Stimulation of Incorporation of Nucleic Acid Precursors into HeLa Cells Caused by Proflavine

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1. The effect of proflavine and other acridines on the incorporation of precursors into the nucleic acids of HeLa cells was examined. 2. Relatively low concentrations ($50\mu\text{M}$) of proflavine completely inhibited incorporation of precursors into DNA, but allowed a small extent of incorporation into RNA. 3. Acridine-resistant incorporation into RNA was unaffected by actinomycin D at $2\mu\text{g./ml.}$ and persisted even at high concentrations ($500\mu\text{M}$) of many acridines. 4. A few combinations of acridine and precursor, notably $250\mu\text{M}$ -proflavine and [^{14}C]adenine, caused a stimulation of incorporation. 5. The proflavine-stimulated incorporation was into alkali-stable di- and tri-nucleotides. 6. It was concluded that the effect was due to the preferential inhibition of degradation of a fraction of RNA that normally turned over, thus allowing small radioactive oligonucleotides to accumulate in the cells.

Many of the acridine dyes, e.g. proflavine, Acridine Orange, Acridine Yellow, interact strongly with nucleic acids to produce metachromatic complexes (Morthland, De Bruyn & Smith, 1954). The effects of the acridines on the metabolism of animal cells broadly resemble those produced by actinomycin D: the synthesis of RNA is strongly inhibited and synthesis of protein is relatively unaffected (Reich, Franklin, Shatkin & Tatum, 1962; Weisner, Acs, Reich & Shafiq, 1965; Scholtissek & Rott, 1964; Watts & Davis, 1966). The acridines inhibit the DNA-dependent synthesis of RNA *in vitro*; it is therefore probable that, as with actinomycin D, inhibition of RNA polymerase is of importance in their action *in vivo* (Hurwitz, Furth, Malamy & Alexander, 1962; Waring, 1964). This is unlikely to be the only point of action, however, since the acridines also inhibit other enzymes (Albert, 1966*b*; Leith, 1963; Weinstein & Finkelstein, 1967) and interact with a variety of polyanions (Bradley & Wolf, 1959; Saunders, 1964).

Although the acridines inhibit the DNA-dependent RNA polymerase, Scholtissek & Rott (1964) have described the synthesis of RNA in chick-embryo cells, which is very resistant to proflavine; the precise nature of the RNA made under these conditions is not known, but Scholtissek (1965) concluded that it was synthesis of RNA, not simply a turnover of end groups. During a study of the effects of different acridines on RNA synthesis in HeLa cells and isolated HeLa-cell nuclei, acridine-resistant RNA synthesis of this type has

been observed, but it became apparent that a few acridines also produced an apparent stimulation of RNA synthesis. Some of these results are reported here.

METHODS

HeLa cells were grown in suspension culture by the methods described by Watts (1964).

Materials. [$8\text{-}^{14}\text{C}$]Adenine (sp. radioactivity 30mc/m-mole), [$\text{G-}^{14}\text{C}$]guanosine (sp. radioactivity 30mc/m-mole), [$\text{G-}^{14}\text{C}$]uridine (sp. radioactivity 30mc/m-mole), [$2\text{-}^{14}\text{C}$]thymidine (sp. radioactivity 30mc/m-mole), [$8\text{-}^{14}\text{C}$]ATP (sp. radioactivity 18mc/m-mole) and [$2,8\text{-}^3\text{H}$]adenine (sp. radioactivity 2c/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Proflavine, 9-aminoacridine, Acridine Orange and acriflavine were obtained from British Drug Houses Ltd., Poole, Dorset; mepacrine and ethidium bromide were from Boots Pure Drug Co. Ltd., Nottingham; rivanol and Acridine Yellow were from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Acridine Orange was recrystallized from aq. 50% (v/v) ethanol. Acriflavine was freed from proflavine by the method described by Albert (1966*a*). Actinomycin D was obtained from Merck, Sharpe and Dohme Ltd., Hoddesdon, Herts. Pancreatic ribonuclease (EC 2.7.7.16), venom phosphodiesterase (EC 3.1.4.1) and spleen phosphodiesterase (EC 3.1.4.1) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Labelling of cells in presence of acridines. Solutions and cultures containing acridines were kept in the dark and exposed only briefly to subdued light. About 5×10^6 cells in 10 ml. of medium were pipetted into 15 ml. conical centrifuge tubes containing the radioactive precursor ($0\text{-}3\mu\text{C/tube}$) and suitable amounts of an aqueous solution of the acridine.

