Effect of Cycloheximide on Protein and Ribonucleic Acid Synthesis in Cultured Human Lymphocytes

By J. E. KAY AND A. KORNER

Department of Biochemistry, University of Cambridge

(Received 1 March 1966)

1. Phytohaemagglutinin stimulates the transformation into blast cells of human lymphocytes incubated *in vitro*. This transformation is accompanied by an increase in the incorporation of [^{14}C]leucine into protein and [^3H]uridine into RNA. 2. The incorporation of [^{14}C]leucine by cultures grown in the presence or absence of phytohaemagglutinin is inhibited to the same extent by cycloheximide, a known inhibitor of protein synthesis. 3. Lymphocytes grown without phytohaemagglutinin synthesize mainly non-ribosomal RNA. [^3H]Uridine incorporation by these cells was increased by cycloheximide. 4. Lymphocytes incubated with phytohaemagglutinin begin to synthesize substantial quantities of ribosomal RNA. Under these conditions [^3H]uridine incorporation was partially inhibited by cycloheximide. This inhibition is shown to be largely a result of inhibition of the synthesis of ribosomal RNA.

PHA*, an extract of the kidney bean, *Phaseolus vulgaris*, causes small lymphocytes, maintained *in vitro*, to be transformed into blast cells, which then enter cell division (Nowell, 1960; Marshall & Roberts, 1965). This transformation is preceded by an early rise in RNA synthesis (McIntyre & Ebaugh, 1962) and protein synthesis (Cooper, 1962) and is accompanied by the initiation of DNA synthesis (Cooper, Barkhan & Hale, 1961). Some of the PHA-stimulated increase in RNA synthesis in lymphocytes can be accounted for by the synthesis of ribosomal RNA (Rubin & Cooper, 1965). Ribosomal RNA is synthesized slowly by unstimulated lymphocytes.

Cycloheximide (Actidione) inhibits protein synthesis in yeast (Kerridge, 1958), mammalian cell cultures (Bennett, Smithers & Ward, 1964) and other cells. It also inhibits the synthesis of ribosomal RNA in yeast (Fukuhara, 1965; de Kloet, 1965) and *Neurospora* (Fiala & Davis, 1965) but it has little effect on RNA synthesis in cultured mammalian cells (Bennett *et al.* 1964; Ennis & Lubin, 1964).

The present paper reports the effect of cycloheximide on protein and RNA synthesis in human lymphocytes and in lymphocytes incubated with PHA.

MATERIALS AND METHODS

Chemicals. [^3H]Uridine (5.0 or 14.3 c/m-mole) and DL-[1- ^{14}C]leucine (32 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Tissue culture media TC199 (single strength) and TC199 without leucine (tenfold concentrated) were obtained from Burroughs, Wellcome and Co., London. The tenfold concentrated solution was diluted with glass-distilled water and NaHCO_3 (final concn. 1.1 g./l.). Sodium benzylpenicillin (final concn. 200 units/ml.) and streptomycin sulphate (final concn. 100 $\mu\text{g.}/\text{ml.}$) were added.

PHA (batch K5680) was obtained from Burroughs, Wellcome and Co. and the solid material was dissolved in 5 ml. of medium TC199 and used at a concn. of 0.018–0.020 ml./ml. of lymphocyte suspension. Cycloheximide (Actidione) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and uridine from British Drug Houses Ltd., Poole, Dorset. Actinomycin D was a gift from Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A., and puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All were dissolved in medium TC199 before use.

Preparation and incubation of lymphocyte cultures. Lymphocytes were prepared by the technique of Coulson & Chalmers (1964) from heparin-treated blood of normal human volunteers. Sterile precautions were employed throughout the procedure. Venous blood (15 ml.) was withdrawn into a heparin-washed syringe, mixed with 5 ml. of 3% (w/v) gelatin in 0.15 M-NaCl in a silicone-treated universal bottle and allowed to sediment at 37° for 30 min. The upper layer, containing lymphocytes, was withdrawn and diluted with 2 vol. of medium TC 199. Samples (0.5 ml.) were transferred from this suspension to 108 mm. $\times$ 17 mm. cylindrical centrifuge tubes that were then gassed with CO_2 +air (5:95) and stoppered with silicone bungs. In experiments in which RNA was extracted or ribosomes were prepared from the cells, 2 ml. cultures were set up. Cultures usually contained 0.8×10^6 – 1.2×10^6 lymphocytes/ml.

