}	0.088

[a] The template was d(TG:AC). After incubation for 30 min the reaction was stopped and the 2′,3′-AMP was isolated as described in Materials and Methods. Usually two to four assay tubes were combined for the isolation of the AMP. [b] These samples were rechromatographed on thin layer, and the ^{32}P was determined in the cellulose in a low-background planchet counter (Nuclear-Chicago). No significant difference was found from the liquid scintillation value reported in the table.

adding 5 μl of the enzyme solution. After incubation for 30 min at 37°, the reaction was stopped with 15 μl of concentrated ammonium hydroxide, and the entire mixture was spotted on the origin of a 2.5 × 57 cm strip of DEAE-cellulose paper. Descending chromatography was then carried out in 0.3 M ammonium formate. Under these conditions the excess of mononucleotides are completely separated from the polyribonucleotides that remain at the origin. The origins of the chromatograms were then cut out and incubated in 0.5 ml of 0.8 M NaOH at room temperature for 15 hr in order to hydrolyze the polyribonucleotides to mononucleotides. The paper was extracted with small portions of water, and the extract was neutralized by the addition of Dowex 50 resin in the pyridine form. After concentration, the neutralized solution was spotted on Whatman 3MM paper, together with 2′,3′-AMP and uridine 2′-(3′)-monophosphate as carriers. These were separated by electrophoresis at pH 2.7 (0.05 M ammonium formate buffer) for 20 min at 200 v/cm. The AMP, which remained near the origin was eluted from the paper with 2% ammonium hydroxide and was deposited on Whatman 1 paper, together with carrier 2′,3′-UMP. Descending chromatography was carried out in the system isobutyric acid–ammonium hydroxide–water (66:2:32) for 18 hr, which also gave a good separation of AMP and UMP. The AMP was again eluted and subjected to a second electrophoresis at pH 7.1 (0.05 M phosphate buffer) for 20 min at 150 v/cm. After this, the AMP was eluted with water, and the solution was dissolved in a scintillation liquid (295 g of naphthalene, 18.4 g of diphenyloxazole, 0.184 g of α-naphthylphenyloxazole, 1400 ml of xylene, 1400 ml of dioxane, and 840 ml of absolute ethanol), and the ^{3}H and ^{32}P radioactivities were determined simultaneously in the Packard Tri-Carb spectrometer.

Results

The experiment shown in Figure 2 was carried out to determine the incorporation of labeled ATP, UTP, and GTP into acid-insoluble polyribonucleotides in the absence of added DNA and in the presence of two levels of *E. coli* DNA primer. A reasonably linear incorporation was found with increasing quantities of template, and extrapolation of the points without the addition of template suggests that not more than 0.002 μg of DNA was present in the enzyme preparation. However, in the case of ATP a considerably higher amount of incorporation was consistently observed, which indicates that there was some poly-A polymerase activity in the enzyme preparation.

This was further investigated in the experiment shown in Figure 3. Here, the time course of the incorporation of labeled ATP and CTP was measured in the absence of added DNA and in the presence of 5 μg of *E. coli* DNA. Comparable amounts of ATP and CTP were incorporated into polyribonucleotides in the presence of the primer, with a linear incorporation for 15 min followed by a diminution in rate. On the other hand, in the absence of DNA, there was essentially no incorporation of the nucleotides until about 30 min when a small, but significant amount of ATP was incorporated, which probably represents the formation of poly-A. Consequently in the subsequent experiments the incubations were carried out for 30 min

to minimize any contribution of poly-A formation to the results.

The main experiments, using the synthetic d(TG:AC) DNA, to determine the possible frequency of FU incorporation into polyribonucleotides in place of C are summarized in Table II. In every case direct comparisons were made of the incorporation of the isotope from [α-^{32}P]FUTP and [α-^{32}P]UTP under identical conditions into the AMP obtained from the alkaline hydrolysis of the acid-insoluble polyribonucleotides, as shown in the scheme in Figure 1. It can be seen that in every experiment there was some incorporation of FUTP into positions adjacent to A in the polynucleotide, but at a frequency that varied from 0.024 to 0.65%. However, this very low frequency of copying "errors" was not unique to FUTP, because in most cases slightly more errors were made in the case of UTP. The apparently higher percentage transfer of ^{32}P to AMP observed with FUTP in expt 4 is not significant because of the very low radioactivities resulting from the extensive decay of the ^{32}P during the course of these experiments.

