Ribonucleic Acid Synthesis in Yeast

THE EFFECT OF CYCLOHEXIMIDE ON THE SYNTHESIS OF RIBONUCLEIC ACID IN *SACCHAROMYCES CARLSBERGENSIS*

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1. Cycloheximide causes the release of the control amino acids have over RNA synthesis in *Saccharomyces carlsbergensis* N.C.T.C. 74. 2. The antibiotic causes a gradual deceleration of RNA formation. After incubation for 60 min. at 30° RNA synthesis usually proceeds at a rate only a few per cent of that of the untreated control. 3. In the presence of cycloheximide two types of RNA accumulate in the cell: soluble RNA and a high-molecular-weight RNA. The latter has a base composition intermediate between those of yeast DNA and yeast ribosomal RNA, and sediments in a sucrose gradient at a rate faster than that of the 23S ribosomal RNA component. 4. Yeast ribosomal RNA contains methylated bases. Judged from the incorporation of [*Me*-¹⁴C]methionine, the extent of methylation of ribosomal RNA is about 20% of that of the 'soluble' RNA fraction. The high-molecular-weight RNA formed in the presence of cycloheximide is less methylated than normal RNA. In this case the sucrose-density-gradient sedimentation patterns of newly methylated and newly synthesized RNA do not coincide. 5. In the presence of cycloheximide, polysomal material accumulates, indicating that messenger RNA is formed. 6. The effect of the antibiotic on protein and RNA synthesis can be abolished by washing of the cells. The RNA that has accumulated during incubation of the cells with the antibiotic is not stable on removal of cycloheximide. 7. The results presented in this study are discussed in relation to the regulation of RNA formation in yeast.

Cycloheximide, an antifungal antibiotic, was first isolated by Whiffen, Bohonas & Emerson (1946) from *Streptomyces griseus*. Cycloheximide is toxic to a broad range of organisms. Thus growth is inhibited in fungi (Whiffen *et al.* 1946), algae, higher plants (Ford, Klomparens & Hamm, 1958) and

Cycloheximide

mammalian cells (Ford & Leach, 1948; Wettstein, Noll & Penman, 1964; Williamson & Schweet, 1965). Bacteria have been found to be insensitive to the antibiotic. Several reports have been made on the activity of cycloheximide on yeasts (Siegel & Sisler, 1964; de Kloet, 1965*a,b*). There are no indications that cycloheximide interferes with respiration or fermentation. Siegel & Sisler (1964)

found that the drug affects the concentration of a number of metabolites; in particular, nucleotides accumulated. The authors concluded that the main action of cycloheximide is an inhibition of protein biosynthesis. The transfer of amino acids from the s-RNA* to the ribosomes was considered to be the primary site of action of the antibiotic. Other effects are probably a secondary result of this interference with protein biosynthesis.

Bennett, Smithers & Ward (1964) found that cycloheximide inhibits DNA synthesis in mammalian cells. Kerridge (1958) reported an inhibition of DNA formation and a smaller effect of the antibiotic on RNA synthesis in yeast. Siegel & Sisler (1964), using *Saccharomyces pastorianus*, observed a stimulatory action of the drug on RNA synthesis.

In bacteria chloramphenicol is a powerful inhibitor of protein biosynthesis, yet RNA synthesis is allowed to continue for a considerable time. Although the RNA synthesized in the presence of

* Abbreviations: s-RNA, 'soluble' or transfer RNA; m-RNA, messenger RNA.

chloramphenicol resembles ribosomal RNA in its base composition (Pardee, Paegin & Prestidge, 1957) and sedimentation properties (Kurland, Nomura & Watson, 1962), Gros, Naono, Woese, Willson & Attardi (1963) and Otaka, Osawa & Sibatani (1964) observed that the RNA formed in the presence of chloramphenicol could stimulate protein synthesis in a cell-free system. Gordon, Boman & Isaksson (1964) observed that this RNA could be methylated with a homologous methylating enzyme preparation, in contrast with normal ribosomal RNA. This indicates that the RNA synthesized in the presence of chloramphenicol is not as normal as had been considered. Further, chloramphenicol tends to release the control that amino acids exert over RNA synthesis (Kurland & Maaløe, 1962). In a previous communication (de Kloet, 1965a) it was found that in yeast cycloheximide causes the accumulation of RNA with an abnormal sedimentation pattern and a base composition intermediate between those of DNA and ribosomal RNA. The present investigation was carried out to study more precisely the action of cycloheximide on RNA synthesis in yeast. The results indicate that there are similarities and differences between the action of cycloheximide and chloramphenicol on RNA synthesis in yeast and *Escherichia coli* respectively.

MATERIALS AND METHODS

Materials. Cycloheximide was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The ^{14}C -labelled compounds and ^{32}P orthophosphate were products of The Radiochemical Centre, Amersham, Bucks. The snail enzyme used in this study was purchased from the Industrie Biologique Française, Gennevilliers, Seine, France. The preparation was centrifuged for 20 min. at 25 000g to remove some contaminating material.

Yeast strain. *Saccharomyces carlsbergensis* strain N.C.T.C. 74 was grown as described by de Kloet, van Wermeskerken & Koningsberger (1961). Protoplasts were made as described in the same paper.

Incorporation of ^{14}C -labelled compounds into RNA of whole cells. The conditions for the incorporation of ^{14}C -labelled precursors are described in the legends to the Figures. When ^{14}C uracil incorporation was studied the incubation medium always contained 12 μg . of each of the four RNA bases/ml. to prevent a premature exhaustion of the medium, which would cause a non-linear incorporation of radioactivity into RNA. Yeast cells and protoplasts were preincubated in unlabelled medium for at least 20 min. to obtain a steady rate of incorporation of ^{14}C uracil. All experiments done with protoplasts always contained in addition 120 mg. of mannitol/ml. as an osmotic stabilizer. Radioactivity was estimated after precipitating and washing of the samples with cold 5% (w/v) trichloroacetic acid, resuspending the precipitate in 0.5 ml. of M-sodium acetate buffer, pH 5.2, and precipitating and washing with ethanol, ether-chloroform-ethanol (1:1:2, by vol.) and acetone. The addition of sodium acetate was necessary

to prevent losses of RNA during washing with lipid-dissolving agents. The samples were plated on stainless-steel planchets and counted with a Philips Geiger-Müller counter at about 3% efficiency.

