The Use of Actinomycin D in Studies of Nucleolar Function

The ability of actinomycin D (AMD) to bind to the DNA molecule has long been recognized as the basis of its biological action and its inhibiting effect on DNA dependent RNA synthesis is well known.

A particularly important property is that at certain concentrations within the range 0.01–0.1 μg/ml (10^{-7}–$10^{-8}M$) AMD appears to inhibit selectively the synthesis of RNA by the nucleolus of mammalian cells without any significant effect upon RNA synthesis in the extra-nucleolar part of the nucleus[1]. Numerous workers using various biological systems have investigated the effects of comparable concentrations of AMD on the passage of cells through the cell cycle[2]. There is an increasing tendency to ascribe the observed disturbances, notably delays in the onset of DNA synthesis and mitosis, to a dependence of these particular events within the cycle upon the continued synthesis of nucleolar RNA and, since the nucleolus is known to be involved in ribosomal RNA (r-RNA) synthesis[3] in terms of a dependence upon a continued supply of ribosomes.

Whatever the nature of the inhibition by AMD of r-RNA synthesis, this effect has been detected primarily because r-RNA is an easily identifiable species of RNA which appears to be made on a morphologically identifiable part of the genome, i.e. the nucleolar associated chromatin. We would like to offer some evidence to suggest that present thinking on nucleolar function and r-RNA in this context may be too narrow.

Materials and methods. A strain of L-cells which has been cultured in this laboratory for many years was maintained in exponential growth by twice weekly transfer. Synchronous cultures when required were prepared by selective harvesting of mitotic cells. For morphological studies, cells were exposed to unlabelled AMD for 6 h, fixed in methanol and stained with dilute Geimsa. All other cells were exposed for 2 h to various concentrations of either unlabelled AMD or ^{3}H AMD at a specific activity of 3.38 Ci/mM. RNA metabolism was assessed by administering a 10 min pulse of generally labelled ^{3}H uridine (20 μCi/ml; 3 Ci/mM) followed by immediate fixation and treatment with DNA ase. Autoradiographs were prepared using Kodak AR 10 stripping film.

Results and discussion. Figure 1 shows the effects on RNA metabolism of a 2 h exposure to AMD. In these experiments the specific concentration range 0.02 μg/ml to 0.08 μg/ml AMD affects nucleolar RNA synthesis without a measurable reduction in nucleolar plasmic RNA synthesis. To cause the same effect GAFFNEY and NARDONE[4] used 0.04 μg/ml on their L-cells, a dose which is within the above concentration range, but the doses of 0.1 μg/ml and 0.01 μg/ml used by FUJIWARA[5] also on L-cells and ARRIGHI[6] on Chinese hamster cells respectively are not. The 2 latter papers show that the dose of 0.04 μg/ml first suggested by PERRY may not be appropriate to every cell system[7] and the specific concentration of AMD which selectively affects nucleolar RNA synthesis should be determined for the particular experimental conditions being used. Any dose of AMD which may result in a substantial reduction in nucleoplasmic RNA label[8] is causing at least 2 effects, either or neither of which may be relevant to other cellular metabolic processes.

The results of experiments in which cultures were fixed immediately after a 2 h exposure to ^{3}H AMD are illustrated in Figure 2. There is negligible cytoplasmic incorporation but at both 0.07 μg/ml and 1.4 μg/ml the drug appears to bind all over the nucleus. Similar studies with synchronous cultures of mouse L-cells at various times during the mitotic cycle showed that cells can bind AMD at all stages of interphase but an approximately 2-fold increase in binding capacity occurs during S-phase suggesting an association with the DNA content of the cell. The presence of label all over the nucleus and the specific binding of the drug to the genome have been recorded in light microscope studies by HAMANN et al.[9] and electron microscope studies by SIMARD[10] but the possibility of selective binding to the nucleolar associated chromatin at a 'nucleolar specific' dose could not be overlooked.

The biological activity of the tritiated drug was checked by observing the effect of a 2 h exposure to ^{3}H AMD upon ^{3}H uridine incorporation. The contribution of bound ^{3}H AMD to the labelling of the nuclei was negligible under the conditions employed and the distribution of grains was very similar to that obtained with unlabelled AMD. All the evidence suggests that the tritiated molecules behaved in exactly the same way as unlabelled molecules of AMD and their presence all over the nucleus must reduce further the likelihood that the nucleolus is the site of all metabolic disturbances caused by a 'nucleolar specific' dose of AMD.