The tubes were closed with rubber stoppers and rotated on a wheel at 15 rev./min. for 30 min. at 37°. The samples were then centrifuged at 300 *g* for 3 min. and the pellet of cells was subjected to one of the following methods of fractionation.

(1) The cells were washed twice with 3 ml. of 0.6 *M*-trichloroacetic acid at 0° and then with 3 ml. of ethanol.

(2) The cells were washed as before with 0.6 *M*-trichloroacetic acid and ethanol and then extracted with 3 ml. of aq. 10% (w/v) NaCl for 1 hr. at 100°. The extract was made 70% (v/v) with respect to acetone and the precipitate collected.

(3) The cells were shaken for 5 min. at 4° with 3 ml. of water that had been saturated with phenol (Harris & Watts, 1962). Ethanol (4 ml.) was then added, and the sample was centrifuged at 2000 *g* for 10 min. The precipitate was washed with 3 ml. of aq. 70% (v/v) ethanol and then with 3 ml. of ethanol.

Measurement of radioactivity. The method described by Watts (1969) was used.

Radioautography. Cells were grown for 30 min. in medium containing [2,8-³H]adenine (5 μ C/ml.) and suitable concentrations of proflavine. The cultures were then centrifuged at 300 *g* for 3 min. and the cells were fixed in 5 ml. of Bouin's solution, washed twice with 5 ml. of fixative and twice with 5 ml. of ethanol. The fixed cells were stored in aq. 70% (v/v) ethanol at 4°. The cells were flattened on glass slides by suspending them in water or aq. 45% (v/v) acetic acid at 4° and flattening them under a cover-glass. The preparation was then frozen with solid CO₂ and the cover-glass levered off. Radioautographs were prepared in the usual way with Kodak AR10 stripping film, exposed for 2 weeks at 4° and developed.

Chromatography. Descending chromatography on sheets of Whatman no. 1 paper was carried out with the following solvents: A, butan-1-ol saturated with water and containing 1% (v/v) of aq. NH₃ (sp.gr. 0.88) (MacNutt, 1952); B, methanol-ethanol-conc. HCl-water (50:25:6:29, by vol.) (Kirby, 1956); C, isobutyric acid-M-NH₃-M-EDTA (100:60:1:6, by vol.) (Krebs & Hems, 1953); D, aq. 70% (v/v) propan-2-ol + 0.35 ml. of aq. NH₃ (sp.gr. 0.88)/l. of tank volume (Markham & Smith, 1952).

The u.v.-absorbing regions on the chromatograms were located with a Hanovia Chromatolite lamp. Radioactive regions were located by radioactivity with Kodak Blue Brand X-ray film.

Chromatography on columns of DEAE-cellulose was essentially by the method of Holley, Everett, Madison & Zamir (1965). HeLa cells (2×10^7) were grown for 30 min. in medium containing [8-¹⁴C]adenine and proflavine and then dispersed in 3 ml. of aq. 2% (w/v) sodium dodecyl sulphate; 3 ml. of aq. 80% (w/v) phenol was added and the mixture was shaken at 4° for 30 min. The sample was centrifuged at 2000 *g* for 10 min., the lower phenol phase discarded and the residue was extracted a second time with 3 ml. of aq. 80% (w/v) phenol and again centrifuged. The upper aqueous phase was collected and acetone (about 1 vol.) gradually added at 0° until a precipitate formed. The precipitate of RNA and DNA was removed by centrifugation and acetone added to the supernatant fluid to a final concentration of 75% (v/v). The precipitate forming overnight at -20° was collected and dissolved in 4 ml. of a solution containing partially hydrolysed yeast RNA. The partial hydrolysate of yeast RNA was prepared by incubating 5 mg. of yeast RNA

(British Drug Houses) for 4 hr. at 37° with 0.5 mg. of pancreatic ribonuclease in 5 ml. of 0.1 *M*-tris-HCl buffer, pH 7.5 (Madison, Everett & King, 1967). The hydrolysate was shaken with an equal volume of aq. 80% (w/v) phenol to remove protein and the upper phase containing the hydrolysed RNA was collected and freed from phenol by extracting it with 2 $\times$ 5 ml. of ether.

