Effects of 5-Fluorouracil and 6-Azauracil on the Synthesis of Ribonucleic Acid and Protein in *Saccharomyces carlsbergensis*

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1. Some effects of 6-azauracil and 5-fluorouracil on protein and RNA synthesis in *Saccharomyces carlsbergensis* were studied. 2. Both analogues caused a severe inhibition of RNA formation, whereas protein synthesis was much less affected. 3. Induced α -glucosidase formation was only slightly impaired. 4. Both analogues caused an inhibition of ribosome formation, although 5-fluorouracil was far more effective. 5. In the presence of the latter analogue abnormal RNA of high molecular weight and of more DNA-like base composition accumulated. On reincubation in medium free of analogue but containing uracil the abnormal RNA disappeared and was replaced by the normally sedimenting high-molecular-weight RNA species.

5-Fluorouracil is known to cause a multiplicity of effects in micro-organisms (Heidelberger, 1965). The analogue is incorporated into RNA, where it may replace about 50% of the uracil (Horowitz & Chargaff, 1959). An inhibition of RNA synthesis has been observed at relatively high concentrations (Horowitz, Saukkonen & Chargaff, 1960), higher than those required for the cessation of DNA synthesis. The latter phenomenon is the result of the inhibition of thymidylate synthetase by fluorodeoxyuridylic acid (Cohen, Flaks, Barner, Loeb & Lichtenstein, 1958). A major effect of the analogue is a severe inhibition of the formation of ribosomes (Aronson, 1961; Kono & Osawa, 1964; Nakada, 1965), though the cells continue to form small amounts of ribosomal RNA with a normal sedimentation pattern and base composition when the values of uracil and 5-fluorouracil are taken together (Andoh & Chargaff, 1965). A definite explanation of this phenomenon has never been given.

Other well-known effects, such as interference with induced enzyme formation (Horowitz *et al.* 1960; Jacob & Monod, 1961; Nakada & Magasanik, 1964), suppression of certain phage mutations (Champe & Benzer, 1962) and phenotypic repair of nonsense mutations in alkaline phosphatase in *Escherichia coli* (Garen & Siddiqi, 1962), have all been explained to be caused by the incorporation of 5-fluorouracil into messenger RNA resulting in miscoding of the amino acid sequence of the proteins. However, though the analogue is extensively incorporated into the RNA of a number of viruses, only very little interference with the infectivity of these viruses

and their progeny has been found (Gordon & Staehelin, 1959).

Effects of 5-fluorouracil on yeast RNA and protein metabolism have been described by Kempner (1961) and Kempner & Miller (1963). These authors observed that, though the composition of the amino acid pool is drastically changed, the amino acid composition of the newly synthesized protein remains unaltered. As in bacteria the formation of ribosomes was found to be strongly affected.

6-Azauracil is not incorporated into RNA (for review see Skoda, 1963), though it is converted into 6-azauridine and 6-azauridylic acid. The latter compound acts as an inhibitor of orotidylate decarboxylase (Handschemacher & Pasternak, 1958). This interference with pyrimidine nucleotide biosynthesis is probably the main reason for the bacteriostatic activity of the analogue.

The present paper describes the results of some experiments on the effect of the analogues on RNA and protein synthesis in yeast. de Kloet & Strijkert (1966) have previously reported that 5-fluorouracil causes a severe inhibition of ribosomal RNA formation and the accumulation of a heterogeneous high-molecular-weight RNA and transfer RNA, both containing up to 20% of the analogue. A comparison of the effects of 5-fluorouracil and 6-azauracil has now been made. The results show that, though both analogues inhibit preferentially the formation of ribosomes, only 5-fluorouracil causes the accumulation of abnormally sedimenting RNA.

MATERIALS AND METHODS

Chemicals. 6-Azauracil was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. 5-Fluorouracil

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was obtained from Serva, Heidelberg, Germany. Cycloheximide was a product of Koch-Light Laboratories Ltd., Colnbrook, Bucks.

[8-¹⁴C]Adenine (specific activity 20.0 mc/m-mole), [U-¹⁴C]lysine (specific activity 180 mc/m-mole) and carrier-free [³²P]orthophosphate were purchased from The Radiochemical Centre, Amersham, Bucks. 5-Fluoro[2-¹⁴C]uracil (specific activity 20.0 mc/m-mole) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. [1-¹⁴C]Leucine (specific activity 3.8 mc/m-mole) was a product of the Commission de l'Energie Atomique, France.

Yeast strain and cultivation. *Saccharomyces carlsbergensis* (N.C.T.C. 74) was grown and the cells were converted into protoplasts with snail enzyme as described by de Kloet, van Wermskerken & Koningsberger (1961). The protoplasts were used on the day of preparation.