Finally the findings of three workers who all worked on L-cells and all selected the appropriate dose of AMD for a 'nucleolar specific' effect in their system will be

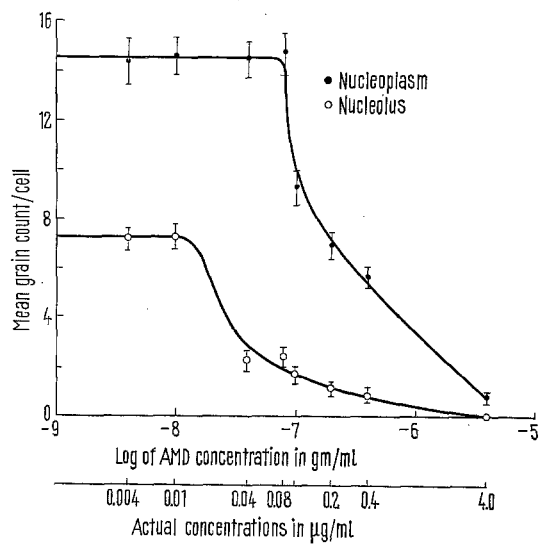

Fig. 1. The effect of actinomycin D at various concentrations on the uptake of ^{3}H uridine into the nucleolus and nucleoplasm of mouse L-cells. Mean ^{3}H uridine grain count per cell after 2 h treatment with AMD at increasing concentrations.

[1] R. P. PERRY, Expl. Cell Res. *29*, 400 (1963).
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[4] E. V. GAFFNEY and R. M. NARDONE, Expl. Cell Res. *53*, 410 (1968).
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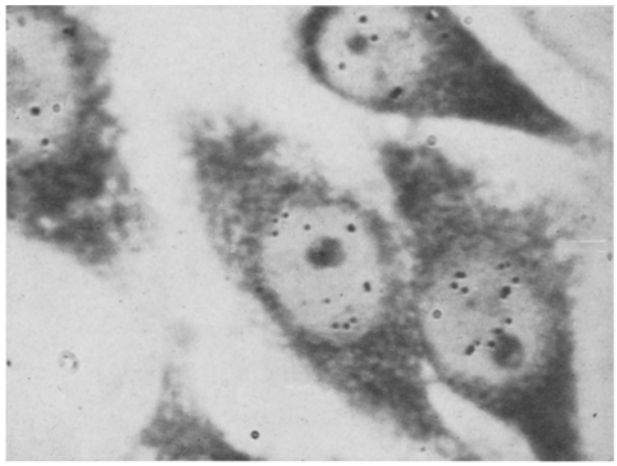
[9] W. HAMANN, W. OEHLERT and M. MEDDERICH, Virchows Arch. path. Anat. Physiol., Abt. B. Zellpath, *1*, 120 (1968).
[10] R. SIMARD, J. Cell Biol. *35*, 716 (1967).



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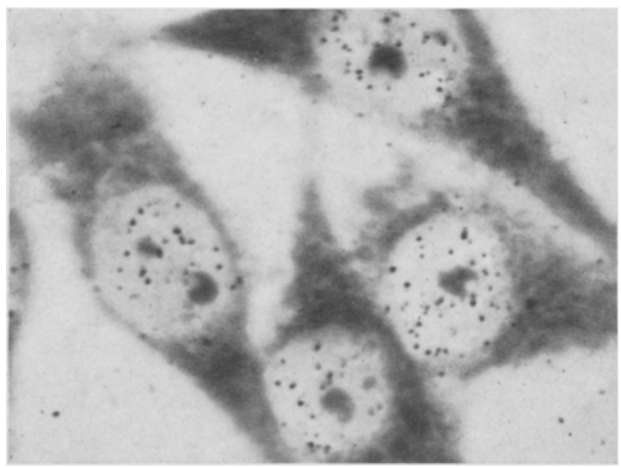
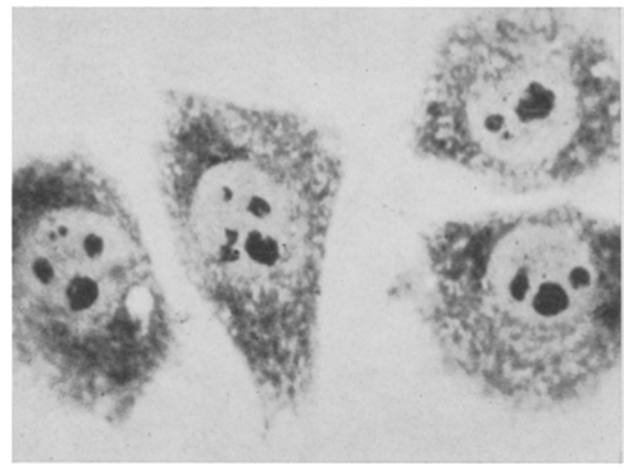


b

Fig. 2. The uptake of ^{3}H AMD into mouse L-cells during a 2 h exposure at (a) 0.07 µg/ml and (b) 1.4 µg/ml. × 1100.