Lymphocyte cultures were maintained in stationary culture at 37°. In many experiments it was necessary to

* Abbreviation: PHA, phytohaemagglutinin.

replace the medium during the incubation. The old medium was removed after gentle centrifugation of the cultures, and replaced by 0.5 ml. or 2.0 ml. portions of fresh medium composed of 20% of bovine serum and 80% of medium TC199, previously warmed to 37°. PHA was added to the bulk lymphocyte suspension before the cultures were set up and other additions were made either to the bulk lymphocyte suspension or to portions of fresh medium before addition to the cultures.

If the medium was replaced after exposure of cultures to PHA for 3 hr. or more, omission of PHA from fresh medium did not affect the subsequent incorporation of [^{14}C]leucine, in agreement with the findings of Newsome (1963).

In some experiments in which [^{14}C]leucine incorporation was measured, medium TC199 without leucine replaced medium TC199. This replacement did not affect the incorporation of [^{14}C]phenylalanine or [^3H]uridine.

Measurement of [^{14}C]leucine incorporation into protein. The culture medium was removed after centrifugation and the cells were washed once with 0.15 M-NaCl. The cell pellet was then precipitated with 0.5 N-HClO₄ containing an excess of unlabelled leucine and the precipitate was collected on Oxoid membranes. The membranes were washed with 0.5 N-HClO₄ containing unlabelled leucine and stuck to cardboard disks. In earlier experiments the membranes were also washed with ethanol-ether (1:1, v/v) and ether, but this procedure did not affect the observed amino acid incorporation and made it more difficult to attach the membranes to the cardboard disks. The radioactivity in the samples was assessed with a Nuclear-Chicago gas-flow end-window counter or by a Nuclear-Chicago low-background gas-flow counter. The efficiency of counting was about 20%. Wherever practicable, sufficient counts were recorded to give a counting error of less than $\pm 3\%$. Three or four replicate cultures were used for each measurement.

Measurement of [^3H]uridine incorporation into RNA. Cultures were chilled in ice and the medium was removed after centrifugation. All procedures were carried out at 4°. The cells were washed once with cold 0.15 M-NaCl, and the RNA was then precipitated with 5 ml. of cold 0.5 N-HClO₄. The precipitate was washed once with 5 ml. of 0.5 N-HClO₄, with 5 ml. of ethanol-ether (1:1, v/v) and with 3 ml. of ether. The precipitate was dissolved in 0.2 ml. of *m*-Hyamine hydroxide 10X in methanol and washed into a vial with 3 ml. of toluene scintillator containing 2,5-diphenyloxazole (3.5 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50 mg./l.). The radioactivity was determined in a Packard or Nuclear-Chicago scintillation spectrometer. The efficiency of counting was 15%. Four or six replicate cultures were used for each measurement.

Preparation of ribosomes. Landschütz ascites cells, grown in female mice of the Porton strain for 6 days and kindly provided by Miss B. L. M. Hogan, were used to provide carrier ribosomes.

A 6 ml. sample of ascitic fluid containing about 3×10^8 cells/ml. was centrifuged at 1000 *g* for 3 min. and the cells were washed twice with Earle's saline (Earle, 1943) and once with medium R [10 mM-KCl-20 mM-tris-HCl (pH 7.5)-1.5 mM-MgCl₂]. The cells were then suspended in 20 ml. of medium R and the cells from three 2 ml. lymphocyte cultures were added to 3 ml. of ascites suspension. The cells were allowed to swell for 10 min. at 0° and then

homogenized in a tightly fitting homogenizer (Munro, Jackson & Korner, 1964) at 0°.

The homogenates were centrifuged at 10000 *g* for 10 min. at 0°, the supernatants were removed and each was diluted to 6 ml. with medium R, layered over 6 ml. of *m*-sucrose in medium R and centrifuged at 105000 *g* for 3 hr. The supernatant was decanted, and the pellet was rinsed with medium R and resuspended in 0.4 ml. of medium R.

Extraction of radioactive RNA. RNA was extracted from cells after they had been suspended in rat-liver post-mitochondrial supernatant, which was used to provide carrier RNA.

Female albino rats, starved overnight to deplete the livers of glycogen, were decapitated and the livers rapidly removed. The tissue was cut up with scissors in ice-cold 0.25 M-sucrose-20 mM-tris buffer (pH 7.4)-3 mM-magnesium acetate and rinsed free of blood. It was homogenized (Munro *et al.* 1964) at 0° in 3 vol. of the same medium and the homogenate centrifuged for 15 min. at 10000 *g* at 0° to remove cell debris, nuclei and mitochondria. The post-mitochondrial supernatant was decanted and diluted to 35 ml./liver with the homogenizing medium.