The solution of radioactive sample with carrier was put on to a column of DEAE-cellulose (Calbiochem, Los Angeles, Calif., U.S.A.) (12.5 cm. $\times$ 0.35 cm.; exchange capacity 0.5 m-equiv./g.) and eluted with a linear gradient formed from 60 ml. of 7 *M*-urea and 60 ml. of 0.6 *M*-sodium acetate in 7 *M*-urea. Fractions (1 ml.) were collected and the *E*₂₆₀ and radioactivity determined. The recovery of radioactivity from the columns was at least 85%.

Loss of RNA from isolated nuclei. Nuclei were prepared by a modification of the method of Fisher & Harris (1962) as described previously (Watts, 1969). The nuclei were used as a suspension in 0.25 *M*-sucrose in 0.1 *M*-tris-HCl buffer (pH 7.5 at 20°). Samples (1 ml.) containing about 2×10^6 nuclei and suitable concentrations of proflavine were incubated for 30 min. at 37° and then centrifuged for 3 min. at 300 *g*. The supernatant was collected, 1 mg. of yeast RNA as carrier was added and both the pellet and supernatant were acidified with 2.5 ml. of 0.6 *M*-trichloroacetic acid at 0°. The samples were centrifuged at 2000 *g* for 5 min. at 0° and the radioactivity in the precipitates was determined.

Synthesis of RNA by isolated nuclei. Nuclei were prepared from non-radioactive cells and incubated with [8-¹⁴C]ATP under conditions similar to those used by Widnell & Tata (1966). Radioactivity continued to be incorporated into acid- and ethanol-insoluble material for at least 1 hr. Each sample (final vol. 0.5 ml.) contained about 6×10^6 nuclei, 5 mM-MgCl₂, 4 mM-MnCl₂, 0.4 *M*-(NH₄)₂SO₄, 0.1 *M*-tris-HCl buffer, pH 7.5, 1 mM-GTP, 1 mM-CTP, 1 mM-UTP, 50 mM-sucrose and 0.05 μ C of [8-¹⁴C]ATP. Various concentrations of proflavine were also present. The samples were incubated for 45 min. at 37° and then cooled to 0° and precipitated with 2.5 ml. of 0.6 *M*-trichloroacetic acid. The precipitate was washed once with water and extracted for 1 hr. at 100° with 3 ml. of aq. 10% (w/v) NaCl. Then 2 vol. of ethanol was added to the extract and the radioactivity in the precipitate was determined.

RESULTS

Effect of proflavine on the incorporation of precursors into RNA. Fig. 1 shows the effect of proflavine on the incorporation of [¹⁴C]uridine. Under the conditions used, most of the radioactivity in the control culture was in nuclear RNA (Watts, 1964). Increasing concentrations up to 40 μ M-proflavine progressively decreased incorporation to about 10% of that observed in absence of acridine. Further increases in concentration up to 500 μ M-proflavine only slowly decreased the extent of residual incorporation, which was clearly very resistant to proflavine. The residual incorporation was also observed when the cells were extracted by methods 2 and 3. Dinitrophenol inhibited proflavine-resistant incorporation, indicating that the

Fig. 1. Incorporation of [8-¹⁴C]adenine and [¹⁴C]uridine into HeLa cells in the presence of proflavine. The cells were grown for 30 min. in medium containing the labelled precursor and various concentrations of proflavine and the acid-precipitable radioactivity was then determined. ●, Incorporation of [¹⁴C]uridine; ○, incorporation of [8-¹⁴C]adenine.

