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EFFECT OF DAUNOMYCIN ON HeLa CELL NUCLEIC ACID SYNTHESIS

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Abstract—In the presence of daunomycin (0·5 μg/ml) HeLa cell DNA synthesis was irreversibly inhibited. RNA synthesis although initially inhibited gradually reversed and returned to normal levels by 24 hr. Analysis showed that the drug selectively inhibited ribosomal RNA and only partially inhibited heterogeneous RNA which was altered in physical characteristics. Inhibition of protein synthesis was a late event indicating the continued synthesis of messenger RNA in the presence of daunomycin. A possible mechanism to explain these differential effects is discussed.

INHIBITION of nucleic acid synthesis by daunomycin is now well established.[1–3] Although a concentration of 1 μg/ml produces considerable inhibition of DNA and RNA synthesis in HeLa cells at 4 hr, protein synthesis is relatively unaffected.[3] Daunomycin *in vitro* has been shown to intercalate with DNA and the observed inhibition of nucleic acid synthesis is thought to be a consequence of this interaction. Inhibition of protein synthesis would therefore be expected to follow as a result of a reduction in the synthesis of messenger RNA. Since the average half-life of HeLa cell messenger RNA is 5–6 hr[4] no effect on protein synthesis by daunomycin would be expected to occur until after this time had elapsed. In order to study these effects it is necessary to extend the period of observation beyond 4 hr. In this paper we report the results of experiments in which HeLa cells were exposed to various concentrations of daunomycin for periods as long as 24 hr. The results obtained show that daunomycin is selective in its action on the synthesis of various species of RNA.

MATERIALS AND METHODS

HeLa cells. The CCL2 line was obtained originally from the American Type Culture Collection Cell Repository and it has been continuously maintained in this laboratory for 2 years. Cultures were used for experiments in the logarithmic phase of growth 2 days after seeding.

Media

(a) *Growth medium.* Eagle's minimal essential medium supplemented with 10 per cent calf serum was used throughout for propagation of cell stocks. An antibiotic mixture was added to give a final concentration of penicillin 50 units, streptomycin 50 μg, neomycin 25 μg, and bacitracin 1·25 units/ml of medium.

(b) *Maintenance medium.* Eagle's minimal essential medium supplemented with 1 per cent or 2 per cent calf serum and the antibiotic mixture was used in all experiments.

Chemicals

All radioactive materials were purchased from the Radiochemical Centre, Amersham, Bucks. These included [^{3}H]uridine 6·7 c/m-mole, 5[^{3}H]thymidine 5·0 c/mole and [^{14}C]leucine 36·6 mc/m-mole. Daunomycin (Brand Daunorubicin) was a gift from May & Baker Ltd.

Incorporation of radioactive precursors

Growth medium from exponentially growing HeLa cells was replaced with maintenance medium containing either 0·2 or 1·0 μc of [3H]uridine or [^{3}H]thymidine/ml. After an appropriate period of incorporation synthesis was stopped by decanting the medium and replacing it with 10 ml of chilled Earle's saline. Subsequently each bottle was shell frozen in an ethylene-glycol bath at $-15°$. After removal from the bath, cells were effectively removed from the glass by shaking the gradually melting Earle's saline. In those experiments where sub-cellular fractionations were performed, cells were removed from the glass by replacing radioactive medium with frozen Earle's saline sticks (10 ml) and shaking vigorously. Such a procedure facilitates the subsequent subcellular fractionation.

Extraction and analysis of nucleic acids

DNA. The extraction method of Marmur[5] modified by Harley *et al.*[6] was used.

RNA. Total cell RNA was extracted by the method of Scherrer and Darnell.[7] Where sub-cellular fractionation was performed prior to RNA extraction the fractionation procedures were based on those of Penman[8] and Penman, Smith and Holzman.[9] Washed cell pellets were swollen in low ionic strength buffer, homogenized in a Dounce-type homogenizer and then treated with mixed detergent to release clean nuclei which were deposited by centrifugation leaving the cytoplasmic fraction in the supernatant. The nuclei were lysed in high ionic strength buffer and separated into nucleoli and nucleoplasm by treatment with deoxyribonuclease. After fractionation RNA was extracted from the cytoplasm by preliminary precipitation with 2·5 vol. of ethanol at $-15°$ and followed by extraction of the precipitate with SDS and cold phenol. The aqueous phase was re-extracted twice with chloroform–isoamyl alcohol before precipitation overnight with 2·5 vol. of potassium acetate–alcohol. The final precipitate was dissolved in a suitable volume of buffer. RNA was extracted from the nucleoplasm in the same way except that phenol extraction was performed at 60°, and similarly from the nucleoli but without preliminary precipitation with ethanol.