Preparation and sucrose-density-gradient centrifugation of high-molecular-weight RNA and sub-ribosomal particles. High-molecular-weight RNA was prepared from protoplasts with sodium dodecyl sulphate and phenol as described previously (de Kloet, 1966). Sub-ribosomal particles were prepared and subjected to sucrose-density-gradient centrifugation as described in the same paper.

Measurement of the incorporation of ¹⁴C-labelled amino acids and bases into protein and nucleic acid. Protein biosynthesis was estimated as the hot-trichloroacetic acid-insoluble radioactivity after incubation of cells or protoplasts with the ¹⁴C-labelled precursors in media as described in the legends to the Figures and the Tables. Nucleic acid formation was measured similarly but as cold-trichloroacetic acid-insoluble material (de Kloet, 1966).

Determination of induced α -glucosidase synthesis. The induced formation of α -glucosidase by yeast protoplasts was determined as described previously (de Kloet *et al.* 1961; Halvorson & Elias, 1958). Maltose was used as the inducer and *p*-nitrophenyl α -D-glucoside as the chromogenic substrate. Units are expressed as μ moles of *p*-nitrophenol released/min. at 30°.

Base analysis of newly formed RNA. The base composition of newly synthesized RNA was determined from the radioactivity of the nucleotides obtained by alkaline hydrolysis of ³²P-labelled RNA (de Kloet, 1966). High-molecular-weight RNA was isolated by centrifugation through a sucrose density gradient. Fractions containing RNA sedimenting with *S* greater than 12s were pooled and the RNA was precipitated by the addition of 2 vol. of cold ethanol.

RESULTS

Time-course and concentration-dependence of the effects of 5-fluorouracil and 6-azauracil on RNA and protein formation. Fig. 1 shows the results of a study on the concentration-dependence of the effects of the analogues on RNA synthesis. The experiments demonstrate that at fairly low concentration (30 μ g./ml.) the incorporation of [8-¹⁴C]-adenine into RNA is severely affected. Increasing the concentration of the analogues caused little more effect on the total amount of incorporated radioactivity. The results of a study on the time-course of the effect of a high concentration of the analogues are shown in Fig. 2(a). It is clear that the analogues cause both a rapid and severe inhibition

Fig. 1. Effect of different concentrations of 5-fluorouracil and 5-azauracil on RNA formation in yeast. Yeast cells (*E*₅₀₀ 1.0) were incubated at 30° in 1 ml. of a medium containing (per ml.): 10 mg. of glucose, 2 mg. of casein hydrolysate, 20 μ moles of KH_2PO_4 - Na_2HPO_4 buffer, pH 6.2, 2 μ moles of MgCl_2 , 30 μ g. of [8-¹⁴C]adenine (5 mc/m-mole) and different amounts of analogue. After 60 min. the incubation was terminated by the addition of 1 ml. of cold 10% trichloroacetic acid and the cold-trichloroacetic acid-insoluble radioactivity was measured. Values are expressed as percentages of the incorporation of samples incubated in the absence of the analogues. O, In the presence of 5-fluorouracil; ×, in the presence of 6-azauracil.

of RNA synthesis. This effect is observed in the presence as well as in the absence of amino acids in the medium and differs in this respect from the effect of cycloheximide on RNA formation in yeast. In the latter case the ultimate rate of RNA synthesis is independent of the amino acid concentration of the medium (de Kloet, 1966).

Quantitatively in a medium containing casein hydrolysate ('casamino acids'; Difco), 5-fluorouracil and 6-azauracil were found to be more inhibitory to RNA formation than cycloheximide was. The simultaneous addition of cycloheximide and 5-fluorouracil resulted in values for RNA formation only slightly higher than when 5-fluorouracil was present in the medium alone.

The experiments described above show that the analogues interfere in a severe way with the formation of RNA. Since this might in fact be the result of an effect on DNA metabolism, the effect of 5-fluorodeoxyuridine was investigated. The latter compound was found to be far less active than 5-fluorouracil. Also, the simultaneous addition of thymidine and 5-fluorouracil did not abolish the effect of the analogue. These observations make it unlikely that the inhibition of RNA formation is

Fig. 2. (a) Time-course of the effect of 5-fluorouracil and 6-azauracil on RNA synthesis in yeast. Yeast cells were incubated in 25 ml. of a medium as described in Fig. 1 in the presence or absence of 5-fluorouracil, 6-azauracil or cycloheximide. Samples (1.5 ml.) were withdrawn at the times indicated and pipetted into 1.5 ml. of cold 10% trichloroacetic acid. Values are expressed as total cold-trichloroacetic acid-insoluble radioactivity incorporated by the samples. ×, Without addition; o, in the presence of cycloheximide (20 µg./ml.); ●, in the presence of 5-fluorouracil (100 µg./ml.); Δ, in the presence of 6-azauracil (100 µg./ml.). (b) Effect of 5-fluorouracil and 6-azauracil on the time-course of [1-¹⁴C]leucine incorporation into protein by yeast cells. Yeast cells were incubated as described in (a), except that [8-¹⁴C]adenine was replaced by [1-¹⁴C]leucine (16 µg./ml.). Values are expressed as total hot-trichloroacetic acid-insoluble radioactivity present in the samples. ×, Without addition; Δ, in the presence of 5-fluorouracil (100 µg./ml.); o, in the presence of 6-azauracil (100 µg./ml.).

connected in some way with an interference with DNA synthesis.