Preparation and sucrose-density-gradient centrifugation of yeast RNA. RNA for sucrose-density-gradient sedimentation analysis was prepared as follows. Protoplasts (from 25 ml. of medium containing approx. 12 mg. of protein) were collected by centrifugation in the cold and lysed by resuspending in 4 ml. of a solution containing (per ml.) 2 μmoles of EDTA (disodium salt), 20 μmoles of tris-HCl buffer, pH 7.6, 100 μmoles of NaCl and 10 mg. of sodium lauryl sulphate. Then 4 ml. of 90% (w/v) phenol was added to the slightly turbid lysate and the mixture was shaken gently at 37° in a Gyrotary water-bath shaker for about 60 min. The phenol layer and the water layer were separated by centrifugation for 20 min. at 3000g. The water layer was pipetted off and shaken a second time with phenol. M-sodium acetate was added to give a final concentration of 0.1 M and the RNA was precipitated by the addition of 2 vol. of ethanol at -20°. After standing at -20° for 30 min., the RNA was collected by centrifugation, redissolved in 2 ml. of a solution containing (per ml.) 100 μmoles of NaCl, 2 μmoles of EDTA (disodium salt) and 20 μmoles of sodium acetate buffer, pH 5.0, and again precipitated by the addition of ethanol. Finally the RNA was dissolved again in 2 ml. of the solvent mentioned above and a 1 ml. sample containing approx. 2 mg. of RNA was layered on top of 29 ml. of linear 20-5% (w/v) sucrose gradient containing (per ml.) 2 μmoles of EDTA (disodium salt), 100 μmoles of NaCl and 20 μmoles of sodium acetate buffer, pH 5.2. Centrifugation was carried out for 16 hr. at 24 000 rev./min. (60 000g) in the SW 25.1 rotor of the Spinco L1 centrifuge at 2°. The rotor was stopped without using the brake. Fractions were collected after puncturing the bottom of the tube. A 0.1 ml. sample was diluted 30-fold for the estimation of $E_{260\text{ m}\mu}$. To the remainder of the fraction 2 mg. of protoplast protein was added as a carrier. Protein and RNA were precipitated by the addition of 5 ml. of cold 5% (w/v) trichloroacetic acid. After washing the samples as described above the radioactivity was measured.

Preparation and sucrose-density-gradient centrifugation of polysomes, ribosomes and ribosomal sub-units. Sucrose-density-gradient centrifugation of ribosomes and ribosomal aggregates was carried out as follows. Protoplasts that had incorporated ^{14}C uracil for an appropriate time were collected by centrifugation and lysed in 0.5 ml. of a buffer containing (per ml.) 10 μmoles of magnesium acetate, 10 μmoles of mercaptoethanol, 50 μmoles of KCl and 20 μmoles of tris-HCl buffer, pH 7.6. Then 0.05 ml. of 10% (w/v) sodium deoxycholate solution was added and 0.2 ml. of the lysate was layered on top of 5 ml. of linear 20-5% (w/v) sucrose gradient containing (per ml.) 10 μmoles of MgCl_2 , 50 μmoles of KCl and 20 μmoles of tris-HCl buffer, pH 7.6. Centrifugation was carried out for 60 min. at 37 000 rev./min. (120 000g) in the SW 39 rotor of the Spinco model L1 preparative ultracentrifuge at 2°. Fractions were again collected after puncturing the bottom of the tube, $E_{260\text{ m}\mu}$ was measured after fivefold dilution and radioactivity of the samples was again estimated after the addition of 2 mg. of carrier yeast protoplast protein and washing as described above.

Ribosomal particles were isolated from deoxycholate-lysed protoplasts by differential centrifugation. After

removal of the heavier cell parts by spinning for 10 min. at 5000g, the ribosomes were collected by centrifugation for 2 hr. at 105000g.

Separation of ribosomal 80s particles into 60s and 40s sub-units was carried out by the method of Henshaw (1964). Ribosomes obtained from 4 ml. of a protoplast suspension ($E_{260\text{m}\mu}$ 1) were resuspended in 0.3 ml. of a solution containing (per ml.) 2 μ moles of MgCl_2 and 50 μ moles of KCl. Then 0.03 ml. of 0.2 M-sodium pyrophosphate buffer, pH 8.0, was added and a 0.2 ml. sample was put on top of 5 ml. of linear 20–5% (w/v) sucrose gradient containing KCl (50 mM). Centrifugation was carried out for 3 hr. at 37000 rev./min. (120 000g) in the SW39 rotor of the Spinco model L1 ultracentrifuge at 2°. The collection of the fractions as well as the estimation of the radioactivity and $E_{260\text{m}\mu}$ were carried out as described for the ribosomal aggregates.

Estimation of the base composition of newly formed RNA with [^{32}P]orthophosphate. The base ratio of newly formed RNA was estimated from the radioactivity of the different nucleotides obtained by alkaline hydrolysis of ^{32}P -labelled RNA extracted from yeast cells (Yčas & Vincent, 1960). After the incorporation of [^{32}P]orthophosphate, the cells were collected by centrifugation. To obtain a partial separation of s-RNA from the remaining RNA (Monier, Stephenson & Zamecnik, 1960) the cells were resuspended in 4 ml. of ice-cold 0.1 M-NaCl in 20 mM- $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 6.3. An equal volume of 90% (w/v) phenol was added and the mixture was shaken for 10 min. at room temperature. After centrifugation for 10 min. at 3000g the water layer containing the soluble RNA was removed carefully. The cells thus lacking at least part of their s-RNA content were further treated with ethanol to remove the phenol, followed by washing with cold 5% (w/v) trichloroacetic acid (five times), 0.1 M-potassium acetate in ethanol, ethanol, boiling chloroform-ether-ethanol (1:1:2, by vol.) and acetone. RNA was extracted by three successive treatments with 10% (w/v) NaCl at 100° for 45 min. The RNA was precipitated in the cold with sodium acetate and ethanol as described above. Residual protein was removed by shaking the RNA dissolved in 4 ml. of 0.1 M-NaCl with an equal volume of 90% (w/v) phenol for 60 min. The RNA was again precipitated with ethanol and dialysed against water for 24 hr. Hydrolysis was carried out by incubating the RNA in 2 ml. of 0.5 N-KOH at 37° for 18 hr. After cooling and neutralization with HClO_4 a portion of the hydrolysate containing approx. 3 mg. of nucleotide material was put on a Dowex 1 (formate form) column (0.8 cm. $\times$ 25 cm.) and the nucleotides were separated by elution with a formic acid gradient as described by Osawa, Takata & Hotta (1958). The peaks were localized by continuous reading of the ultraviolet extinction with the aid of a Uvicord spectrophotometer (LKB Produktor, Stockholm, Sweden). The fractions were pooled, the volumes of each nucleotide fraction were adjusted to 25 ml. and $E_{260\text{m}\mu}$ and $E_{280\text{m}\mu}$ were measured. These data were used as a control on the quantitative elution of the nucleotides from the column. The following molar extinction coefficients at 260 m μ were used: adenylic acid, 14.2×10^3 ; guanylic acid, 11.8×10^3 ; uridylic acid, 10.0×10^3 . For cytidylic acid, 13.0×10^3 was used as the molar extinction coefficient at 280 m μ . The radioactivity of the nucleotides was estimated after adsorption on charcoal (type SXS30; Norit Mij, Amsterdam, The

Netherlands). A 10 ml. portion of the nucleotide solution was shaken in the cold with 20 mg. of acid-washed charcoal for 45 min. The charcoal was collected by centrifugation, washed twice with 10 ml. of water, plated on stainless-steel planchets, dried and counted with a Philips Geiger-Müller counter.

s-RNA was isolated from the water layer obtained after phenol extraction of the whole cells as described above. After precipitation with ethanol, the RNA was dialysed against frequent changes of water. Bovine serum albumin (5 mg.) was added as a carrier to the solution containing the RNA. s-RNA and serum albumin were precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. Washing was carried out as described above. The RNA was hydrolysed and counted after the acetone washing. Separation of the nucleotides was carried out as described above.

RESULTS

Effect of cycloheximide on the rate of RNA synthesis. In yeast the synthesis of RNA is under the control of amino acids (Yčas & Brawerman, 1957). Fig. 1 shows the results of experiments in which the effect of the medium on RNA synthesis in *Saccharomyces carlsbergensis* N.C.T.C. 74 was investigated. The results show that the addition of casein hydrolysate ('casamino acids'; Difco) as a source of nitrogen results in a 10–20-fold stimulation of the rate of RNA formation, as measured by the incorporation of [^{14}C]uracil. The casein hydrolysate could be replaced by an artificial mixture of the naturally occurring amino acids. A further study showed that under the conditions chosen for the experiments 1 mg. of casein hydrolysate/ml. suffices to cause maximal stimulation of RNA synthesis. Since in all experiments the four bases were included in the incubation media, an enhanced synthesis of RNA due to an increased formation of nucleic acid precursors can be excluded.