Cells from three to six 2 ml. cultures were suspended in 10-15 ml. of post-mitochondrial supernatant. Sodium dodecyl sulphate was added (0.1% final concn.) and the suspension was shaken with freshly redistilled phenol (saturated with water) at 63° for 3 min. and then rapidly cooled. In some experiments the extraction was carried out at 4° without sodium dodecyl sulphate and the aqueous layer after separation was made 0.1% with respect to sodium dodecyl sulphate. After centrifugation the aqueous layer was removed and protein re-extracted twice more with phenol at room temperature. Sodium acetate, pH 5.2 (final concn. 2%, w/v), was added to the aqueous material and the RNA was precipitated overnight at -15° with 2.5 vol. of ethanol. The precipitate was washed twice with 96% (v/v) ethanol and once with ether and dissolved in 0.4 ml. of 0.1 M-NaCl-0.01 M-sodium acetate, pH 5.2. Residual ether was blown off with air.

Analysis of RNA and ribosomes by sucrose-density-gradient centrifugation. Linear gradients were prepared and fractionated as described by Munro *et al.* (1964). For ribosomes the gradients were 10-30% (w/v) sucrose in medium R and 0.2 ml. samples of ribosome suspension were used. The gradients were developed by centrifugation at 37000 rev./min. for 1 hr. in the SW39 rotor of the Spinco model L centrifuge. For RNA the gradients were 5-20% (w/v) sucrose in 0.1 M-NaCl-0.01 M-sodium acetate, pH 5.2, and the centrifugation time was 3-5 hr. Fractions (3 drops) were collected and each was diluted with 0.9 ml. of glass-distilled water before determination of E_{260} . Determination of the radioactive RNA in each fraction was as described by Munro *et al.* (1964).

RESULTS

Effect of cycloheximide on [^{14}C]leucine incorporation into protein. Table 1 compares the incorporation of [^{14}C]leucine into protein by lymphocytes incubated with or without PHA for 4 days. Incorporation by unstimulated cells falls slowly throughout the period of culture, whereas in the presence of PHA amino acid incorporation rises to

a maximum at 48–60 hr. At this time incorporation is more than five times the initial rate and more than 10 times the rate shown by unstimulated lymphocytes. Fig. 1 shows the effect of various cycloheximide concentrations on [¹⁴C]leucine incorporation by unstimulated cultures and by cultures incubated for 44 hr. with PHA. No difference was detectable between the two: both showed marked inhibition. In subsequent experiments cycloheximide was used at a concentration of 25 µg./ml., which inhibits [¹⁴C]leucine incorporation by 95%.

Effect of cycloheximide on [³H]uridine incorporation into RNA. Table 2 shows the effect of cycloheximide on [³H]uridine incorporation during 2 hr. periods by cultures incubated with or without PHA for 0·5, 25 or 45 hr. In the absence of cycloheximide the incorporation by cultures incubated without PHA fell during the incubation period,

Table 1. *Effect of PHA on [¹⁴C]leucine incorporation into protein by lymphocyte cultures during 3 hr. incorporation periods*

Lymphocyte cultures (0·5 ml.) were incubated with or without PHA. At the times indicated the medium was replaced by fresh medium containing [¹⁴C]leucine (1·0 µC/ml.) and the cultures were incubated for a further 3·0 hr. before harvesting.

Time (hr.)	[¹⁴ C]Leucine incorporated (counts/min./culture)	
	Without PHA	With PHA
1	171	202
5	195	307
21	169	581
49	115	1205
73	97	781
93	75	609

whereas that by cultures incubated with PHA rose rapidly. A stimulation was usually apparent within 1 hr. of the addition of PHA and, after a 4–6 hr. lag, the rate of incorporation rose rapidly to a maximum at about 48 hr. At this time it is 10–15 times the initial rate and 20–40 times the rate shown by cultures incubated without PHA.

Fig. 1. Effect of cycloheximide on [¹⁴C]leucine incorporation into protein. The culture medium was replaced by 0·5 ml. of fresh medium containing [¹⁴C]leucine (1 µC/ml.) and cycloheximide. The cultures were incubated for a further 2·0 hr. ○, Freshly prepared cultures incubated without PHA; in this experiment medium TC199 without leucine replaced medium TC199. Δ, Cultures that had been incubated with PHA for 44 hr.

Table 2. *Effect of cycloheximide on [³H]uridine incorporation into RNA by lymphocyte cultures during 2 hr. incorporation periods*

Lymphocyte cultures (0·5 ml.) were incubated with or without PHA. At the time indicated the medium was replaced by fresh medium containing [³H]uridine (3·3 µC/ml.) with or without cycloheximide (25 µg./ml.) and cultures were incubated at 37° for a further 2·0 hr. before harvesting.