Discussion

The main conclusion that can be drawn from these experiments is that in the system studied, using a synthetic DNA template and purified *E. coli* RNA polymerase, the frequency of the incorporation of fluorouracil into RNA during the transcription process is less than 0.03%, which means that less than 1 in 3000 cytosines in the RNA are replaced by FU. It has not been possible to push the system to any higher resolution than this because, although the small amount of poly-A polymerase in the enzyme preparation does not significantly affect these results, the equivalent incorporation of UTP and FUTP into the polyribonucleotides limits the resolution of the system. This very low level of incorporation of UTP and FUTP, rather than reflecting errors in base pairing, is more probably a consequence of the minute amount of DNA that is still present in the purified RNA polymerase preparation, as shown in Figure 2. However, very comparable results were obtained in a similar experiment using a highly purified RNA polymerase preparation from *Azotobacter vinelandii* generously provided by Dr. Robert Warner of New York University. Hence, it does not seem possible to obtain a higher degree of resolution in a system of this sort, and the question of the errors of base pairing of FU during transcription remains open.

The proposition that the shutoff of enzyme induction and the mutants found in tobacco mosaic and polio virus is a consequence of the incorporation of FU into RNA in place of C still remains an attractive one. It is impossible to decide at present among the three alternatives: (1) the hypothesis is wrong; (2) a frequency of error less than 1 in 3000 could account for the biological results; or (3) this *in vitro* system is not strictly analogous to the process of transcription and viral replication in intact cells.

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References

Brenner, S., Stretton, A. O. W., and Kaplan, S. (1965), *Nature 206*, 994.

Bussard, A., Naono, S., Gros, F., and Monod, J. (1960), *Compt. Rend. Acad. Sci. 250*, 4049.

Chamberlin, M., and Berg, P. (1962), *Proc. Natl. Acad. Sci. U. S., 48*, 83.

Champe, S. P., and Benzer, S. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 532.

Chaudhuri, N. K., Montag, B. J., and Heidelberger, C. (1958), *Cancer Res. 18*, 318.

Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1004.

Cooper, P. D. (1964), *Virology 22*, 186.

Duschinsky, R., Pleven, E., and Heidelberger, C. (1957), *J. Am. Chem. Soc. 79*, 4559.

Furlong, N. B., Williams, N. L., and Willis, D. P. (1965), *Biochim. Biophys. Acta 103*, 341.

Gordon, M. P., and Staehelin, M. (1959), *Biochim. Biophys. Acta 36*, 351.

Grunberg-Manago, M., and Michelson, A. M. (1964), *Biochim. Biophys. Acta 87*, 593.

Harbers, E., and Heidelberger, C. (1959), *Biochim. Biophys. Acta 35*, 381.

Hartmann, K-U., and Heidelberger, C. (1961), *J. Biol. Chem. 236*, 3006.

Heidelberger, C. (1965), *Progr. Nucleic Acid Res. Mol. Biol. 4*, 1.

Heidelberger, C., Harbers, E., Leibman, K. C., Takagi, Y., and Potter, V. R. (1956), *Biochim. Biophys. Acta 20*, 445.

Hoard, D. E., and Ott, G. (1965), *J. Am. Chem. Soc. 87*, 1785.

Horowitz, J., and Chargaff, E. (1959), *Nature 184*, 1213.

Horowitz, J., Saukkonen, J. J., and Chargaff, E. (1960), *J. Biol. Chem. 235*, 3266.

Houlabek, V. (1962), *J. Mol. Biol. 6*, 164.

Josse, J., Kaiser, A. D., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 864.

Kramer, G., Wittmann, H. G., and Schuster, H. (1964), *Z. Naturforsch. 19b*, 46.

Munyon, W., and Salzman, N. P. (1962), *Virology 18*, 95.

Nakada, D., and Magasanik, B. (1964), *J. Mol. Biol. 8*, 105.

Naono, S., and Gros, F. (1960), *Compt. Rend. Acad. Sci. 250*, 3889.

Pfitzner, K. E., and Moffatt, J. G. (1964), *Biochem. Biophys. Res. Comm. 17*, 146.

Remy, D. C., Sunthankar, A. V., and Heidelberger, C.