When similar experiments are carried out in the presence of cycloheximide the controlling effect of the amino acids on the rate of RNA synthesis is abolished. In the presence of casein hydrolysate (or any other source of amino acids) cycloheximide causes an inhibition of RNA formation; in the absence a stimulation can be observed, resulting in a release of the rate of RNA synthesis from the controlling effect of the amino acids in the medium (Fig. 1). The rate then solely depends on the nutritional value (e.g. glucose content) of the medium.

Protein synthesis in *Saccharomyces carlsbergensis* N.C.T.C. 74 is very sensitive to cycloheximide. In previous studies it had been found that a 50% inhibition of protein biosynthesis occurs *in vivo* as well as *in vitro* when the antibiotic is present at a concentration of 0.1 $\mu\text{g.}/\text{ml.}$ (de Kloet, 1965b). Fig. 2 shows that similar low concentrations of the antibiotic have an effect on RNA formation and

Fig. 1. Effect of cycloheximide and casein hydrolysate on RNA synthesis in yeast. Yeast cells ($E_{500m\mu}$ 1) were incubated at 30° in 25 ml. of medium containing (per ml.) 10 mg. of glucose, 20 μ moles of $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 6.3, 2 μ moles of MgCl_2 and 12 μ g. of unlabelled uracil, guanine, cytosine and adenine. After preincubation for 20 min. with or without casein hydrolysate (0.2%), 14 μ g. of [^{14}C]uracil (specific activity 32 mc/m-mole) was added, followed after 5 min. (zero time) by cycloheximide (10 μ g./ml.). Samples (2 ml.) were withdrawn at times indicated in the Figure, washed and counted as described in the Materials and Methods section. Values are expressed as counts/min./mg. dry wt. of material. x—x, With casein hydrolysate without cycloheximide; Δ — Δ , with casein hydrolysate with cycloheximide; x---x, without casein hydrolysate without cycloheximide; Δ --- Δ , without casein hydrolysate with cycloheximide.

that RNA synthesis becomes virtually independent of the amino acids at concentrations of cycloheximide that inhibit protein synthesis completely.

A further study of the time-course of the effect of the antibiotic on RNA synthesis revealed that there was a gradual deceleration of the rate of RNA formation. Usually after 2 hr. (about one generation time in the casein hydrolysate medium) RNA synthesis proceeds at a rate only a few per cent of that of control cells incubated without the antibiotic (see Fig. 8). Further, the release of RNA synthesis from its amino acid control on the addition of cycloheximide occurs only after a lag (Fig. 1), in contrast with the effect of the antibiotic on protein synthesis, which is nearly immediate. A closer study revealed that the lag time occurring in the absence of amino acids could be abolished by the addition of an amino acid mixture together with the antibiotic, indicating that the

Fig. 2. Effect of different cycloheximide concentrations on the incorporation of [^{14}C]uracil and [^{14}C]leucine by yeast. Yeast cells were incubated in 2 ml. of medium as described in Fig. 1 with the addition of casein hydrolysate (0.2%). The samples contained either 6 μ g. of DL-[^{14}C]leucine (specific activity 36 mc/m-mole) or 2 μ g. of [^{14}C]uracil. The incubation was terminated after 60 min. by the addition of 2 ml. of cold 10% (w/v) trichloroacetic acid. Samples containing [^{14}C]leucine were heated for 10 min. at 90° before the usual washing procedure. Values are expressed as the percentage of the radioactivity of a cell suspension incubated in the absence of cycloheximide. x, [^{14}C]uracil; Δ , [^{14}C]leucine.

lag probably represents the time needed by the cell to provide amino acids in some way (synthesis or proteolysis). DNA synthesis was also found to be strongly inhibited by the antibiotic. This observation agrees with that made by Bennett *et al.* (1964), in experiments on mammalian cells, and by Kerridge (1958), also with *Saccharomyces carlsbergensis*. This effect of the antibiotic was not studied in detail. It should be pointed out that yeast contains about 30 times as much RNA as DNA and that in these experiments the incorporation of ^{14}C into DNA after incubation of the cells with [^{14}C]uracil in the absence of cycloheximide never amounted to more than about 2% of that taken up by the RNA. Hence it is very unlikely that the low rate of incorporation of [^{14}C]uracil after a prolonged incubation with cycloheximide is due to DNA synthesis. The amount of label remaining insoluble in trichloroacetic acid after 18 hr. treatment with 0.5N-potassium hydroxide at 37° was taken as a measure of the incorporation into DNA.

To obtain further information on the nature of the RNA formed in the presence of cycloheximide several molecular properties were investigated. A preliminary account of some of these properties has been given (de Kloet, 1965a).

Fig. 3. Effect of cycloheximide on $[^{14}\text{C}]$ uracil and $[^{14}\text{C}]$ methyl incorporation into RNA of yeast protoplasts. Protoplasts ($E_{600 \text{ m}\mu}$ 1) were incubated at 30° in 25 ml. of medium containing (per ml.) 120 mg. of mannitol, $20 \mu\text{moles}$ of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 6.3, 10 mg. of glucose, 2 mg. of casein hydrolysate, $2 \mu\text{moles}$ of MgCl_2 and $12 \mu\text{g.}$ of each of unlabelled uracil, adenine, guanine and cytosine. After preincubation for 5 min. with or without cycloheximide ($10 \mu\text{g./ml.}$), $14 \mu\text{g.}$ of $[^{14}\text{C}]$ uracil (specific activity 30 mc/m-mole) or $50 \mu\text{g.}$ of $[Me\text{-}^{14}\text{C}]$ -methionine (specific activity 30 mc/m-mole) was added. For the 3 min. pulse experiment (a) the unlabelled uracil was omitted from the vessel. In the other experiments the reaction was terminated after 45 min. by rapid cooling. RNA was isolated and subjected to sucrose-density-gradient analysis as described in the Materials and Methods section. (a) 3 min. with $[^{14}\text{C}]$ uracil; (b) 45 min. with $[^{14}\text{C}]$ uracil without cycloheximide; (c) 45 min. with $[^{14}\text{C}]$ uracil with cycloheximide; (d) 45 min. with $[Me\text{-}^{14}\text{C}]$ methionine without cycloheximide; (e) 45 min. with $[Me\text{-}^{14}\text{C}]$ methionine with cycloheximide. —, $E_{260 \text{ m}\mu}$; x---x, radioactivity.