Time (hr.)	[³ H]Uridine incorporated					
	Without PHA			With PHA		
	(counts/min./culture)			(counts/min./culture)		
	Control	Cycloheximide	Cycloheximide/ control ratio	Control	Cycloheximide	Cycloheximide/ control ratio
0·5	3196	4011	1·26	3478	3517	1·01
25	2364	2435	1·03	25272	16304	0·64
45	2011	1918	0·95	42693	28856	0·67

Table 3. *Effect of cycloheximide and puromycin on [³H]uridine incorporation by freshly prepared lymphocyte cultures*

The medium of freshly prepared 0.5 ml. cultures was replaced by fresh medium containing [³H]uridine (3.3 μ C/ml.) and cycloheximide or puromycin. Results are expressed as the ratio of [³H]uridine (counts/min.) incorporated by cultures containing antibiotics to [³H]uridine (counts/min.) incorporated by control cultures. The results are given as means $\pm$ s.d. with the numbers of experiments in parentheses.

Time of incubation.....	[³ H]Uridine incorporated (antibiotic/control ratio)		
	1 hr.	2 hr.	3 hr.
Without PHA			
Cycloheximide (0.1 μ g./ml.)	1.13 $\pm$ 0.02 (2)	1.06 $\pm$ 0.03 (3)	—
Cycloheximide (25 μ g./ml.)	1.33 $\pm$ 0.09 (8)	1.33 $\pm$ 0.09 (10)	1.11 $\pm$ 0.10 (4)
Cycloheximide (100 μ g./ml.)	1.21 $\pm$ 0.14 (2)	0.98 $\pm$ 0.06 (3)	—
Puromycin (10 μ g./ml.)	1.30 $\pm$ 0.05 (2)	1.10 $\pm$ 0.05 (3)	—
Puromycin (50 μ g./ml.)	0.91 $\pm$ 0.07 (4)	0.83 $\pm$ 0.14 (3)	0.46 $\pm$ 0.06 (3)
With PHA			
Cycloheximide (25 μ g./ml.)	1.18 $\pm$ 0.04 (3)	1.11 $\pm$ 0.10 (6)	—

Fig. 2. Effect of cycloheximide on [³H]uridine incorporation into RNA of unstimulated cultures. Freshly prepared 2 ml. cultures without PHA were incubated with [³H]uridine (6.6 μ C/ml.) with or without cycloheximide (25 μ g./ml.) for 2.0 hr. RNA was extracted at 63° from six 2 ml. cultures and analysed by sucrose-density-gradient centrifugation on a linear 5–20% (w/v) sucrose gradient in 0.1 M-NaCl–0.01 M-sodium acetate, pH 5.2. (a) No cycloheximide; (b) cycloheximide. O, E_{260} ; Δ , radioactivity. Material sedimented from right to left.

Table 2 also shows that the rate of [³H]uridine incorporation by cultures incubated with PHA for 25–45 hr. was inhibited about 35% by cycloheximide whereas the incorporation by cultures incubated without PHA was not inhibited. Similar experiments showed that the inhibitory effect of cycloheximide became apparent at the same time as the rapid increase of [³H]uridine incorporation.

Cycloheximide caused a stimulation of [³H]uridine incorporation by freshly prepared lymphocyte cultures incubated without PHA (Table 2). This stimulation was consistently observed for 2–3 hr. after the addition of 25 μ g. of cycloheximide/ml. (Table 3), but was diminished when PHA was added together with the cycloheximide. No stimulation of RNA labelling by cycloheximide was seen when cells were incubated without PHA for 24 hr. or more.

Less stimulation of [³H]uridine incorporation was seen when 0.1 μ g. of cycloheximide/ml. was used and the stimulatory effect was abolished at high doses of cycloheximide (100 μ g./ml.).

A similar, but transitory, stimulation of [³H]uridine incorporation into RNA was seen when the cells were incubated in the presence of 10 μ g. of puromycin/ml. (Table 3). This concentration of puromycin inhibited [¹⁴C]leucine incorporation by 85%. Again, higher concentrations of puromycin abolished the stimulatory effect on [³H]uridine incorporation.

Analysis of labelled RNA by sucrose-density-gradient centrifugation. Fig. 2(a) shows that the RNA synthesized by unstimulated cultures during the first 2 hr. of incubation *in vitro* has a heterogeneous sedimentation pattern, with a peak between the 4s and 18s components of rat-liver cytoplasmic RNA. This pattern was not markedly affected by the presence of cycloheximide in the incubation medium (cf. Figs. 2a and 2b).