(1961), *J. Org. Chem. 27*, 2491.
Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. 1*, 14.
Rosen, B. (1965), *J. Mol. Biol. 11*, 845.
Sutic, D., and Djordjevic, B. (1964), *Nature 203*, 434.
Tener, G. M. (1961), *J. Am. Chem. Soc. 83*, 159.
Wahba, A. J., Gardner, R. S., Basilio, C., Miller, R. S., Speyer, J. F., and Lengyel, P. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 116.
Wells, R. D., Ohtsuka, E., and Khorana, H. G. (1965), *J. Mol. Biol. 14*, 222.
Wittmann, H. G. (1964), *Z. Vererbungslehre 95*, 333.

Inhibition of Protein Synthesis by Amicetin, a Nucleoside Antibiotic*

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ABSTRACT: Amicetin is a nucleoside antibiotic in which an aminoacyl residue is attached to the amino group of cytosine, rather than to the carbohydrate moiety as is the case in the other known aminoacyl antibiotics, all of which have been shown to interfere with protein synthesis. At concentrations of amicetin which in cultures of *Escherichia coli* K12 completely prevented protein synthesis, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis continued. In a cell-free *E. coli* system, the antibiotic interfered with the polyuridylate-directed incorporation of L-phenylalanine into polyphenylalanine. Amicetin did not significantly prevent the formation of phenylalanyl-RNA, but interfered with the transfer of the soluble ribonucleic acid (s-RNA) bound amino acid to the polypeptide. These observations show that the attachment of the aminoacyl residue to the heterocyclic moiety of a nucleoside instead of to the carbohydrate portion can result in a molecule capable of interfering with protein synthesis.

A number of recently discovered antibiotics contain one or more amino acids attached to the carbohydrate moiety of a nucleoside (Fox *et al.*, 1966). The aglycone may be adenine, as in puromycin and homocitrullylaminoadenosine, or it may be cytosine, as in gougerotin and Blasticidin S. In the adenine-containing antibiotics the amino acids are attached to a 3-amino-3-deoxy-β-D-ribofuranosyl moiety (Figure 1a,b). When cytosine is the base, the amino acids are linked to a 4-amino-4-deoxyhexopyranose moiety of the D configuration (Figure 1c,d). Despite these differences, all of these antibiotics have been shown to interfere with protein synthesis (Yarmolinsky and De la Haba, 1959; Guarino *et al.*, 1963; Clark and Gunther, 1963; Nathans, 1964; Yamaguchi *et al.*, 1965).

In 1953, the antibiotic amicetin was first isolated from *Streptomyces fasciculatus* (McCormick and Hoehn, 1953) and from *Streptomyces vinaceus drappus* (DeBoer *et al.*, 1953; Caron and DeBoer, 1953). Two additional antibiotics, isolated in 1955 and named allomycin and sacromycin, were subsequently found to be identical with amicetin (Tatsuoka *et al.*, 1955; Hinuma *et al.*, 1955). The antibiotic was also found in a culture broth of *Streptomyces plicatus*, where it occurs together with two other structurally related aminoacyl nucleosides (Haskell *et al.*, 1958; Haskell, 1958). The structure of amicetin is shown in Figure 1e (Flynn *et al.*, 1953; Stevens *et al.*, 1956, 1962; Hanessian and Haskell, 1964). The α-methylseryl-*p*-aminobenzoyl residue is attached to the amino group of cytosine, rather than to the carbohydrate moiety, which consists of two hexopyranose units. Thus, amicetin differs structurally from the other known aminoacyl nucleoside antibiotics.

Early studies showed amicetin to be a strong inhibitor of mycobacteria both *in vitro* and *in vivo* (DeBoer *et al.*, 1953; Tatsuoka *et al.*, 1955). It also inhibited a number of gram-positive organisms (DeBoer *et al.*, 1953), and demonstrated intermediate activity against the KB strain of human epidermoid carcinoma cells (Smith *et al.*, 1959). Amicetin prolonged the survival time of mice with leukemia-82 (Burchenal *et al.*, 1954), but was inactive against acute leukemia in children who had previously developed resistance to methotrexate, 6-mercaptopurine, and steroids (Tan and Burchenal, 1956).

Little information concerning the mode of action of amicetin is available. Like a number of other structurally unrelated antibiotics, amicetin interfered with the resynthesis of M protein which had been removed with trypsin from a strain of *Streptococcus pyogenes* group A (Brock, 1963). Since the same concentration

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