Sedimentation properties of RNA formed in the presence of cycloheximide. The studies on the sedimentation behaviour of yeast RNA were carried out with RNA isolated from yeast protoplasts, because protoplasts are a convenient source for preparing undegraded RNA. Control experiments demonstrated that cycloheximide has similar inhibitory and stimulatory effects on RNA synthesis depending on the presence of amino acids in the medium, as had been observed with whole cells. Fig. 3(a) shows the sedimentation pattern of yeast RNA isolated from protoplasts that had been incubated for 3 min. with [^{14}C]uracil. As has been found in a number of similar cases (Scherrer, Latham & Darnell, 1963; Hiatt, 1962), the rapidly labelled RNA sediments as heterogeneous material, with its main peak of radioactivity sedimenting in front of the ribosomal 23s RNA. The 260m μ -absorbing material representing the stable RNA species shows the usual pattern of ribosomal 23s and 16s RNA together with s-RNA. The present method of isolating high-molecular-weight yeast RNA does not yield material sedimenting at about 14s, a value sometimes reported for bacterial m-RNA (Gros *et al.* 1963). The addition of cycloheximide does not result in a different sedimentation pattern in this short-term labelling experiment. As shown previously (de Kloet, 1965a), a continued incubation of the protoplasts with [^{14}C]uracil (or a 'pulse' and 'chase' experiment) in the presence of cycloheximide results in the accumulation of this rapidly sedimenting RNA, together with RNA of lower molecular weight (Figs. 3b and 3c).

When [^{32}P]orthophosphate was used instead of [^{14}C]uracil, similar results were obtained. Analysis showed that the high-molecular-weight RNA possesses a base composition intermediate between those of yeast DNA and yeast ribosomal RNA, and that the low-molecular-weight RNA has the base composition of yeast s-RNA (Table 1). The latter finding indicates that there occurs no gross degradation of the high-molecular-weight RNA to material with the sedimentation properties of s-RNA during the course of the experiment, although excessively long incubations result in degradation of the high-molecular-weight RNA (Fig. 9b). Since the experiments shown in Fig. 3 were carried out in a medium containing amino acids, cycloheximide inhibits RNA formation (see Fig. 1). A calculation shows that in this case the synthesis of the high-molecular-weight RNA is more inhibited than the formation of s-RNA (e.g. 75% versus 25%). This has also been shown in experiments in which the effect of the antibiotic on the formation of s-RNA obtained by a direct treatment of whole yeast cells with phenol was measured (see the Materials and Methods section). In a particular experiment, the synthesis of s-RNA was inhibited after 30 min. by 30%, after 70 min. by 60% and after 120 min. by about 80%, whereas the synthesis of the total RNA was inhibited at the same times by respectively 50, 85 and 95%. Both in the presence as well as in the absence of cycloheximide, the RNA fraction occurring in the water phase obtained after treatment of the yeast cells with phenol has the base composition of yeast

Table 1. *Effect of cycloheximide on the base composition of newly synthesized yeast RNA*

Protoplasts were incubated in medium as described in Fig. 3 except for the reduction of the phosphate concentration to 2 $\mu\text{moles/ml}$. After preincubation for 5 min. at 30°, cycloheximide (5 $\mu\text{g./ml.}$) was added followed after 5 min. by 0.25 mc of carrier-free [^{32}P]orthophosphate. The incubation was carried out for 90 min. The isolation of the RNA and the sucrose-density-gradient centrifugation were carried out as described in the Materials and Methods section. The fractions containing material sedimenting faster than about 12s (high-molecular-weight RNA) and those that were slower (low-molecular-weight RNA) were pooled, dialysed to remove the sucrose, washed and hydrolysed with 5 mg. of carrier yeast RNA, and the nucleotides were separated as described in the text. The values for yeast ribosomal RNA and s-RNA were obtained from protoplasts prepared from cells labelled overnight with [^{32}P]orthophosphate. The values for yeast DNA were obtained from Zamenhof & Chargaff (1950).

	Base composition (moles/100 moles)			
	CMP	AMP	GMP	UMP(T)
Without cycloheximide				
Low-molecular-weight RNA	27.0	22.6	28.0	22.4
High-molecular-weight RNA	19.4	25.8	27.2	26.3
With cycloheximide				
Low-molecular-weight RNA	27.5	23.0	27.4	22.6
High-molecular-weight RNA	19.1	30.3	22.8	27.9
Yeast DNA	18	32	18	32
Yeast ribosomal RNA	19.3	26.3	27.7	26.6
Yeast s-RNA	28.5	21.6	27.9	22.0

s-RNA, leaving no doubt about the nature of this RNA fraction (Table 2).

Further studies demonstrated that in the presence of cycloheximide the omission of casein hydrolysate from the medium did not result in a different sedimentation pattern of the labelled RNA, again supporting the assumption that cycloheximide abolished the controlling effect of the amino acids on RNA synthesis in yeast. This experiment demonstrates also that cycloheximide stimulates in the absence of amino acids the synthesis of both high-molecular-weight RNA and s-RNA. This observation was confirmed when the effect of the antibiotic on the formation of s-RNA obtained by treatment of whole yeast cells with phenol was investigated. The base composition of the two components was equally found not to be affected by the omission of the amino acids from the medium. A study on the effect of different cycloheximide concentrations revealed that quantities as small as $0.1 \mu\text{g./ml.}$ (which cause 50% inhibition of protein synthesis; de Kloet, 1965b) show an effect on the sedimentation properties of the newly synthesized RNA (Fig. 4). Figs. 3 and 4 show further that the $E_{260 \text{ m}\mu}$ pattern does not change as a result of the incubation of the protoplasts with the antibiotic, indicating that cycloheximide has no influence on the stability of pre-existing RNA.

Effect of cycloheximide on the methylation of yeast RNA. Further evidence for the abnormal properties of the RNA formed in the presence of cycloheximide was found when the effects of the antibiotic on the methylation of RNA was studied. Figs. 3(d) and 3(e) show the results of experiments in which yeast protoplasts were incubated for 45 min. with [$\text{Me-}^{14}\text{C}$]methionine in the absence or presence of cycloheximide, after which the RNA was extracted and subjected to sucrose-density-gradient centri-

fugation as described above. The results demonstrate that in yeast as in other cases both ribosomal and s-RNA are methylated. As shown in Fig. 3(d), the specific activity of the s-RNA is about fivefold higher than that of the ribosomal RNA, indicating a much more extensive methylation of the s-RNA than of the ribosomal RNA, an observation similar to that made with bacterial ribosomal RNA (see e.g. Gordon & Boman, 1964; Starr & Fefferman, 1964). Several control experiments were carried out to ensure that the incorporated [^{14}C]methyl label was present as nucleotide material. Essentially all the label was soluble in hot trichloroacetic acid, or could be made soluble in cold trichloroacetic acid after digestion with ribonuclease or alkaline hydrolysis. Approx. 95% of the label in this cold trichloroacetic acid solution could be adsorbed on charcoal. Column chromatography on Dowex 1 (formate form) showed the label to be present mainly in the methylated nucleotide fractions, emerging from the column at distinctly different places from the main nucleotides.

The addition of cycloheximide ($5 \mu\text{g./ml.}$) to the incubation medium results in a qualitatively and quantitatively different sedimentation pattern of [^{14}C]methyl- and [^{14}C]uracil-labelled RNA (Figs. 3c and 3e). Qualitatively the results show that the main fraction of newly methylated high-molecular-weight RNA sediments close to the ribosomal 23s RNA component, whereas the [^{14}C]uracil-labelled material sediments as a broad band of heterogeneous material. Quantitatively the antibiotic has a much more severe inhibitory action on the methylation than on the synthesis of high-molecular-weight RNA (85% versus 70% inhibition). These results demonstrate that the RNA formed in the presence of cycloheximide is distinctly less methylated compared with normal RNA and that it is probably heterogeneous, although, since methylation is

Fig. 4. Effect of different cycloheximide concentrations on the sedimentation pattern of newly synthesized yeast RNA. The experimental conditions were as described for the incorporation of [^{14}C]uracil in Fig. 3. (a) 90 min. incubation with $0.1 \mu\text{g.}$ of cycloheximide/ml. of medium; (b) 90 min. incubation with $0.5 \mu\text{g.}$ of cycloheximide/ml. of medium. —, $E_{260 \text{ m}\mu}$; × — — — ×, radioactivity.