Polyacrylamide gel electrophoresis

The method of Loening[10] was used. 2·4 per cent acrylamide gels after clearing were loaded with 100 μl of sample and electrophoresis was carried out for 3 hr at 125 V. After scanning at 265 mμ in the Joyce–Loebl u.v. scanner the gels were frozen in CO_2 snow before slicing in the Mickle Laboratories gel slicer. Two slices of approximately 0·9 mm comprised each sample for scintillation counting. These were hydrolysed in

0·35 ml N NaOH at 37° for 3–4 hr before the addition of 10 ml BBOT scintillation fluid.[11,12] Samples were counted in the Packard Tricarb scintillation counter.

RESULTS

To determine whether inhibition of nucleic acid synthesis by daunomycin is followed by a secondary inhibitory effect on protein synthesis, HeLa cell monolayers were treated with daunomycin at a concentration of 0·5 μg/ml and the incorporation of radioactive thymidine, uridine, and leucine into DNA, RNA, and protein, respectively, was measured at regular intervals. Observations over a 24-hr time period showed that DNA synthesis was significantly inhibited throughout the period of the experiment. In contrast, RNA synthesis although initially inhibited soon began to recover and its recovery was complete by 24 hr. Under these conditions no inhibition of protein synthesis was observed.

In the absence of any long lasting inhibition of RNA synthesis it is not possible to detect any secondary inhibition of protein synthesis. Therefore, higher concentrations of daunomycin were employed in an effort to prolong the inhibition of RNA synthesis. At a concentration of 10 μg/ml, inhibition of RNA synthesis persisted throughout the 24-hr period. Under these conditions, a significant fall in protein synthesis was observed at 12 hr which developed and was almost complete at 24 hr

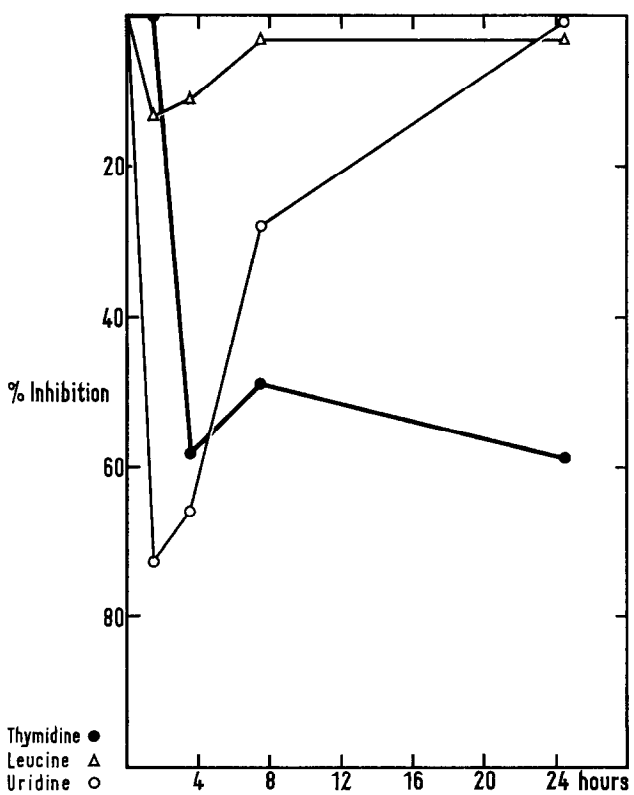

FIG. 1.