Previous studies (de Kloet & Strijkert, 1966) have demonstrated that protein synthesis in *S. carlsbergensis* is much less affected by 5-fluorouracil than is RNA formation, in agreement with observations on other organisms (Kempner & Miller, 1963; Horowitz *et al.* 1960). The results in Fig. 2(b) show that a similar effect is observed with 6-azauracil, the latter being a little more inhibitory than 5-fluorouracil. Studies on the concentration-dependence of the effect of the analogues on protein synthesis showed, as had been found for RNA synthesis, that low concentrations of the analogues caused a near-maximum inhibition, higher concentrations having little more effect.

Effect of 5-fluorouracil and 6-azauracil on the induced formation of α-glucosidase in yeast protoplasts. It has been shown that in bacteria 5-fluorouracil interferes with the formation of induced enzymes (Horowitz *et al.* 1960; Jacob & Monod, 1961). Fig. 3 shows the results of some experiments on the effect of the analogues on the induced formation of α-glucosidase in yeast protoplasts. The results demonstrate that the formation of the

enzyme is only weakly affected, even at the very high concentrations of the analogues (100 µg./ml.) employed in these experiments. A further investigation of the enzyme formed in the presence of 5-fluorouracil revealed that it had an unaltered heat-denaturation curve as well as pH optimum and electrophoretic pattern. α-Glucosidase synthesis is not unique in its response to 5-fluorouracil, since the induced formation of β-galactosidase in *Saccharomyces fragilis* is also not affected by the analogue (P. J. Strijkert, personal communication). These observations lead to the tentative conclusion that at least some induced enzymes formed in yeast in the presence of 5-fluorouracil are not essentially different from the native enzymes, despite the replacement of about 70% of the uracil residues in the newly synthesized RNA by the fluorinated derivative (de Kloet & Strijkert, 1966; Table 1). The results in Fig. 3 show also that a 60 min. preincubation of the protoplasts with 5-fluorouracil before the addition of the inducer does not influence the results greatly.

Effect of 5-fluorouracil and 6-azauracil on the formation of ribosomal sub-units. Although 5-fluorouracil and 6-azauracil do not seem to affect

Fig. 3. Effect of 5-fluorouracil and 6-azauracil on induced α -glucosidase formation by yeast protoplast. Yeast protoplasts (E_{500} 1.0) were incubated at 30° in 35 ml. of a medium containing (per ml.): 30 mg. of maltose, 5 mg. of glucose, 2 mg. of casein hydrolysate, 20 μ moles of KH_2PO_4 - Na_2HPO_4 buffer, pH 6.2, 2 μ moles of MgCl_2 , 120 mg. of mannitol and 5-fluorouracil or 6-azauracil. Samples (1.5 ml.) were withdrawn at the times indicated and cooled to stop enzyme synthesis. Protoplasts were collected by centrifugation in the cold and lysed in 1 ml. of cold distilled water. The lysate was centrifuged for 5 min. to remove heavy cell debris. A 0.2 ml. sample of the supernatant was used for determination of the enzymic activity (de Kloet *et al.* 1961). Values are expressed as total units of activity present in the samples. x, Without addition; ○, in the presence of 5-fluorouracil (100 $\mu\text{g./ml.}$); ●, in the presence of 6-azauracil (100 $\mu\text{g./ml.}$); Δ, after 60 min. preincubation with 5-fluorouracil (100 $\mu\text{g./ml.}$).

total protein synthesis in yeast cells to a very large extent the analogues were found to inhibit specifically the formation of ribosomal sub-units. In the experiment shown in Fig. 4, 5-fluorouracil and 6-azauracil inhibited total protein synthesis by 25 and 35% respectively, whereas the formation of ribosomal sub-units was impaired by 90 and 80% respectively. The observed effect of 5-fluorouracil is in agreement with the results obtained with other micro-organisms (Aronson, 1961; Kono & Osawa, 1964). However, the results in Fig. 4 show also that the incorporation of ^{14}C -labelled amino acids into the protein of the ribosomal sub-units is not completely inhibited by the analogues. Since, however, a number of proteins found in ribosomal preparations might in fact be cytoplasmic contaminants (Neu & Heppel, 1964), the latter observation is difficult to evaluate.