Fig. 5. Effect of cycloheximide on the accumulation of polysomes in yeast. The incubation was carried out in 8 ml. of medium as described for the incorporation of [^{14}C]uracil in Fig. 3. After 75 min. the reaction was terminated by rapid cooling, and the protoplasts were collected by centrifugation, lysed with sodium deoxycholate and subjected to sucrose-density-gradient centrifugation as described in the Materials and Methods section. A 0.4 ml. portion of 60% (w/v) sucrose solution containing MgCl_2 (1 mM) and KCl (50 mM) was layered on the bottom of the tube under the gradient. (a) Without cycloheximide; (b) with cycloheximide (5 $\mu\text{g./ml.}$); (c) as (a), digested for 20 min. at 20° with ribonuclease (10 $\mu\text{g./ml.}$); (d) as (b) but digested with ribonuclease; (e) $E_{260 \text{ m}\mu}$ pattern at the beginning of the experiment. —, $E_{260 \text{ m}\mu}$; $\times$ — $\times$, radioactivity.

known to occur at the polynucleotide level (Fleissner & Borek, 1962), a methylation of pre-existing RNA cannot be excluded. Cycloheximide was found to

have approximately equal effects on the methylation and synthesis of the s-RNA fraction. This confirms the conclusion of Kjellin-Stråby & Boman

(1965) that the s-RNA formed in the presence of cycloheximide in a methionine-requiring strain of *Saccharomyces cerevisiae* is methylated normally.

Cellular localization of the RNA synthesized in the presence of cycloheximide. It has been shown above that cycloheximide causes the accumulation of a DNA-like RNA that might be m-RNA. Therefore an investigation was carried out on the effect of the antibiotic on the formation of polysomes. As shown in Fig. 5(b), the addition of cycloheximide (5 $\mu\text{g./ml.}$) to the medium during the incubation results in the nearly complete disappearance of the free 80s particles normally found in yeast cells or protoplasts. Further, all radioactivity sediments either in the form of low-molecular-weight material

or as material of a very heavy and complex nature to the bottom of the tube, leaving the 80s region nearly devoid of ^{14}C -labelled RNA. Incubation with trace amounts of ribonuclease results in the reappearance of the free 80s particles (Fig. 5d). This yields strong evidence that cycloheximide causes the accumulation of polysomes and hence of m-RNA. Control experiments showed that the antibiotic did not interfere directly with the sedimentation of the ribosomes. As has been shown by de Kloet, Barnett, Bloemers & Koningsberger (1963), yeast protoplasts incubated in the absence of cycloheximide also contain a considerable part of their ribosomes in the form of this heavy polysomal material (Figs. 5a and 5c).

Fig. 6. Effect of cycloheximide on the incorporation of ^{14}C uracil into ribosomal sub-units. Yeast protoplasts were incubated in 4 ml. of medium as described for the incorporation of ^{14}C uracil in Fig. 3, except for the omission of the unlabelled uracil in the 5 min. pulse experiment (a). At the times indicated the vessels containing the samples were cooled and the protoplasts collected by centrifugation. A crude ribosomal fraction was prepared as described in the Materials and Methods section and 40s and 60s ribosomal sub-units were separated by sucrose-density-gradient centrifugation by the method of Henshaw (1964). (a) 5 min. without cycloheximide; (b) 75 min. with cycloheximide (5 $\mu\text{g./ml.}$); (c) as (b) but without cycloheximide. —, $E_{260 \text{ m}\mu}$; $\times$ — $\times$, radioactivity.

From the results presented in Fig. 5 it cannot in fact be concluded directly whether the RNA present in the polysomes after the incubation with cycloheximide is present as a stable RNA component of the sub-units or as m-RNA. In both cases the ribosomal structure can give some protection against the degradation by ribonuclease (Takanami & Zubay, 1964). Therefore the polysomes or the ribosomal fraction isolated as described in the Materials and Methods section were subjected to sucrose-density-gradient analysis by the method of Henshaw (1964). This treatment results in dissociation of the polysomes and the ribosomes into their sub-units (Fig. 6).

Fig. 6(b) shows that the radioactivity incorporated into the polysomes in the presence of cycloheximide is not a part of the stable RNA of the 60s and 40s sub-units of the yeast ribosomes. The pattern of the radioactivity resembles that of the pulse-labelled RNA (Fig. 6a). Fig. 6(c) demonstrates that in the absence of the antibiotic the pattern of radioactivity closely coincides with the $E_{260\text{ m}\mu}$ pattern, indicating that in this case the [^{14}C]uracil becomes readily incorporated into the RNA of the 60s and 40s particles. These results hence yield strong evidence that a major fraction

of the RNA synthesized and accumulated in the presence of cycloheximide is m-RNA and show that no measurable quantities of 40s and 60s ribosomal sub-units are formed.

Base composition of the RNA synthesized in the presence of cycloheximide. The results presented above on the sedimentation properties of RNA formed in the presence of cycloheximide demonstrated that cycloheximide causes the accumulation of RNA with a base composition intermediate between those of yeast DNA and yeast ribosomal RNA, together with s-RNA. To obtain more information about the effect of the antibiotic on the formation of m-RNA and ribosomal RNA, RNA used for base composition analysis was extracted from whole cells previously depleted of at least part of their s-RNA, by treatment with phenol, rather than isolating it by means of gradient centrifugation. Treatment of whole yeast cells with phenol does not extract all the s-RNA present in the cell. s-RNA attached to the growing peptide chain will probably be retained by the cells in the phenol layer. The results in Table 2 show, however, that the applied method has the expected effect of separating the RNA of the cells into two fractions with clearly different base compositions, one of

Table 2. *Effect of cycloheximide on the base composition of newly synthesized yeast RNA*

Yeast cells were preincubated for 10 min. in 25 ml. of medium containing (per ml.) 10 mg. of glucose, 2 mg. of casein hydrolysate, 2 μmoles of MgCl_2 and 2 μmoles of $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 6.2. Cycloheximide (10 $\mu\text{g./ml.}$) and [^{32}P]orthophosphate (0.01 mc/ml.) were added at zero time except where indicated. The extraction of the labelled RNA and the base composition analysis were carried out as described in the Materials and Methods section. Analyses were made at the times indicated. The base composition of yeast ribosomal RNA was estimated with RNA retained by phenol from cells labelled overnight with [^{32}P]orthophosphate; the base composition of yeast s-RNA was estimated with RNA released by water from the same cells. The DNA base ratio was obtained from Zamenhof & Chargaff (1950).