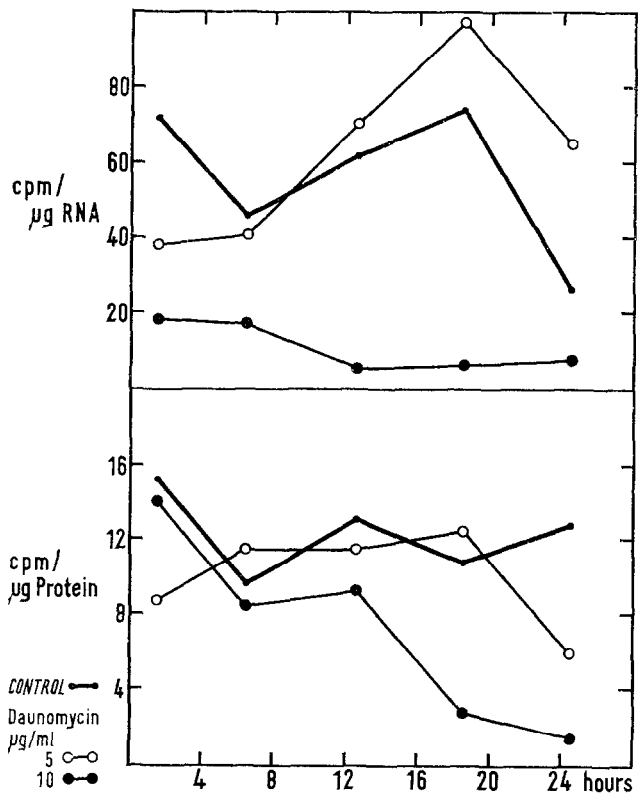

FIG. 2.

(Fig. 2). If this fall in protein synthesis was secondary to an inhibitory effect on mRNA synthesis its onset would have been expected earlier in the course of the experiment, since the half life of HeLa cell messenger RNA is 5–6 hr. Any secondary inhibition of protein synthesis should, therefore, become apparent at this time, as occurs with actinomycin D.[4]

Since messenger RNA appeared to be functionally unaffected by daunomycin a possible effect on ribosomal RNA synthesis was considered. HeLa cells untreated and treated with daunomycin (5 μg/ml) were incubated for 1·5 hr and afterwards labelled with [^{3}H]uridine for 40 min. After subcellular fractionation, RNA was extracted from the cytoplasmic, nucleolar and nucleoplasmic fractions and analysed by polyacrylamide gel electrophoresis. The results obtained are illustrated in Fig. 3. In Fig. 3a, the characteristic 45 and 32 S ribosomal RNA precursors can be seen in the radioactive and optical density profiles of the nucleolar fraction from untreated cells, and in Fig. 3c the characteristic 28 and 18 S ribosomal RNA can be seen in the radioactive profile of the cytoplasmic fraction of these cells. In contrast, the profiles of the nucleolar and cytoplasmic fractions from treated cells show an almost complete cessation of ribosomal RNA synthesis (Figs. 3b and c).

In the nucleoplasmic fraction of untreated cells (Fig. 3d) the radioactive label is seen to be incorporated in the nucleoplasmic heterogeneous RNA[13,14] but some label

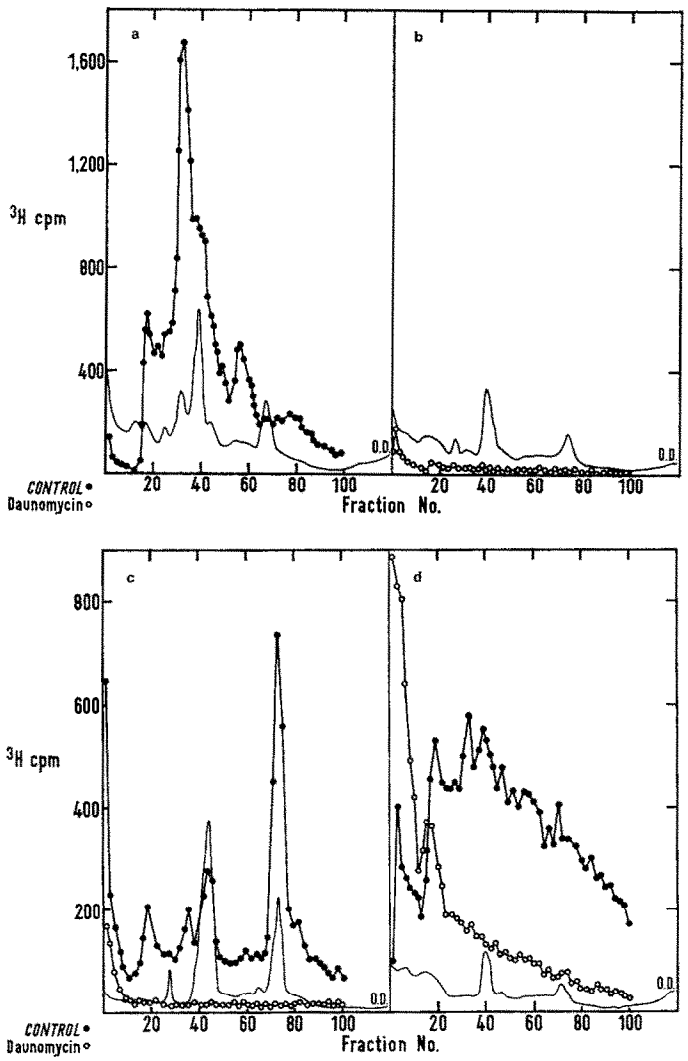

FIG. 3.