Effect of 5-fluorouracil and 6-azauracil on the sedimentation pattern of newly synthesized RNA.

In a previous paper (de Kloet & Strijkert, 1966) it was shown that the RNA formed in the presence of 5-fluorouracil sediments as heterogeneous material of high molecular weight together with transfer RNA. Since both 5-fluorouracil and 6-azauracil inhibit the formation of ribosomal particles it was decided to investigate also the effect of the latter analogue on the sedimentation pattern of the newly synthesized RNA. The results in Fig. 5 show, however, that RNA formed in the presence of 6-azauracil sediments as the normal stable RNA components of the cell. This is also illustrated by Fig. 6, where the effect of the analogues on the incorporation of $[8\text{-}^{14}\text{C}]\text{adenine}$ into sub-ribosomal particles was investigated. The data show that RNA formed in the presence of 5-fluorouracil is not incorporated into mature particles whereas that synthesized in the presence of 6-azauracil is.

Quantitatively the analogues cause different degrees of inhibition of the high- and low-molecular-weight RNA species. Whereas 6-azauracil causes about the same degree of inhibition of the synthesis of both types of RNA, 5-fluorouracil impairs the formation of high-molecular-weight RNA to a greater extent than low-molecular-weight RNA (Fig. 5). (Since the data in Fig. 6 are the results of different experiments these should not be compared on a quantitative basis.)

Base composition of RNA synthesized in the presence of 5-fluorouracil and 6-azauracil. Previous studies (de Kloet & Strijkert, 1966) with $[32\text{P}]\text{-orthophosphate}$ have shown that in the presence of 5-fluorouracil only transfer RNA and high-molecular-weight RNA with a relatively high content of adenylic acid and uridylic acid are formed. The experiments demonstrated that in both classes of RNA as much as 70% of the uridylic acid residues may be replaced by the fluorinated analogue. In the Dowex-formic acid system employed in these studies for the base analysis, 5-fluorouridylic acid is eluted after uridylic acid (Horowitz & Chargaff, 1959) and fractions containing this nucleotide were detected as a routine by monitoring the column eluate. An analysis of yeast RNA labelled with 5-fluoro $[2\text{-}^{14}\text{C}]\text{uracil}$ revealed that the radioactivity appears essentially as fluoro $[2\text{-}^{14}\text{C}]\text{uridylic acid}$, a finding in agreement with the results of studies on other organisms (Horowitz & Chargaff, 1959). Hence, though yeast can convert uracil into cytosine (approx. 35% of added exogenous $[^{14}\text{C}]\text{uracil}$ is recovered as $[^{14}\text{C}]\text{cytosine}$ from an alkaline digest of RNA), the organism is apparently unable to convert 5-fluorouracil into the corresponding cytosine derivative. Table 1 shows the base composition of high-molecular-weight RNA synthesized in the presence of 6-azauracil and 5-fluorouracil. The data show that even a low concentration of 5-fluorouracil

Fig. 4. Effect of 5-fluorouracil and 6-azauracil on the formation of ribosomal particles in yeast. Yeast protoplasts (E_{500} 1.0) were incubated at 30° in 5 ml. of a medium containing (per ml.): 10 mg. of glucose, 120 mg. of mannitol, 2 mg. of casein hydrolysate, $20 \mu\text{moles}$ of $\text{KH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$ buffer, pH 6.2, $2 \mu\text{moles}$ of MgCl_2 , $1.5 \mu\text{g.}$ of $[\text{U-}^{14}\text{C}]$ -lysine, $160 \mu\text{g.}$ of $[\text{U-}^{14}\text{C}]$ -leucine and 5-fluorouracil or 6-azauracil. After 60 min. the vessels were cooled and the protoplasts were collected by centrifugation in the cold. Preparation of the ribosomal particles and the sucrose-density-gradient centrifugation of the ribosomal sub-units were carried out as described previously (de Kloet, 1966; Henshaw, 1964). The E_{260} was read after sixfold dilution and the radioactivity was measured as described previously (de Kloet, 1966). (a) Without addition; (b) in the presence of 6-azauracil (100 $\mu\text{g./ml.}$); (c) in the presence of 5-fluorouracil (100 $\mu\text{g./ml.}$). —, E_{260} ; $\times$ — $\times$, radioactivity.

(22 $\mu\text{g./ml.}$) causes the replacement of about 70% of the uridylic acid residues by 5-fluorouridylic acid. When equal amounts (100 $\mu\text{g./ml.}$) of both uracil and 5-fluorouracil are present in the medium, about 20% of the uridylic acid is replaced by the analogue. This result is in agreement with that obtained by Kempner & Miller (1963), who also observed a preferential uptake of uracil compared with 5-fluorouracil.