	Base composition (moles/100 moles)			
	CMP	AMP	GMP	UMP(T)
RNA retained by phenol				
(a) 3 min. without cycloheximide	20.2	27.9	23.7	28.2
(b) 45 min. without cycloheximide	19.7	26.0	27.6	26.6
(c) 120 min. without cycloheximide	19.6	26.4	28.2	26.6
(d) As (c) but medium lacking amino acids	20.3	27.1	26.0	27.2
(e) 45 min. with cycloheximide	18.6	30.9	22.8	27.7
(f) As (e) but 120 min. preincubation with cycloheximide before addition of [^{32}P]orthophosphate	18.6	32.3	21.6	26.3
(g) 120 min. with cycloheximide	19.6	30.6	21.8	28.0
(h) As (g) but medium lacking amino acids	19.6	29.8	22.4	27.2
Yeast ribosomal RNA	19.3	26.3	27.7	26.6
Yeast DNA	18	32	18	32
RNA released in water phase				
(a) 120 min. without cycloheximide	27.5	22.0	28.0	22.4
(b) 120 min. with cycloheximide	28.5	21.1	27.8	22.6
Yeast s-RNA	28.5	21.6	27.9	22.0

ribosomal RNA and the other of s-RNA. As calculated from $E_{260\text{ m}\mu}$ data or from experiments in which yeast cells had grown for several generations with [^{14}C]uracil the yield of s-RNA obtained by treatment of whole cells with phenol was about 50% of the yield obtained with the sucrose-gradient method of isolating s-RNA. In the latter case about 15% of the total cellular RNA is found to be s-RNA.

An analysis has been made of the base composition of the newly synthesized RNA extracted after different times of growth in the presence of [^{32}P]orthophosphate in a medium rich in amino acids and in a buffer not promoting growth, both in the presence and absence of cycloheximide. The results are presented in Table 2. The results show that in growing yeast the base composition of the newly synthesized RNA, which at first has a base composition intermediate between those of DNA and ribosomal RNA, soon attains the base composition of yeast ribosomal RNA, a conclusion in accordance with that of Mitchison & Gross (1965), obtained in their studies on *Schizosaccharomyces pombe*. In yeast incubated in a glucose-containing buffer RNA synthesis proceeds at a very low rate. The RNA formed in this case has a base composition intermediate between those of ribosomal RNA and yeast DNA (Kitazume & Yças, 1963). Under these circumstances the addition of cycloheximide results in a strong stimulation of the synthesis of RNA with a base composition even more distorted towards that of DNA. The addition of cycloheximide to a yeast-cell suspension in a medium containing amino acids results in the accumulation of a similar type of RNA (de Kloet, 1965a). Under these circumstances preincubation of the cells with cycloheximide for 120 min. before the addition of [^{32}P]orthophosphate hardly affected the result, although RNA synthesis was inhibited by about 95%. It was found, further, that incubation of the cells with or without cycloheximide or amino acids did not affect the base composition of the newly formed water-soluble RNA obtained after treatment of the yeast cells with phenol, as the base composition of the latter was found to remain unchanged like that of s-RNA.

Stability of the RNA formed in the presence of cycloheximide after removal of the antibiotic. On removal of cycloheximide after preincubation of the yeast cells with the antibiotic, the organism still has the ability to carry out protein biosynthesis. The removal of the antibiotic in the experiments described in Figs. 7 and 8 was carried out by filtration and washing of the cells over a Millipore filter in the cold.

Fig. 7 shows that the resumption of protein formation occurs after a lag. This lag was found to be rather independent of the time of preincuba-

tion of the cells with the antibiotic (between 5 and 60 min. at 30°) and the concentration of the drug (between 5 and 100 $\mu\text{g./ml.}$). In all cases there occurred a similar gradual increase in the rate of [^{14}C]leucine incorporation. A simple explanation for this lag period would be that part of the antibiotic is bound very tightly inside the yeast cell and is washed out only very slowly during the subsequent incubation. When the recovery of [^{14}C]uracil incorporation after preincubation for 60 min. with cycloheximide was studied, after removal of the antibiotic a similar gradual increase

Fig. 7. Recovery of protein synthesis after preincubation of yeast cells with cycloheximide. Yeast cells ($E_{500\text{ m}\mu}$ 1) were incubated at 30° in 25 ml. of medium as described in Fig. 1 in the presence or absence of cycloheximide (10 $\mu\text{g./ml.}$) for 60 min. The samples were cooled and filtered over a Millipore filter (diam. 25 mm., pore size 0.45 μ) in the cold. The cells were washed with three successive 16 ml. quantities of cold medium free of antibiotic. Finally the cells were resuspended in 25 ml. of medium containing (per ml.) 2 $\mu\text{g.}$ of DL-[1- ^{14}C]leucine (specific activity 36.6 mc/m-mole) with or without cycloheximide. Samples (2 ml.) were withdrawn at the times indicated, precipitated and washed as described in the Materials and Methods section. Values represent counts/min./mg. dry wt. of material. Δ, Preincubated and reincubated without cycloheximide; ○, preincubated with cycloheximide and reincubated without cycloheximide; ×, incubated with cycloheximide.

Fig. 8. Effect of preincubation of yeast cells with cycloheximide on the incorporation of $[^{14}\text{C}]$ uracil after removal of the antibiotic. Yeast cells were preincubated with or without cycloheximide ($10\text{ }\mu\text{g./ml.}$) and washed as described in Fig. 7. After removal of the antibiotic the cells were resuspended in fresh medium containing (per ml.) $0.5\text{ }\mu\text{g.}$ of $[^{14}\text{C}]$ uracil (specific activity 30 mc/m-mole) with or without cycloheximide. Samples were withdrawn at the times indicated and washed as described in the Materials and Methods section. Values are expressed as counts/min./mg. dry wt. of material. O, Preincubated and reincubated without cycloheximide; Δ , preincubated without and incubated with cycloheximide ($10\text{ }\mu\text{g./ml.}$); x, preincubated with and incubated without cycloheximide; $\square$, preincubated with and incubated with cycloheximide ($10\text{ }\mu\text{g./ml.}$).

in activity was observed (Fig. 8), though with a longer lag than observed for $[^{14}\text{C}]$ leucine incorporation.

When cells preincubated with $[^{14}\text{C}]$ uracil and cycloheximide are washed free of label and antibiotic, and the incubation is continued in unlabelled medium containing $[^{12}\text{C}]$ uracil, the label is retained in the cell in a cold-trichloroacetic acid-insoluble form. Only after a prolonged incubation (over 2 hr.) with cycloheximide is there some decrease in cold-trichloroacetic acid-precipitable radioactivity. The absence or presence of either amino acids or cycloheximide did not affect this phenomenon. Since the RNA formed in the presence of cycloheximide possesses some abnormal properties it

was investigated whether there occurred a change in sedimentation pattern and base composition during the recovery phase. Figs. 9(a) and 9(c) show that a profound change in the sedimentation properties of the RNA indeed occurs on reincubation of the protoplasts in cycloheximide-free medium. Removal of the antibiotic was in this case obtained by careful washing of the protoplasts by repeated centrifugation in the cold. Table 3 shows that the base composition of the RNA also changes from the cycloheximide-induced type to the normal ribosomal composition. This change was, as could be expected, prevented when cycloheximide was included in the medium. The results show that the RNA that accumulates in the presence of cycloheximide is in fact unstable after the removal of the antibiotic. Apparently this instability is not accompanied by degradation of the radioactive RNA into such products that can be diluted by newly synthesized nucleic acid precursors.

DISCUSSION

The RNA formed in the presence of cycloheximide differs from the normal RNA synthesized in yeast in many respects, e.g. base composition, methylation and sedimentation pattern. Analysis showed that two components are formed: one sedimented like pulse-labelled RNA and had a base composition intermediate between those of yeast DNA and yeast ribosomal RNA, and the other component was found to be s-RNA. A somewhat similar accumulation of abnormal high-molecular-weight RNA was observed by Holland (1963) and Tamaoki & Mueller (1963) in their studies on the effect of puromycin on RNA synthesis in HeLa cells. The formation of RNA with abnormal sedimentation properties in the presence of cycloheximide has been shown to occur in *Neurospora crassa* by Fiala & Davis (1965) and in *Saccharomyces cerevisiae* by Fukuhara (1965).