The RNA synthesized by cultures incubated with PHA for 48 hr. and then incubated with [³H]uridine for a further 2 hr. shows peaks of radioactivity corresponding to the 4s, 18s and 28s E_{260} peaks of rat-liver cytoplasmic RNA (Fig. 3a). When cycloheximide was added simultaneously with the [³H]uridine, the pattern of radioactivity

Fig. 3. Effect of cycloheximide on $[^3\text{H}]$ uridine incorporation into RNA of cultures stimulated by incubation with PHA. Cultures (2 ml.) were incubated with PHA for 48 hr. The medium was then replaced by fresh medium containing $[^3\text{H}]$ uridine ($6.6 \mu\text{C}/\text{ml.}$) with or without cycloheximide ($25 \mu\text{g.}/\text{ml.}$) and the cultures were incubated for a further 2.0 hr. RNA from three 2 ml. cultures was extracted and analysed as given in Fig. 2. (a) No cycloheximide; (b) cycloheximide. $\circ$, E_{260} ; Δ , radioactivity.

sedimenting at 28s and 18s is not observed but replaced by a broad band at approx. 4–35s. Similar results are obtained after 1 hr. or 3 hr. incubation with $[^3\text{H}]$ uridine. When RNA was extracted at 4° in the absence of sodium dodecyl sulphate, incubation with cycloheximide inhibited the incorporation into the 18s and 28s RNA by about 90% whereas incorporation into 4s RNA was only slightly inhibited (Fig. 4).

Effect of cycloheximide on the incorporation of $[^3\text{H}]$ uridine into ribosomes. Ribosomes were prepared from lymphocytes that had been incubated with PHA for 48 hr. Ascites cells were used to provide carrier ribosomes (see the Materials and Methods section). The profile of ascites-cell ribosomes obtained after centrifuging them through a 10–30% (w/v) sucrose density gradient was not affected by the added lymphocytes. Incorporation of $[^3\text{H}]$ uridine, present during the last 2 hr. of incubation, is shown in Fig. 5. When cycloheximide was added simultaneously with the $[^3\text{H}]$ uridine, incorporation into particles sedimenting at or above 80s was decreased to 40% of the level in control cultures.

Fig. 4. Effect of cycloheximide on $[^3\text{H}]$ uridine incorporation into RNA extracted in the absence of sodium dodecyl sulphate from cultures stimulated by incubation with PHA. Incubation conditions were as given in Fig. 3. RNA was extracted from three 2 ml. cultures at 4° without sodium dodecyl sulphate. Sucrose-density-gradient centrifugation was as given in Fig. 2. (a) No cycloheximide; (b) cycloheximide. $\circ$, E_{260} ; Δ , radioactivity.

Stability of $[^3\text{H}]$ uridine-labelled RNA. To study the stability of RNA labelled by $[^3\text{H}]$ uridine in the presence or absence of cycloheximide, the medium of cultures, labelled with $[^3\text{H}]$ uridine for 1.5 hr., with or without cycloheximide, was replaced by fresh medium containing $10 \mu\text{g.}$ of actinomycin/ml. and non-radioactive uridine (0.1 mM). Actinomycin or uridine at these concentrations, added at the same time as the $[^3\text{H}]$ uridine, inhibited incorporation by 90% or more.

Table 4 shows that, for unstimulated cells, the same percentage of $[^3\text{H}]$ uridine-labelled material remained after a 2 hr. 'chase' with actinomycin and uridine, whether cycloheximide had been added to the culture or not. When cultures were incubated with PHA for 45 hr. before exposure to $[^3\text{H}]$ uridine the material incorporated in the absence of cycloheximide was more stable than that incorporated in its presence.

In some 'chase' experiments unlabelled uridine alone was used. Even when the uridine concentration was as high as 10.0 mM , a concentration that, when added together with $[^3\text{H}]$ uridine, inhibited its incorporation more than 99%, the loss of labelled material was very much slower than that observed with an actinomycin 'chase'.

Table 5 shows the results of experiments in

which the stability of [^3H]uridine-labelled RNA to the presence of cycloheximide during the 'chase' period was examined. The presence of cycloheximide during the 'chase' period did not affect the stability of the labelled RNA whether the

cultures were grown in the presence or absence of PHA.