RNA formed in the presence of 6-azauracil has a base composition differing only slightly from that of normal high-molecular-weight RNA. The slight difference indicates that apparently in the presence of this analogue some abnormal RNA having a DNA-like base composition accumulates.

Recovery of RNA synthesis by yeast cells previously incubated with 5-fluorouracil or 6-azauracil. Previous studies have shown that yeast cells recover readily from a treatment with cycloheximide (de Kloet, 1966). Fig. 7 shows that after a preincubation with 6-azauracil the organism resumes its high rate of RNA synthesis within a reasonably short time, when the reincubation medium contains uracil. Omission of the uracil from the reincubation medium results in a strongly diminished capacity of the cells to recover. Cells preincubated with 5-fluorouracil react strikingly differently. Even a prolonged incubation with a high concentration of uracil does not result in a significantly higher rate of RNA formation compared with cells reincubated in a

Fig. 5. Sedimentation pattern of RNA synthesized by yeast protoplasts in the presence of 5-fluorouracil and 6-azauracil. Yeast protoplasts were incubated as described in Fig. 4 except that the medium contained [8- ^{14}C]-adenine (40 $\mu\text{g./ml.}$; 10 mc/m-mole) instead of labelled amino acids. After 60 min. the incubation was terminated by cooling the vessels. The preparation of high-molecular-weight RNA was carried out as described previously (de Kloet, 1966). A 200 $\mu\text{g.}$ sample of the RNA was centrifuged for 4.5 hr. at 37000 rev./min. in the Spinco XW 39.1 rotor through a sucrose gradient composed of 5–20% (w/v) sucrose in 0.05 M-NaCl–2 mM-EDTA–0.02 M-sodium acetate buffer, pH 5.0. Fractions (about 0.25 ml.) were collected after puncturing the bottom of the tube. The E_{260} was read after sixfold dilution and the radioactivity was measured as described previously (de Kloet, 1966). (a) Pattern without analogue; (b) pattern after incubation with 6-azauracil (100 $\mu\text{g./ml.}$); (c) pattern after incubation with 5-fluorouracil (100 $\mu\text{g./ml.}$). —, E_{260} ; $\times$ — $\times$, radioactivity.

medium lacking uracil or containing the analogue. In all these cases the cells continue to accumulate RNA at a low rate. A further study showed that this effect occurs after a short time of preincubation with the analogue (within 10 min.).

Metabolic stability of the RNA formed in the presence of 5-fluorouracil. Earlier studies on the RNA formed in the presence of cycloheximide had shown that this RNA is unstable on removal of the antibiotic and is converted into the normal ribosomal RNA species (de Kloet, 1966). Since the

RNA formed in the presence of 5-fluorouracil is also abnormal and resembles that accumulated in the presence of cycloheximide, the metabolic stability of this RNA was also investigated.

Table 2 shows that yeast cells labelled with fluor[2- ^{14}C]uracil or [8- ^{14}C]adenine lose some cold-trichloroacetic acid-soluble radioactivity on continued incubation in unlabelled medium containing the analogue. Reincubation in medium containing uracil does not result in such a decrease of radioactivity. The observation that about 17% loss of

Fig. 6. Effect of 5-fluorouracil and 6-azauracil on the incorporation of [8-¹⁴C]adenine into the RNA of sub-ribosomal particles of yeast protoplasts. Yeast protoplasts were incubated in a medium as described in Fig. 5. After 60 min. the vessels were cooled and the protoplasts collected by centrifugation in the cold. The preparation of the ribosomal fraction and the sucrose-density-gradient centrifugation of the sub-ribosomal particles were carried out as described previously (de Kloet, 1966). (a) Pattern without addition; (b) pattern after incubation in the presence of 6-azauracil (100 μ g./ml.); (c) pattern after incubation in the presence of 5-fluorouracil (100 μ g./ml.). —, E_{260} ; $\times$ — $\times$, radioactivity.

Table 1. Base composition of yeast RNA synthesized in the presence of 5-fluorouracil and 6-azauracil

Yeast protoplasts (E_{500} 1.0) were incubated for 60 min. at 30° in 25 ml. of a medium containing (per ml.): 10 mg. of glucose, 2 mg. of casein hydrolysate, 120 mg. of mannitol, 0.5 μ mole of KH_2PO_4 – Na_2HPO_4 buffer, pH 6.2, 2 μ moles of MgCl_2 , 0.016 mc of carrier-free [³²P]orthophosphate and 5-fluorouracil (FU) or 6-azauracil (AZAU). High-molecular-weight RNA (HMW RNA) was isolated and separated from low-molecular-weight material as described previously (de Kloet, 1966), as was the determination of the base composition from the radioactivity of the nucleotides in the alkaline hydrolysate. Nucleotides were adsorbed on charcoal before their radioactivity was measured. Values for the base composition of normal high-molecular-weight yeast DNA were taken from a previous paper (de Kloet, 1966). The yeast DNA base composition was obtained from the literature (Zamenhof & Chargaff, 1950). Values are expressed as moles/100 moles.