Table 1. *Effect of dinitrophenol on incorporation of [8-¹⁴C]adenine in the presence of proflavine*

Cells were grown for 30 min. in medium containing [8-¹⁴C]adenine and various concentrations of proflavine, with and without the addition of 2 mM-dinitrophenol. The acid-precipitable radioactivity was then determined.

Concn. of proflavine (μM)	Concn. of dinitrophenol (mM)	Radioactivity incorporated (c.p.m./10 ³ cells)
0	0	21.3
	2	11.3
160	0	3.49
	2	1.22
310	0	3.38
	2	2.44
470	0	6.29
	2	4.56
760	0	11.1
	2	6.98

process was energy-dependent; Table 1 shows the effect of dinitrophenol on incorporation of [8-¹⁴C]adenine in the presence of proflavine. It is thus unlikely that the effect was due to a non-specific absorption of label. This result agrees with the observations of Scholtissek & Rott (1964) on the effect of proflavine on the incorporation of [¹⁴C]uridine into chick-embryo cells. It therefore seems probable that a proflavine-resistant synthesis of RNA may be a general feature of animal cells.

Adenine possesses a number of advantages over pyrimidine derivatives as a precursor for labelling RNA (Watts, 1964), and the effect of proflavine on the incorporation of [8-¹⁴C]adenine was therefore examined. Fig. 1 shows that the lower concentrations of proflavine produced effects similar to those found with [¹⁴C]uridine: incorporation of labelled adenine fell to a value that was about 10% of that into the control culture. In contrast, however, higher concentrations of proflavine produced a large increase in the incorporation of [8-¹⁴C]adenine, with a maximum at about 250 μM-proflavine. The maximum extent of stimulated incorporation was usually about 50% of that observed in the absence of proflavine, although on some occasions the incorporation was actually greater than that in the control culture. The effect was sensitive to the conditions of culture, particularly pH, the optimum value for which was about 7.4. The concentration of proflavine producing maximum incorporation normally lay in the range 200–600 μM.

The effect of proflavine on the incorporation of [¹⁴C]guanosine was qualitatively similar to that on the incorporation of [¹⁴C]adenine. The lower concentrations of proflavine (up to 100 μM) decreased incorporation to about 2% of that into the control culture and this rose to about 9% of the control value at about 230 μM-proflavine.

Since there is evidence that some acridines may inhibit the synthesis of phosphorylated precursors of RNA (Scholtissek & Becht, 1966), the effect of proflavine on the incorporation of [¹⁴C]uridine was studied by introducing the acridine after the cells had already grown for 30 min. in medium containing [¹⁴C]uridine. Under these conditions there was a large radioactive intracellular pool of phosphorylated pyrimidine precursors available for incorporation into RNA. Again, however, there was no evidence of stimulation of incorporation of [¹⁴C]uridine.

The incorporation of uridine and adenine in the absence of proflavine is under these conditions almost entirely into RNA. The stimulated incorporation might, however, have been due to labelling of DNA. The effect of proflavine on the incorporation of a precursor into DNA was therefore examined. Proflavine (50 μM) decreased incorporation of [2-¹⁴C]thymidine to about 1% of that into the control culture and there was no evidence that there was increased incorporation at higher concentrations of acridine.

The incorporation of [¹⁴C]uridine and [8-¹⁴C]adenine in the presence of other acridines was also examined, but none of the compounds listed in the Methods section produced an effect on the scale obtained with proflavine and [8-¹⁴C]adenine. There was a 27% increase in incorporation of [¹⁴C]uridine

Fig. 2. Time-course of incorporation of [8-¹⁴C]adenine into HeLa cells in the presence of proflavine. The cells were grown in medium containing [8-¹⁴C]adenine and proflavine, harvested at intervals and the acid-precipitable radioactivity was determined. ●, Incorporation in the absence of proflavine; ○, 760 μM-proflavine present; ▲, 80 μM-proflavine present.

in the presence of 25 μM-Acridine Orange, and 9-aminoacridine and rivanol produced small effects on the incorporation of [8-¹⁴C]adenine. The phenanthridine ethidium bromide, which interacts with nucleic acids (Elliott, 1963), was also examined, but produced no stimulation of incorporation.