DISCUSSION

Transformation of lymphocytes into blast cells is accompanied by an increase in the rate of protein and RNA synthesis (Cooper, 1962; McIntyre & Ebaugh, 1962; Epstein & Stohman, 1964; Mueller & Le Mahieu, 1966). Our findings confirm these observations. It is not surprising that cycloheximide inhibited protein synthesis in cultures grown with or without PHA (Fig. 1), for it inhibits protein synthesis in other cells and tissues (Kerridge, 1958; Bennett *et al.* 1964).

There is evidence that much of the RNA synthesized by lymphocytes in the presence of PHA is ribosomal RNA. Electron-microscopic studies have shown that blast cells contain larger numbers of ribosomes and more prominent nucleoli than are found in small lymphocytes (Tanaka, Epstein, Brecher & Stohman, 1963; Johnson & Roberts, 1964). Rubin & Cooper (1965) found that lymphocyte cultures exposed to PHA synthesize significant quantities of ribosomal RNA as well as increased amounts of unstable RNA.

Our results confirm these findings and demonstrate that cycloheximide inhibited [^3H]uridine incorporation into RNA by cultures incubated for 24 or 48 hr. with PHA. Much of the inhibitory effect was on the synthesis of ribosomal RNA, for labelling of ribosomes (Fig. 5) and of the 18s and 28s peaks of ribosomal RNA (Figs. 3 and 4) were decreased by incubation of the cultures with cycloheximide. The RNA labelled in the presence of cycloheximide was less stable than that synthesized in control cultures. Much of the unstable

Fig. 5. Effect of cycloheximide on [^3H]uridine incorporation into ribosomes of cultures stimulated by incubation with PHA. Incubation conditions were as given in Fig. 3. Ribosomes were prepared from three 2 ml. cultures and analysed by sucrose-density-gradient centrifugation on linear 10–30% (w/v) sucrose gradients in medium R. (a) No cycloheximide; (b) cycloheximide. $\circ$, E_{260} ; Δ , radioactivity.

Table 4. Stability of [^3H]uridine incorporated in the presence of cycloheximide

Lymphocyte cultures (0.5 ml.) were incubated with [^3H]uridine ($3.3 \mu\text{C}/\text{ml}$.) with or without cycloheximide ($25 \mu\text{g}/\text{ml}$.) for 1.5 hr. The medium was then replaced by fresh medium containing actinomycin ($10 \mu\text{g}/\text{ml}$.) and uridine (0.1 mM) and the cultures were incubated further as indicated. A: Freshly isolated cultures, without PHA. B: Cultures that had been incubated with PHA for 48 hr. before the addition of fresh medium containing [^3H]uridine.

		[^3H]Uridine incorporated			
	Time at harvesting (hr.)	Control		Cycloheximide	
		(counts/min./culture)	(% of radioactivity in stable form)	(counts/min./culture)	(% of radioactivity in stable form)
A	1.5	3646	(100)	5303	(100)
	2.5	2079	57	3150	59
	3.5	1899	52	2561	48
B	49.5	25313	(100)	16999	(100)
	50.5	12771	50	7105	41
	51.5	11392	45	6379	37

Table 5. *Effect of cycloheximide present during an actinomycin 'chase' on the stability of previously incorporated [³H]uridine-labelled RNA*

Freshly prepared lymphocyte cultures (0.5-ml.), with or without PHA, were incubated with [³H]uridine (3.3 μ C/ml.) for 2.5 hr. The medium was then replaced by fresh medium containing actinomycin (10 μ g./ml.) and uridine (0.1 mM) with or without cycloheximide (25 μ g./ml.) and the cultures were incubated further as indicated.

		[³ H]Uridine incorporated			
		Control		Cycloheximide	
	Time at harvesting (hr.)	(counts/min./culture)	(% of radio-activity in stable form)	(counts/min./culture)	(% of radio-activity in stable form)
With PHA	2.5	5431	(100)	—	—
	3.5	2933	54	3130	57
	4.5	2480	45	2447	45
Without PHA	2.5	4184	(100)	—	—
	3.5	2475	59	2381	57
	4.5	1950	46	1904	45

RNA is not ribosomal precursor (Rubin & Cooper, 1965). This result can be explained in terms of cycloheximide inhibition of ribosomal RNA synthesis, for this would leave a higher proportion of the newly synthesized RNA in an unstable component.