	Base composition (%)				
	CMP	AMP	GMP	UMP	Fluoro-UMP
Yeast DNA	18	32	18	32*	
Yeast HMW RNA	19.3	26.3	27.7	26.6	
HMW RNA without FU	19.4	26.2	27.7	26.6	
HMW RNA with FU (22 μ g./ml.)	19.3	28.2	24.6	8.2	19.6
HMW RNA with FU (100 μ g./ml.)	18.9	28.9	24.5	8.3	19.4
HMW RNA with FU (200 μ g./ml.)	18.9	29.9	22.7	7.7	20.8
HMW RNA with FU (100 μ g./ml.) and uracil (100 μ g./ml.)	19.9	28.1	26.2	21.4	4.4
HMW RNA with 6-AZAU (100 μ g./ml.)	19.4	27.8	26.4	26.4	

* Thymidylic acid.

Fig. 7. Recovery of RNA synthesis by yeast cells previously incubated in the presence of 5-fluorouracil or 6-azauracil. Yeast cells (*E*₅₀₀ 1-0) were incubated at 30° in 25 ml. of a medium as described in Fig. 1 in the absence of radioactivity but containing 6-azauracil or 5-fluorouracil. After 60 min. the cells were collected on a Millipore filter as described in Table 2, washed and reincubated in 25 ml. of medium containing 30 µg. of [8-¹⁴C]adenine (5 mc/m-mole) in the presence or absence of uracil. Samples were withdrawn at the times indicated. Cold-trichloroacetic acid-insoluble radioactivity was measured as usual. Values are expressed as total radioactivity incorporated by the samples. Δ, Preincubated in the absence of analogue, reincubated with (—) or without (----) uracil; ×, preincubated with 6-azauracil (100 µg./ml.), reincubated with (—) or without (----) uracil; ○, preincubated with 5-fluorouracil (100 µg./ml.), reincubated with (—) or without (----) uracil.

radioactivity occurs during the reincubation with 5-fluorouracil might be fortuitous, but it might also be an indication that RNA synthesized in the presence of the analogue is not a stable end product. Therefore the metabolic stability of the RNA formed in the presence of 5-fluorouracil was studied more closely by fractionation of the cellular RNA and sub-ribosomal particles.

A fractionation of the RNA of yeast cells can be obtained by phenol treatment of whole yeast cells. This procedure is known to release low-molecular-weight RNA (mainly transfer RNA) into the water phase, whereas high-molecular-weight RNA remains in the cells (Monier, Stephenson & Zamecnik, 1960; de Kloet, 1966). Table 3 shows that reincubation of cells labelled with 5-fluoro[2-¹⁴C]uracil in a medium containing unlabelled 5-fluorouracil results in a severe loss of radioactivity from the

Table 2. *Metabolic stability of the RNA synthesized in the presence of 5-fluorouracil*

Yeast cells (*E*₅₀₀ 1-0) were incubated at 30° in 15 ml. of a medium containing (per ml.): 10 mg. of glucose, 2 mg. of casein hydrolysate, 20 µmoles of KH₂PO₄-Na₂HPO₄ buffer, pH 6.2, 2 µmoles of MgCl₂, 50 µg. of 5-fluorouracil, 100 µg. of adenine and 5 µg. of 5-fluoro[2-¹⁴C]uracil ([¹⁴C]FU) (0.0008 mc) or 2.5 µg. of [8-¹⁴C]adenine ([¹⁴C]A) (0.0004 mc). After 60 min. the samples were cooled and the cells collected by filtration on nitrocellulose filters (Millipore HAWP; 0.45 mµ pore size). The cells were washed in the cold with three 16 ml. portions of cold unlabelled medium lacking analogue. Subsequently, the cells were reincubated for 2 hr. in 15 ml. of medium containing 5-fluorouracil (FU) (50 µg./ml.) or uracil (U) (100 µg./ml.). Samples (2 ml.) were taken before and after the reincubation. Cold-trichloroacetic acid-insoluble radioactivity was measured after the usual washing procedure. Values represent the total radioactivity incorporated by the samples.