The properties of the high-molecular-weight RNA formed in the presence of cycloheximide (DNA-like base composition and sedimentation behaviour differing from ribosomal RNA) are properties usually ascribed to polycistronic m-RNA, although precursor ribosomal RNA sediments in the same region (Scherrer *et al.* 1963). The possibility thus exists that the antibiotic inhibits ribosomal RNA synthesis completely and allows m-RNA and s-RNA formation to continue for some time. In this case the observed inequality of the base composition of RNA formed in the presence of cycloheximide and yeast DNA might reflect an unequal base composition of the two DNA strands, of which only one is copied into RNA. On the other hand, several authors have shown that the

Fig. 9. Effect of continued incubation in the absence of cycloheximide on the sucrose-density-gradient pattern of yeast RNA synthesized in the presence of the antibiotic. Yeast protoplasts were preincubated with cycloheximide (5 $\mu\text{g./ml.}$) and 14 $\mu\text{g.}$ of [^{14}C]uracil (specific activity 30 mc/m-mole) in 20 ml. of medium as described for the incorporation of [^{14}C]uracil in Fig. 3. After 45 min. the samples were cooled, and the protoplasts were collected and washed by resuspending and centrifuging three times in medium without antibiotic. The protoplasts were resuspended in fresh medium with or without cycloheximide and reincubated for 120 min. RNA was isolated and subjected to sucrose-density-gradient centrifugation as described in the text. (a) Pattern before reincubation; (b) pattern at the end of the reincubation with cycloheximide (5 $\mu\text{g./ml.}$); (c) as (b), but without cycloheximide. —, $E_{260\text{ m}\mu}$; $\times$ — $\times$, radioactivity.

Table 3. *Effect of continued incubation in the absence of cycloheximide on the base composition of RNA formed in the presence of the antibiotic*

Yeast cells were incubated for 45 min. in 25 ml. of medium as described in Table 2 in the presence of cycloheximide (10 $\mu\text{g./ml.}$) and [^{32}P]orthophosphate (0.01 mc/ml.). The cells were washed free of the antibiotic as described in Fig. 8 and reincubated in the same medium containing 50 μmoles of $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 6.2, for 2 hr.

	Base composition (moles/100 moles)			
	CMP	AMP	GMP	UMP
Before reincubation	18.6	30.9	22.8	27.7
Reincubation with cycloheximide	18.4	29.8	23.0	28.9
Reincubation without cycloheximide	19.4	26.5	27.0	26.6

pulse-labelled RNA of a number of bacteria, which has also a base composition intermediate between those of DNA and RNA, is in fact a mixture of m-RNA and (precursor) ribosomal RNA (Bolton & McCarthy, 1962; Armstrong & Boezi, 1965; Midgley & McCarthy, 1962). The observation that cycloheximide causes the accumulation of polysomal material is a strong indication that m-RNA does indeed accumulate, although it has yet to be shown that this m-RNA is functional in the synthesis of specific proteins. The finding that m-RNA accumulates is in agreement with observations by other authors who demonstrated that cycloheximide inhibits polysomal breakdown in reticulocytes (Williamson & Schweet, 1965) and rat liver (Wettstein *et al.* 1964) and with the observation (de Kloet, 1965b) that cycloheximide does not interfere with the binding of yeast ribosomes, phenylalanyl-s-RNA and polyuridylic acid into a ternary complex.

The observed non-coincidence between the sedimentation patterns of [^{14}C]methyl- and [^{14}C]uracil-labelled RNA synthesized in the presence of cycloheximide might indicate heterogeneity of the newly formed RNA, although methylation of pre-existing ribosomal RNA cannot be excluded. Actual separation of the two types of RNA by hybridization with DNA will be necessary to find out whether the RNA produced in the presence of cycloheximide is indeed a mixture.

The RNA produced in the presence of cycloheximide is not stable on removal of the inhibitor. The observation that this instability is not accompanied by a loss in trichloroacetic acid-precipitable radioactivity should not be taken as a direct evidence that the RNA produced in the presence of cycloheximide is an obligatory precursor of the other RNA types in the cell and that the conversion of the RNA produced in the presence of cycloheximide into ribosomal RNA occurs at the polynucleotide level. Too little is known about the different pools of nucleic acid precursors, known to be present in yeast. It might well be that the yeast cell degrades the RNA produced in the presence of cycloheximide to use preferentially the nucleotides that are released during degradation for the formation of ribosomal RNA.

The present results show, further, that cycloheximide interferes severely with the mechanism by which the synthesis of RNA is regulated in yeast.

(1) The antibiotic causes the release of the control amino acids normally exert on RNA synthesis in yeast (Figs. 1 and 2). This observation might explain the contradictory results obtained by Siegel & Sisler (1964), who observed a stimulation, and Kerridge (1958), who found an inhibitory action on RNA formation by the drug. A species-

specific effect cannot, however, be excluded since these authors used different strains of yeast, which might show different sensitivities to the antibiotic (Coursen & Sisler, 1960).

Although some direct effect of cycloheximide on the cell nucleus or the RNA polymerase resulting in a total disregulation of RNA synthesis cannot be excluded, the stimulation of RNA synthesis by the antibiotic in a medium lacking amino acids might well be explained in a way similar to that suggested by Kurland & Maaløe (1962) for a similar effect of chloramphenicol on RNA synthesis in *E. coli*. Again, s-RNA acting as an inducer for RNA formation might arise from the action of the antibiotic, as cycloheximide inhibits protein synthesis at the ribosomal level and is supposed not to affect proteolysis, a part of the normal process of protein turnover (Stent & Brenner, 1961; Tissières, Bourgeois & Gros, 1963; Halvorson, 1958).

(2) There is a gradual deceleration of RNA synthesis in yeast in the presence of the antibiotic. Again, this effect cannot be explained at present. Since, however, comparable effects are noted at high concentrations of chloroamphenicol in *E. coli* (Kurland & Maaløe, 1962) and puromycin in HeLa cells (Holland, 1963), a direct effect of the antibiotic on RNA formation is rather unlikely. Hence it seems more reasonable that this effect of the antibiotic is also secondary to its inhibitory action on protein biosynthesis. Thus it might be that yeast contains a labile RNA polymerase or some other protein that needs continuous resynthesis for normal RNA formation. Since, however, a major part of the RNA that is synthesized is m-RNA and since polysomes accumulate and free ribosomes disappear, it might also be that the rate of turnover of the polysomes and the content of free ribosomes have some effect on the rate of synthesis of this particular type of RNA. The possibility that the deceleration of RNA synthesis might be caused by cell death can be rejected since cycloheximide acts like a bacteriostatic rather than a bactericidal agent. However, an interference of the antibiotic with the nucleoside triphosphate-synthesizing systems cannot be excluded at present.