The time-course of incorporation of [8-¹⁴C]adenine in the presence of proflavine was consistently of the form shown in Fig. 2. The higher concentration of proflavine used was that which gave maximum stimulation of incorporation. Incorporation was approximately linear for the first 40 min. and then fell sharply, suggesting that an essential metabolite had been exhausted.

Attempts to localize by radioautography the site of incorporation of [³H]adenine in the presence of high concentrations of proflavine were only partially successful. Most of the radioactivity was in the nucleus, but there was diffusion of label out of the cells during preparation of the radioautographs, which impaired resolution.

Effect of actinomycin D on the incorporation of [14C]adenine in the presence of proflavine. Actinomycin D is an efficient inhibitor of RNA polymerase (Hurwitz *et al.* 1962); at a concentration of 2 μg./ml., however, it had virtually no effect on the ability of proflavine to stimulate incorporation of [8-¹⁴C]adenine (Fig. 3). Similarly, low concentrations of mepacrine (50 μM), which when used alone greatly decreased the incorporation of labelled precursors,

Fig. 3. Effect of actinomycin D on the incorporation of [8-¹⁴C]adenine into HeLa cells in the presence of proflavine. HeLa cells were grown for 30 min. in medium containing [8-¹⁴C]adenine and proflavine, with and without the addition of actinomycin D (2 μg./ml.). The acid-precipitable radioactivity in the cells was then determined. ●, Incorporation without actinomycin D; ○, incorporation with actinomycin D.

failed to inhibit the stimulation of incorporation produced by proflavine.

Nature of the incorporation stimulated by proflavine. The extraction of HeLa cells with a cold aqueous solution of phenol separates the nuclear and cytoplasmic fractions of RNA (Harris & Watts, 1962). When the experiments shown in Fig. 1 were repeated with the use of phenol (method 3) to fractionate the cells and the acid-precipitable radioactivity was determined, there was no detectable stimulation of incorporation of [8-¹⁴C]adenine. The material into which radioactivity was incorporated was thus either acid-soluble or very sensitive to acid after the cells had been disrupted with phenol. Cells were also extracted with hot aq. 10% (w/v) sodium chloride (method 2) and the radioactivity precipitated by aq. 70% (v/v) acetone was determined. In this case stimulation of incorporation was observed, although the sodium chloride method of extraction gave only 50% recovery of radioactivity. The material into which the radioactivity was incorporated therefore appears to have a relatively low molecular weight.

Chromatography of the material into which incorporation of [8-¹⁴C]adenine was stimulated by proflavine. A measure of the chain length of the material into which stimulated incorporation occurred was obtained by chromatography. Cells

that had been labelled with [8-¹⁴C]adenine in the presence of a concentration of proflavine that stimulated incorporation were extracted as described in the Methods section and chromatographed on a column of DEAE-cellulose. Fig. 4 shows the pattern of elution of radioactivity; the radioactivity was present in oligonucleotides containing up to three residues, with most of the label in trinucleotides.

Fig. 4. Chromatography of the radioactivity incorporated into HeLa cells in the presence of proflavine. The experimental details are given in the text; HeLa cells were grown for 30 min. in medium containing [8-¹⁴C]adenine and 400 μ M-proflavine. The cells were then harvested and the radioactive material was extracted and chromatographed on a column of DEAE-cellulose. —, E_{260} ; ●, radioactivity.

Paper chromatography was used to obtain additional information about the material labelled in the presence of proflavine (Table 2). Cells that had been labelled with [8-¹⁴C]adenine in the presence of proflavine were extracted in the usual way with trichloroacetic acid and ethanol and then hydrolysed for 18 hr. at 37° with 0.3 M-potassium hydroxide. The hydrolysate was acidified with m-perchloric acid at 0° and the precipitate of potassium perchlorate, protein and DNA removed by centrifugation. The supernatant fluid was examined by chromatography on Whatman no. 1 paper with the solvent systems described in the Methods section. Chromatography in solvent C showed that the radioactivity from the control and from cells grown in the presence of 50 μ M-proflavine was almost entirely in the 2'- and 3'-phosphates of adenosine. All the radioactivity in the samples that showed a stimulated incorporation was found in two regions; most of the radioactivity was localized in a region that had an R_F approximately equal to that of GMP, the residue in a region with an R_F slightly greater than that of GMP. When the hydrolysate was heated with m-hydrochloric acid at 100° for 30 min. to liberate the purines and then chromatographed in solvents A and B, the radioactivity was all found in the region where adenine was localized. Thus the material obtained by alkaline hydrolysis was an adenine derivative, but not a simple mononucleotide. When the alkaline hydrolysate was chromatographed in solvent D the radioactivity was found in the region where di- or tri-nucleotides would be expected to run. It seems likely therefore that the radioactivity incorporated into the cells in the presence of high concentrations of proflavine