was detectable in the contaminant ribosomal RNA precursors. After treatment, daunomycin produced some inhibition in the total amount of radioactive uridine incorporated into heterogeneous RNA, but most striking was the altered distribution of the incorporated label which became located predominantly in the early fractions representing slow moving RNA.

DISCUSSION

Since intercalation of daunomycin with the DNA helix is non specific,[15–17] transcription of both DNA and RNA from the template should be equally affected. However, the results of this investigation and that of Kim *et al.*[3] show that daunomycin inhibits

DNA synthesis *in vivo* more than RNA synthesis. If DNA transcription is initiated at a single site and the whole of the DNA template is required for DNA replication, even a single intercalated molecule by interrupting the base sequence would interfere with DNA replication. In contrast, RNA chains are of variable length so that transcription would require shorter nucleotide sequences and would be initiated from multiple sites. If so, more intercalated molecules would be required to completely prevent transcription of RNA than DNA. The reversibility of the inhibitory effect on RNA synthesis of low concentrations of daunomycin suggests that intercalation of the drug with the DNA helix must also be at least partially reversible.

In circumstances where inhibition of RNA synthesis by daunomycin is not complete, preferential inhibition of ribosomal RNA is striking and similar to that produced by small doses of actinomycin D and mitomycin C.[14] This suggests that ribosomal genes are read sequentially so that transcription requires the reading of a long uninterrupted base sequence and a single or only a few primary initiation sites. If so, their transcription would be more sensitive to intercalating agents which interrupt the base sequence than transcription of single genes comprising only short base sequences.

The selective inhibition of ribosomal RNA synthesis by daunomycin offers an explanation for the late onset of the inhibitory effect on protein synthesis. Since the half life of ribosomal RNA is considerably longer than the average half life of HeLa cell messenger RNA,[4,18] the delayed inhibition of protein synthesis may be related to the failure of ribosomal RNA synthesis rather than to any effect on mRNA.

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REFERENCES

1. A. DI MARCO, R. SILVESTRINI, S. DI MARCO and T. DASDIA, *J. cell. Biol.* **27,** 545 (1965).
2. A. RUSCONI and E. CALENDI, *Biochim. biophys. Acta* **119,** 413 (1966).
3. J. H. KIM, A. S. GELBARD, B. DJORDJEVIC, S. H. KIM and A. G. PEREZ, *Cancer Res.* **28,** 2437 (1968).
4. S. PENMAN, K. SCHERRER, Y. BECKER and J. DARNELL, *Proc. natn. Acad. Sci. U.S.A.* **49,** 654 (1963).
5. J. MARMUR, *J. molec. Biol.* **3,** 208 (1961).
6. E. H. HARLEY, K. R. REES, and A. COHEN, *Biochim. biophys. Acta* **213,** 171 (1970).
7. K. SCHERRER and J. F. DARNELL, *Biochem. biophys. Res. Commun.* **7,** 486 (1962).
8. S. PENMAN, *J. molec. Biol.* **17,** 117 (1966).
9. S. PENMAN, I. SMITH and E. HOLTZMAN, *Science* **154,** 786 (1966).
10. U. LOENING, *Biochem. J.* **102,** 251 (1967).
11. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
12. C. F. GORDON and A. L. WOLFE, *Analyt. Chem.* **32,** 574 (1960).
13. H. HARRIS, *Prog. Nucleic Acid Res.* **2,** (1963).
14. S. PENMAN, C. VESCO and M. PENMAN, *J. molec. Biol.* **34,** 49 (1968).
15. W. KERSTEN and H. KERSTEN, *Biochem. Z.* **341,** 174 (1964).
16. E. CALENDI, A. DI MARCO, M. REGGIANI, B. SCARPINATO and L. VALENTINI, *Biochim. biophys. Acta* **103,** 25 (1965).
17. W. FULLER and T. PIGRAM, *Nature, Lond.* in press (1971).
18. M. H. VAUGHAN, J. R. WARNER and J. E. DARNELL, *J. molec. Biol.* **25,** 235 (1967).