(3) Cycloheximide causes a complete inhibition of the synthesis of ribosomal 16s and 23s RNA components. This might mean that there occurs a specific RNA polymerase for the synthesis of ribosomal RNA that is specifically inhibited by the antibiotic. On the other hand, cycloheximide might again act indirectly by inhibiting the synthesis of special proteins involved in the synthesis of ribosomal RNA. The possibility that the observed inhibition of ribosomal RNA formation is only apparent and caused by rapid degradation of the latter as a result of the absence of protecting

ribosomal protein is rather unlikely since no degradation products could be detected. Therefore in yeast also ribosomal protein might be required for the synthesis of ribosomal RNA, a suggestion made by Kurland & Maaløe (1962) for ribosomal RNA formation in *E. coli*. A great difference between yeast and *E. coli* is, however, that in this bacterium inhibition of protein synthesis by chloramphenicol allows the synthesis of 16s and 23s RNA for a considerable time, whereas in yeast cycloheximide causes the immediate cessation of these particular types of RNA, though some synthesis of a precursor ribosomal RNA with different sedimentation properties cannot be excluded.

(4) The formation of s-RNA is affected qualitatively in the same way as the formation of high-molecular-weight RNA by the antibiotic. Again, cycloheximide abolishes the controlling effect of the medium, suggesting a regulatory function of amino acids in this case also, and again there is gradual deceleration as a result of the action of the drug. Quantitatively this deceleration is slower; however, no explanation can be given of this effect, though it might also be related to the ability of a cell to vary the s-RNA/total RNA ratio only to a certain limit (Kjeldgaard & Kurland, 1963). Again an interference of the antibiotic with nucleoside triphosphate metabolism cannot be excluded.

Finally, some comments should be made on the action of cycloheximide in yeast as compared with the effect of chloramphenicol on *E. coli*, for there are similarities and differences. Similarities occur in the observation that the presence of the antibiotics causes in the respective sensitive organisms a disturbed methylation of RNA (Gordon, Boman & Isaksson, 1964). The antibiotics inhibit the degradation of m-RNA (Fan, Higa & Levinthal, 1964; Wettstein *et al.* 1964) and allow the synthesis of this particular type of RNA (Gros *et al.* 1963). Further, there is a gradual deceleration of RNA synthesis that is quantitatively different for s-RNA and the remaining RNA.

As discussed above, a great difference occurs in the type of RNA that accumulates in the presence of the antibiotic. In yeast it is RNA with a more DNA-like base composition, and the formation of ribosomal 23s and 16s RNA is completely inhibited. In *E. coli* ribosomal RNA is formed for a considerable time (Kurland *et al.* 1962). Further investigation is needed to find out whether the observed differences reflect a difference in the mode of action of the antibiotics or whether they reflect a fundamental difference in the mode of RNA synthesis in the different organisms, bacteria and yeast.

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REFERENCES

- Armstrong, R. L. & Boezi, J. A. (1965). *Biochim. biophys. Acta*, **103**, 60.
- Bennett, L. L., Smithers, D. & Ward, C. T. (1964). *Biochim. biophys. Acta*, **87**, 60.
- Bolton, E. T. & McCarthy, B. J. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1390.
- Coursen, B. W. & Sisler, H. D. (1960). *Amer. J. Bot.* **47**, 541.
- de Kloet, S. R. (1965a). *Biochem. biophys. Res. Commun.* **19**, 582.
- de Kloet, S. R. (1965b). *Proc. Kon. ned. akad. Wet., Amsterdam*, Series B, **68**, 266.
- de Kloet, S. R., Barnett, L. B., Bloemers, H. P. J. & Koningsberger, V. V. (1963). *Proc. Kon. ned. akad. Wet., Amsterdam*, Series B, **66**, 332.
- de Kloet, S. R., van Wermeskerken, R. K. A. & Koningsberger, V. V. (1961). *Biochim. biophys. Acta*, **47**, 138.
- Fan, D. P., Higa, A. & Levinthal, C. (1964). *J. molec. Biol.* **8**, 210.
- Fiala, E. & Davis, F. F. (1965). *Biochem. biophys. Res. Commun.* **18**, 115.
- Fleissner, E. & Borek, E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1199.
- Ford, J. H., Klomprens, W. & Hamm, C. L. (1958). *Plant Dis. Repr.* **42**, 680.
- Ford, J. H. & Leach, B. E. (1948). *J. Amer. chem. Soc.* **70**, 1223.
- Fukuhara, H. (1965). *Biochem. biophys. Res. Commun.* **18**, 153.
- Gordon, J. & Boman, H. G. (1964). *J. molec. Biol.* **9**, 638.
- Gordon, J., Boman, H. G. & Isaksson, L. A. (1964). *J. molec. Biol.* **9**, 831.
- Gros, F., Naono, S., Woese, C., Willson, C. & Attardi, G. (1963). In *Symposium on Informational Macromolecules*, p. 387. Ed. by Vogel, H. J., Bryson, V. & Lampen, J. O. New York: Academic Press Inc.
- Halvorson, H. (1958). *Biochim. biophys. Acta*, **27**, 255.
- Henshaw, E. C. (1964). *J. molec. Biol.* **9**, 610.
- Hiatt, H. H. (1962). *J. molec. Biol.* **5**, 217.
- Holland, J. J. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 436.
- Kerridge, D. (1958). *J. gen. Microbiol.* **19**, 497.
- Kitazume, Y. & Yčas, M. (1963). *Biochim. biophys. Acta*, **76**, 391.
- Kjeldgaard, N. O. & Kurland, G. C. (1963). *J. molec. Biol.* **6**, 341.
- Kjellin-Stråby, K. & Boman, H. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1346.
- Kurland, C. G. & Maaløe, O. (1962). *J. molec. Biol.* **4**, 193.
- Kurland, C. G., Nomura, M. & Watson, J. D. (1962). *J. molec. Biol.* **4**, 388.
- Midgley, J. E. M. & McCarthy, B. J. (1962). *Biochim. biophys. Acta*, **61**, 696.
- Mitchison, J. M. & Gross, P. R. (1965). *Exp. Cell Res.* **37**, 259.
- Monier, R., Stephenson, M. L. & Zamecnik, P. C. (1960). *Biochim. biophys. Acta*, **43**, 1.
- Osawa, S., Takata, K. & Hotta, Y. (1958). *Biochim. biophys. Acta*, **28**, 271.
- Otaka, E., Osawa, S. & Sibatani, A. (1964). *Biochem. biophys. Res. Commun.* **15**, 568.

- Pardee, A. B., Paegin, K. & Prestidge, L. S. (1957). *Biochim. biophys. Acta*, **24**, 215.
- Scherrer, K., Latham, H. & Darnell, J. E. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 240.
- Siegel, M. R. & Sisler, H. D. (1964). *Biochim. biophys. Acta*, **87**, 70.
- Starr, J. L. & Fefferman, R. (1964). *J. biol. Chem.* **239**, 3460.
- Stent, G. S. & Brenner, S. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 2005.
- Takanami, M. & Zubay, G. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 834.
- Tamaoki, T. & Mueller, G. C. (1963). *Biochem. biophys. Res. Commun.* **11**, 404.
- Tissières, A., Bourgeois, S. & Gros, F. (1963). *J. molec. Biol.* **7**, 100.
- Wettstein, F. O., Noll, H. & Penman, S. (1964). *Biochim. biophys. Acta*, **87**, 525.
- Whiffen, A. J., Bohonas, J. N. & Emerson, R. L. (1946). *J. Bact.* **52**, 610.
- Williamson, A. R. & Schweet, R. (1965). *J. molec. Biol.* **11**, 358.
- Yčas, M. & Brawerman, G. (1957). *Arch. Biochem. Biophys.* **68**, 118.
- Yčas, M. & Vincent, W. S. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 804.
- Zamenhof, S. & Chargaff, E. (1950). *J. biol. Chem.* **187**, 1.