Table 2. Paper chromatography of the incorporation of [8-¹⁴C]adenine in the presence of proflavine

Cells were grown for 30 min. in medium containing [8-¹⁴C]adenine and two concentrations of proflavine, the higher of which stimulated incorporation. The acid-precipitable radioactivity was then prepared, hydrolysed with 0.3 M-KOH and subjected to paper chromatography in different solvents by the methods given in the text. In some cases the sample was further hydrolysed for 30 min. with m-HCl at 100° to liberate purines. The relative intensities of radioactive regions are in the order 0.65 > 0.65 > 0.65.

Solvent	Concn. of proflavine (μ M)	R_F values			
		Radioactive regions	3'-AMP	3'-GMP	Adenine
A	0	0	0	0	
	150	0			
	600	0			
	600*	0.42			0.42
B	600*	0.50			0.50
C	0	0.42; 0.46; 0.65	0.65	0.42	
	150	0.42; 0.65			
	600	0.41; 0.45			
D	600	0.49†	1.0	0.67	

* After hydrolysis with m-HCl.

† Relative to 3'-AMP.

Fig. 5. Effect of proflavine on the loss of labelled RNA from isolated nuclei. Samples containing 2×10^6 nuclei, prepared from HeLa cells that had been grown for 30 min. in medium containing $[8\text{-}^{14}\text{C}]$ adenine, were incubated for 30 min. at 37° in 0.25M -sucrose in 0.1M -tris-HCl buffer, pH 7.5. The acid-precipitable radioactivity in the nuclei and incubation medium was then determined. ●, Radioactivity in the nuclei; ○, radioactivity in the medium; ▲, total radioactivity in nuclei and medium; ----, total radioactivity in nuclei + medium before incubation.

was in alkali-resistant di- and tri-nucleotides. Some confirmation of this possibility was obtained by enzymic hydrolysis. The alkaline hydrolysate was treated with venom diesterase in 0.01M -tris-hydrochloric acid buffer, pH 8.5, containing 0.01M -magnesium chloride, or spleen diesterase in 0.01M -tris-hydrochloric acid buffer, pH 7.0, for 4 hr. at 37° . Both enzymic hydrolysates now gave three radioactive regions when chromatographed in solvent *C*. The new component had an R_F similar to that of AMP. Venom diesterase preferentially hydrolysed the faster-moving of the two original radioactive regions, whereas spleen diesterase hydrolysed the other.

Effect of proflavine on the loss of RNA from isolated nuclei. The ability of proflavine to stimulate incorporation of $[8\text{-}^{14}\text{C}]$ adenine is unlikely to be due to a real increase in the rate of synthesis of RNA; it is much more likely to be due to inhibition of degradation of RNA. Indirect support for this idea comes from the studies of the degradation of RNA in isolated nuclei (Watts, 1969). Cells were grown for a brief period in medium containing $[8\text{-}^{14}\text{C}]$ adenine and the nuclei were then isolated and incubated at 37° with different concentrations of proflavine. Fig. 5 shows that in the control nuclei there was a

Fig. 6. Effect of proflavine on the incorporation of $[8\text{-}^{14}\text{C}]$ -ATP into the RNA of isolated nuclei. Samples containing 6×10^6 nuclei were incubated with $[8\text{-}^{14}\text{C}]$ ATP and unlabelled GTP, CTP and UTP under the conditions described in the text and the radioactivity in the RNA was determined.

rapid loss of labelled RNA, both by degradation and by diffusion of RNA out of the nuclei. This loss was progressively inhibited by increasing concentrations of proflavine until, at about $230\text{ }\mu\text{M}$, the loss of labelled RNA was completely suppressed. Thus the concentration suppressing degradation of RNA was that which stimulated incorporation of $[8\text{-}^{14}\text{C}]$ adenine into intact cells.