Inhibition of synthesis of ribosomal RNA by cycloheximide has been reported in yeast (Fukuhara, 1965; de Kloet, 1965) and *Neurospora* (Fiala & Davis, 1965). There is an accumulation of RNA with a DNA-like base ratio in yeast treated with cycloheximide (de Kloet, 1965). Unlike the results with yeast, previous reports with mammalian cells have shown little effect of cycloheximide on RNA synthesis. Bennett *et al.* (1964) found some inhibition of RNA labelling in human epidermoid-carcinoma cells between 2 and 4 hr. after cycloheximide, but little inhibition between 4 and 24 hr. Ennis & Lubin (1964) reported that cycloheximide caused no inhibition of uridine incorporation into RNA of L cells during a 1 hr. period but some inhibition was observed after 90–120 min. In these experiments, isotope incorporation alone was measured and no examination of the sedimentation characteristics of the labelled RNA was carried out. It is possible that ribosomal RNA was not synthesized in any quantity under the conditions of their experiments so that any inhibition of ribosomal RNA synthesis was balanced by stimulation of the synthesis of relatively more unstable RNA.

We have observed inhibition of ribosomal RNA labelling when cultures that had been incubated with PHA for 48 hr. were incubated with puromycin, which inhibits ribosomal RNA synthesis in HeLa cells (Tamaoki & Mueller, 1963; Holland, 1963; Latham & Darnell, 1965). The inhibition of ribosomal RNA synthesis by cycloheximide and puromycin is unlikely to be direct. These drugs

might inhibit the synthesis of a specific protein needed for ribosomal RNA synthesis or stability, or inhibition of protein synthesis in general may be able to cause inhibition of the synthesis of ribosomal RNA.

Tables 2 and 3 show that cycloheximide, at certain doses, stimulates the labelling of RNA of lymphocytes grown without PHA. Rubin & Cooper (1965) reported that lymphocytes grown without PHA synthesize little ribosomal RNA. We found that the sedimentation pattern after a 2 hr. labelling period with [³H]uridine was heterogeneous and did not show peaks characteristic of ribosomal RNA (Fig. 2). The sedimentation pattern and the stability of the labelled RNA were unaltered when the cells were grown in cycloheximide, but the incorporation of [³H]uridine was markedly increased.

This effect of cycloheximide could be explained in three ways. It could stimulate the preferential utilization, for RNA synthesis, of a pool of uridine to which the labelled uridine had access. Alternatively it could stimulate the rate of RNA synthesis, or it could stabilize the synthesized RNA.

Bennett *et al.* (1964) suggested that, in epidermoid-carcinoma cells, cycloheximide might influence purine biosynthesis indirectly by inhibition of DNA synthesis, which would allow negative feedback control of nucleotide synthesis by the accumulated nucleotides. This effect is unlikely to apply to cultures of small lymphocytes, for they do not synthesize DNA (Bond, Cronkite, Fliedner & Schork, 1958). But some other effect of cycloheximide on the choice or availability of precursors for RNA synthesis cannot be ruled out.

It is possible that cycloheximide could stabilize RNA. It increases the stability of polysomes in cell-free systems from rat liver (Wettstein, Noll &

Penman, 1964) and rabbit reticulocytes (Williamson & Schweet, 1965). It can inhibit polysome breakdown brought about by actinomycin or ethionine in mouse liver (Trakatellis, Montjar & Axelrod, 1965) and by puromycin in reticulocytes (Colombo, Felicetti & Baglioni, 1965). These reports suggest that cycloheximide might stabilize at least cytoplasmic messenger RNA. We found no difference in stability of RNA during an actinomycin 'chase' whether cycloheximide was present or absent during the 'chase' period (Table 5). Further, we found that puromycin, which increases polysome breakdown in reticulocytes (Marks, Burka, Rifkind & Danon, 1963), also caused an increase in the incorporation of [3 H]uridine into RNA in cells grown without PHA. Stabilization of RNA by cycloheximide in our experiments is possible but improbable.

If actinomycin itself stimulated RNA degradation, as suggested by Acs, Reich & Valanju (1963), the interpretation of 'chase' experiments with actinomycin would be misleading. Our finding that more RNA appeared to be stable when uridine alone was used as a 'chase' material, instead of actinomycin, could be interpreted as evidence in favour of this possibility. However, the evidence is against a direct degradative effect of actinomycin and in favour of the alternative explanation that nucleotides from degraded RNA are preferentially reutilized for RNA synthesis (Levinthal, Fan, Higa & Zimmerman, 1963; Harris, 1964).

Uridine incorporation by small lymphocytes is predominantly nuclear (Hayhoe & Quaglino, 1965; Winter & Yoffey, 1965) and the nuclear RNA of other mammalian cells is rapidly degraded while still in the nucleus (Harris, 1963) to materials that can be reutilized for RNA synthesis. It seems probable to us that the stimulation by cycloheximide of the incorporation of [3 H]uridine is a secondary effect of inhibition of protein synthesis on the metabolism of nuclear RNA.