	Radioactivity (counts/min.)		
	Before reincubation	After reincubation	
		With FU	With U
Cells labelled with [¹⁴ C]FU	1250	1020	1300
Cells labelled with [¹⁴ C]A	725	610	740

Table 3. *Metabolic stability of RNA fractions synthesized in the presence of 5-fluorouracil*

Yeast cells were incubated with fluoro[2-¹⁴C]uracil as described in Table 2. After 60 min. the cells were collected by centrifugation and washed twice with 10 ml. of cold medium lacking analogue. Subsequently the cells were reincubated for 2 hr. in medium lacking radioactivity but containing 5-fluorouracil (FU) or uracil (U) as described in Table 2. The RNA was fractionated as described previously by phenol treatment of whole cells (de Kloet, 1966). Water-phase RNA was precipitated with cold trichloroacetic acid after the addition of 5 mg. of yeast protoplast protein as a carrier. The radioactivity was measured after the usual washing procedure. Samples (2 ml.) were taken before and after the reincubation. Values are expressed as the total radioactivity incorporated by the samples.

	Radioactivity (counts/min.)		
	Before reincubation	After reincubation	
		With FU	With U
Phenol-retained RNA	1198	447	907
Water-phase RNA	365	1098	869

RNA retained by the cells, showing that the high-molecular-weight RNA is unstable under these conditions. Reincubation of the cells in medium

Fig. 8. Metabolic stability of the RNA synthesized by yeast protoplasts in the presence of 5-fluorouracil. Yeast protoplasts were incubated for 60 min. in 15 ml. of a medium as described in Fig. 5 in the presence of 5-fluorouracil (50 µg./ml.) and [8- 14 C]adenine (40 µg./ml.; 10 mC/m-mole). The protoplasts were collected by centrifugation in the cold and washed twice with 25 ml. of cold medium lacking analogue and radioactivity. The protoplasts were then reincubated in 15 ml. of a medium containing 5-fluorouracil or uracil. RNA was prepared and subjected to sucrose-density-gradient centrifugation as described previously (de Kloet, 1966). (a) Pattern before reincubation; (b) pattern after reincubation with 5-fluorouracil (50 µg./ml.); (c) pattern after reincubation with uracil (100 µg./ml.). —, E_{260} ; x — x, radioactivity.

containing uracil also results in a loss of radioactivity from this particular RNA fraction, though this loss is less severe than in the experiment mentioned above. In both cases the RNA found in

the water phase gains radioactivity. Whether this is due to the synthesis of transfer RNA or to the appearance of larger degradation products of the high-molecular-weight RNA is not known. The

fact that the total cold-trichloroacetic acid-insoluble radioactivity accumulated by the cells increases somewhat during reincubation is merely due to the fact that centrifugation is a far less efficient washing procedure than filtration through Millipore filters (compare Table 2).

Fig. 8 shows that the sedimentation pattern of the abnormal RNA formed in the presence of 5-fluorouracil changes to a pattern coinciding more with that of the normal stable cellular RNA species on reincubation in a medium containing uracil, though some radioactivity tended to sediment in front of

the heaviest stable RNA component. A continued incubation in medium containing 5-fluorouracil resulted in a gradual loss of radioactivity. The sedimentation pattern of the high-molecular-weight RNA remained abnormal, indicating that under these conditions no conversion into stable ribosomal RNA takes place. When the same phenomenon was studied by sucrose-density-gradient centrifugation of sub-ribosomal particles the results showed that there indeed occurred a gradual incorporation of 5-fluoro[2- 14 C]uracil or [8- 14 C]adenine into these particles during the reincubation

Fig. 9. Metabolic stability of the RNA synthesized by yeast protoplasts in the presence of 5-fluorouracil. Yeast protoplasts were incubated in 5 ml. of a medium as described in Fig. 8, containing 5-fluorouracil (50 μ g./ml.) and [8- 14 C]adenine (40 μ g./ml.; 5 mc/m-mole). The preparation of the ribosomal fractions and the sucrose-density-gradient centrifugation of the sub-ribosomal particles were carried out as described previously (de Kloet, 1966). (a) Pattern before reincubation; (b) pattern after reincubation in medium containing 5-fluorouracil (50 μ g./ml.); (c) pattern after reincubation in medium containing uracil (100 μ g./ml.); (d) as (c), but the protoplasts were pre-incubated with 5-fluoro[2- 14 C]uracil (10 mc/m-mole) instead of [8- 14 C]adenine. —, E_{260} ; x — x, radioactivity.

with uracil (Fig. 9). A reincubation in medium containing 5-fluorouracil yielded only a small amount of radioactivity in the particles; most radioactivity sedimented as material with *S* less than 40s (Fig. 9b). Fig. 9 shows further that there is more radioactivity incorporated into the 40s particle than into the 60s particle. Similar observations can be made when protoplasts are incubated in a medium containing both 5-fluorouracil and uracil, in which case the newly formed RNA contains only 4.5% of 5-fluorouridylic acid (Table 1 and Fig. 10). This might indicate that the formation of the RNA component of the 40s particle is less sensitive to the analogue than is the formation of the 60s particle. Another possibility is that the RNA sedimenting with the 40s particle is in fact a mixture of the true stable RNA component of this particle and high-molecular-weight non-particulate RNA sedimenting with a similar velocity.