Effect of proflavine on the synthesis of RNA in isolated nuclei. Nuclei were isolated and then incubated under conditions that permitted incorporation of $[^{14}\text{C}]$ ATP into RNA. Fig. 6 shows that synthesis of RNA *in vitro* was considerably less sensitive to proflavine than was degradation of rapidly labelled RNA (Fig. 5). Incorporation of $[^{14}\text{C}]$ ATP into nuclear RNA was still about 50% of that into the control at a concentration of proflavine ($230\text{ }\mu\text{M}$) that was sufficient to inhibit degradation of labelled RNA in isolated nuclei. Low concentrations of proflavine actually stimulated incorporation of $[^{14}\text{C}]$ ATP into isolated nuclei. This was a reproducible effect observed with most of the acridines examined and presumably was also due to the preferential inhibition of degradative enzymes, rather than to a real increase in the rate of synthesis of RNA.

DISCUSSION

It is convenient to distinguish two types of incorporation of RNA precursors in the presence of acridines. The first is the residual, acridine-resistant, incorporation of precursors into RNA described by Scholtissek & Rott (1964). This is a fairly general effect observed with many acridines and RNA precursors and the products of alkaline

hydrolysis of the labelled material are similar to those obtained from normal RNA, i.e. predominantly riboside 2'- and 3'-monophosphates. Incorporation in this case is relatively insensitive to acridine concentration and is into RNA molecules that are large enough to be precipitated by trichloroacetic acid or aq. 70% (v/v) ethanol. The second type, the acridine-stimulated incorporation described here, may be related to the first class, but showed several major differences. It did not occur with all combinations of acridines and RNA precursors and was strongly dependent on the acridine concentration. The proflavine-stimulated incorporation of [^{14}C]adenine was into small fragments that were resistant to hydrolysis by alkali. The smallness of the fragments was obscured because trichloroacetic acid did not extract them unless the cell structure was first disrupted with phenol or detergent. This indicates that the fragments were in a matrix of acid-precipitable material, e.g. protein. The resistance to alkali of the oligonucleotides shows that the 2'-hydroxyl group of the sugar moiety was either absent, as in deoxyribose, or substituted, as in 2'-O-methylribose (Smith & Dunn, 1959). In view of the virtually complete inhibition of DNA synthesis at these concentrations of proflavine, the latter of these possibilities is the more probable, although there is no evidence as to the nature of the substituent in the 2'-position. Alkali-resistant di- and tri-nucleotides containing 2'-O-methylribose are obtained mainly from ribosomal RNA and the site of methylation is believed to be the nucleolus (Greenberg & Penman, 1966). This material may be nucleolar in origin, but it is difficult to see how it could be part of a precursor of ribosomal RNA since synthesis of ribosomal RNA involves DNA-dependent RNA polymerase. Actinomycin D and the acridines inhibit RNA polymerase. Both forms of incorporation in the presence of acridines were unaffected by actinomycin D and the mechanism of the acridine-resistant incorporation must therefore be different from that concerned with the synthesis of the bulk of the cellular RNA.

It is unlikely that acridines would stimulate incorporation of RNA precursors and the evidence obtained with isolated nuclei supports the view that the effect was due to the inhibition of degradation of RNA. It is necessary to assume that there is a fraction of RNA that turns over rapidly and whose synthesis is less sensitive to proflavine than is its degradation. Thus over a narrow range of proflavine concentration, products of degradation accumulate,

giving the impression that incorporation has increased.

The properties of this fraction into which stimulated incorporation occurred resemble in some ways those of a component of RNA found in HeLa cells growing normally. This RNA turned over very rapidly and was largely lost when the cells were extracted with phenol, but not when they were extracted with trichloroacetic acid (Watts, 1964).

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