The stimulation of RNA labelling by cycloheximide was not observed in cultures grown without PHA for more than 24 hr. before the addition of labelled compound. Cultures grown for this time may exhibit some spontaneous transformation (Sabesin, 1965), and large phagocytic cells, distinguishable from blast cells, appear (Berman & Stulberg, 1962). These large cells would be expected to synthesize RNA at a more rapid rate than small lymphocytes, and much of the RNA synthesized would be ribosomal. Under these conditions any stimulation of RNA synthesis by cycloheximide would be obscured.

We are grateful to the Medical Research Council and to the British Empire Cancer Campaign for financial support of this work. We thank the Science Research Council for

a Studentship for J. E. K. and Professor F. G. Young for encouragement.

REFERENCES

- Acs, G., Reich, E. & Valanju, S. (1963). *Biochim. biophys. Acta*, **76**, 68.
- Bennett, L. L., jun., Smithers, S. & Ward, C. T. (1964). *Biochim. biophys. Acta*, **87**, 60.
- Berman, L. & Stulberg, C. S. (1962). *Lab. Invest.* **11**, 1322.
- Bond, V. P., Cronkite, E. P., Fliedner, T. M. & Schork, P. (1958). *Science*, **128**, 202.
- Colombo, B., Felicetti, L. & Baglioni, C. (1965). *Biochem. biophys. Res. Commun.* **18**, 389.
- Cooper, E. H. (1962). *Brit. J. Haemat.* **8**, 304.
- Cooper, E. H., Barkhan, P. & Hale, A. J. (1961). *Lancet*, **ii**, 210.
- Coulson, A. S. & Chalmers, D. G. (1964). *Lancet*, **i**, 468.
- de Kloet, S. R. (1965). *Biochem. biophys. Res. Commun.* **19**, 582.
- Earle, W. R. (1943). *J. nat. Cancer. Inst.* **4**, 165.
- Ennis, H. L. & Lubin, M. (1964). *Science*, **146**, 1474.
- Epstein, L. B. & Stohlman, F. (1964). *Blood*, **24**, 69.
- Fiala, E. S. & Davis, F. F. (1965). *Biochem. biophys. Res. Commun.* **18**, 115.
- Fukuhara, H. (1965). *Biochem. biophys. Res. Commun.* **18**, 297.
- Harris, H. (1963). *Nature, Lond.*, **198**, 184.
- Harris, H. (1964). *Nature, Lond.*, **202**, 249.
- Hayhoe, F. G. T. & Quaglino, D. (1965). *Nature, Lond.*, **205**, 151.
- Holland, J. J. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 436.
- Johnson, F. R. & Roberts, K. B. (1964). *J. Anat., Lond.*, **98**, 303.
- Kerridge, D. (1958). *J. gen. Microbiol.* **19**, 497.
- Latham, H. & Darnell, J. E. (1965). *J. molec. Biol.* **14**, 13.
- Levinthal, C., Fan, D. P., Higa, A. L. & Zimmerman, R. A. (1963). *Cold Spr. Harb. Symp. quant. Biol.* **28**, 183.
- McIntyre, O. R. & Ebaugh, F. C., jun. (1962). *Blood*, **19**, 443.
- Marks, P. A., Burka, E. R., Rifkind, R. & Danon, D. (1963). *Cold Spr. Harb. Symp. quant. Biol.* **28**, 223.
- Marshall, W. H. & Roberts, K. B. (1965). *Quart. J. exp. Physiol.* **50**, 361.
- Mueller, G. C. & Le Mahieu, M. (1966). *Biochim. biophys. Acta*, **114**, 100.
- Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
- Newsome, J. (1963). *Lancet* **ii**, 91.
- Nowell, P. C. (1960). *Cancer Res.* **20**, 462.
- Rubin, A. D. & Cooper, H. L. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 469.
- Sabesin, S. M. (1965). *Science*, **149**, 1385.
- Tamaoki, T. & Mueller, G. C. (1963). *Biochem. biophys. Res. Commun.* **11**, 404.
- Tanaka, Y., Epstein, L. B., Brecher, G. & Stohlman, F. (1963). *Blood*, **22**, 614.
- Trakatellis, A. C., Montjar, M. & Axelrod, A. E. (1965). *Biochemistry*, **4**, 2065.
- Wettstein, F. O., Noll, H. & Penman, S. (1964). *Biochim. biophys. Acta*, **87**, 525.
- Williamson, A. R. & Schweet, R. (1965). *J. molec. Biol.* **11**, 358.
- Winter, G. C. B. & Yoffey, J. M. (1965). *Nature, Lond.*, **208**, 1018.