A closer comparison of the data obtained with 5-fluoro[2-¹⁴C]uracil and [8-¹⁴C]adenine (Figs. 9c and 9d) after a 'chase' with uracil revealed that with [8-¹⁴C]-adenine the ratio of the radioactivity incorporated into the 60s and 40s particles exceeds that obtained with 5-fluoro[2-¹⁴C]uracil (0.57 versus 0.26), indicating that the analogue-containing RNA is converted into the more stable RNA species with a simultaneous loss of 5-fluorouracil.

Fig. 10. Sedimentation pattern of yeast RNA formed in the presence of a mixture of 5-fluorouracil and uracil. Yeast protoplasts were incubated in 5 ml. of a medium as described in Fig. 5. The medium contained 5-fluorouracil (100 μ g./ml.) and uracil (100 μ g./ml.). The preparation of the ribosomal fraction and the sucrose-density-gradient centrifugation of the sub-ribosomal particles were carried out as described previously (de Kloet, 1966). —, E_{260} ; x—x, radioactivity.

The latter observation makes it likely that during the conversion the abnormal RNA is extensively degraded and that the degradation products are reutilized for the synthesis of normal stable ribosomal RNA with a simultaneous exclusion of 5-fluorouridylic acid (cf. Iwabuchi, Otaka, Kono & Osawa, 1966) and the incorporation of uridylic acid, which is used preferentially compared with the analogue (Table 1; Kempner, 1961).

DISCUSSION

The present study shows that there are similarities and differences between the effects of 5-fluorouracil and 6-azauracil in yeast. Both analogues cause a substantial inhibition of RNA synthesis, leaving the formation of total protein and of at least some induced enzymes relatively unaffected. Differences occur, however, in the sedimentation pattern of the high-molecular-weight RNA accumulating in the cells during incubation with the analogues. The observation that 5-fluorouracil does not affect the induced formation of α -glucosidase differs sharply from observations on the effect of the analogue on the induced formation of β -galactosidase in *E. coli*. The slightly lower rate of enzyme formation in the presence of 5-fluorouracil compared with the control might well be due to the inhibitory effect of the analogue on ribosome formation, resulting in a diminished cellular capacity for protein formation. This non-sensitivity of enzyme formation to 5-fluorouracil in yeast resembles the absence of an effect of the analogue on the formation of viral-coat protein (Gordon & Staehelin, 1959; Holoubek, 1963) and on enzyme synthesis in plants (Key & Ingle, 1964).

The nature of the miscoding caused by 5-fluorouracil in bacteria is unknown (Heidelberger, 1965; Bujard & Heidelberger, 1966), as is the reason for the smaller effect of the analogue on enzyme formation in yeast and higher organisms. The latter might be related to the finding that mammalian cell-free systems containing 80s ribosomes are less sensitive to factors influencing the correct reading of the genetic code than are bacterial systems containing 70s particles (Weinstein, Ochoa & Friedman, 1966).

In yeast a major effect of 6-azauracil and 5-fluorouracil is a severe inhibition of the rate of RNA formation. Since both analogues interfere in some way with pyrimidine nucleotide biosynthesis (Anderson & Brockman, 1964; Bresnick & Blatchford, 1964; Handschumacher & Pasternak, 1958), this impaired nucleotide formation might well be a primary reason for the lower rate of RNA synthesis. Though both analogues inhibit RNA synthesis to about the same extent, a striking difference occurs particularly in the sedimentation pattern of the

high-molecular-weight RNA formed. Since 6-azauracil is not incorporated into RNA (Skoda, 1963) whereas 5-fluorouracil is, it is likely that the accumulation of abnormal RNA containing the fluorinated analogue is due to the incorporation of the latter. The reason for this effect of 5-fluorouracil is not clear. It might well be that the accumulated RNA is a precursor of ribosomal RNA that cannot be converted into the normal stable RNA species as a result of the synthesis of faulty miscoded proteins. This seems unlikely, since the experiments on the effect of 5-fluorouracil on induced α -glucosidase synthesis suggest that little miscoding occurs in yeast. On the other hand, since polynucleotides containing 5-fluorouracil are known to have physical and chemical properties differing from polymers containing uracil (Grunberg-Manago & Michelson, 1964; Sueoka & Yamane, 1963), the introduction of the analogue into a precursor of ribosomal RNA might alter the properties of this precursor in such a way that it can no longer be converted into ribosomal RNA.

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