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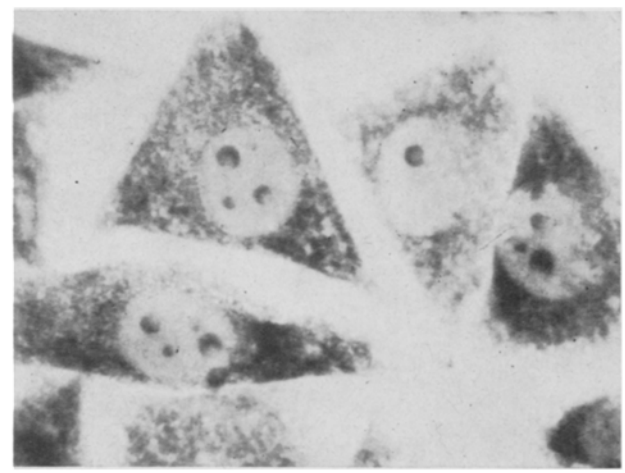

b

Fig. 3. The morphological appearance of mouse L-cells stained with Giemsa. (a) Control and (b) after 6 h incubation in AMD at 0.07 µg/ml. × 1100.

compared. FUJIWARA[5] and RICKINSON[11] both found that a 2 h exposure to AMD during early G_1 delayed the onset of the DNA synthetic phase, and whatever the relevance of the nucleolus or the ribosomes, the importance of this observation when assessing the factors controlling the onset of DNA synthesis must not be minimized. However, FUJIWARA further suggested that a 2 h exposure to AMD during early G_1 also reduces the rate of synthesis of early replicating DNA. RICKINSON used the same exposure time to AMD at the same stage in the G_1 phase but was unable to detect any disturbance, even in cells with an extended G_1 phase, once the DNA synthetic process had commenced. Completely different results have been presented by GAFFNEY and NARDONE[4] who found that none of the cell cycle parameters was disturbed for at least 1.5 mitotic cycles (25–30 h) after a half-hour exposure to AMD. Several discrepancies of this type appear in the literature. Some may be due to variations in the time of exposure to AMD and some to different control mechanisms operating in different cell types but the possibility remains that, for a particular cell type, the dose of AMD required to reduce specifically the nucleolar RNA label is not related to the dose required to cause disturbances in the cell cycle.

These pieces of evidence suggest alternatives to the idea that all the effects of a 'nucleolar specific' concentration of AMD are mediated though reduced production of ribosomes by the nucleolus. We have regularly observed that the nucleoli become much less prominent in fixed and stained cells which have been treated for a few hours with a 'nucleolar specific' concentration of AMD (Figures 3a and b). The nature of the damage is difficult to ascertain under the light microscope and electron microscope studies similar to those reviewed by BERNHARD and GRANBOULIN[12] but at slightly lower doses of AMD are required. A structural disruption of the nucleolus would probably damage all its metabolic functions which are unlikely to be limited to the synthesis of precursor r-RNA and the transport of sub-ribosomal particles to the cytoplasm. Other functions, such as the synthesis of specific

[11] A. B. RICKINSON, Ph.D. Thesis, University of Cambridge (1969).

[12] W. BERNHARD and N. GRANBOULIN, in '*The Nucleus*' *Ultrastructure in Biological Systems* (Eds. A. J. DALTON and F. HAGVENAU; Academic Press, New York 1968), vol. 3, p. 81.

histones suggested by the work of HNLICA et al.[13] on isolated nucleoli and BERLOWITZ and BERNSTAL[14] on the anucleolate mutant *Xenopus laevis*, may be affected.

Alternatively the binding studies with ^{3}H AMD suggest that the 'nucleolar specific' concentration of AMD might also inhibit the synthesis of quantitatively minor components of RNA on regions of the extranucleolar chromatin which are particularly sensitive to AMD binding. The transcription of such components at certain times in the cell cycle might be critical events in maintaining growth.

An independent approach to the study of nucleolar dependence can be made by the technique of microbeam irradiation[15]. The suggestion that the effects of AMD are mediated through injury to the nucleolus will be strongly supported if a particular action of the drug at low concentration can also be caused by specific nucleolar irradiation of similar cells[16].

Résumé. Dans quelques publications récente les pertubations du cycle mitotique qui se produisent après un traitement à l'Actinomycine D (AMD) on été attribuées à une perte de ribosomes. On se demande si on aurait considéré la fonction du nucléole et les effets de l'AMD comme trop restreintes.

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[13] L. S. HNILICA, M. C. LIAU and R. B. HURLBERT, Science *152*, 521 (1966).
[14] L. BERLOWITZ and M. L. BIRNSTIEL, Science *156*, 78 (1967).
[15] R. E. ZIRKLE, Adv. Biol. med. Phys. *5*, 103 (1957).
[16] We thank the Head of the Department, Professor J. S. MITCHELL, for his support of this work.
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Gonadal Pigment of Sea-Cucumber (*Holothuria leucospilota* Brandt)

The pigmentary colours[1] in the integument of echinoderms are also entirely due to the presence of naphthoquinone, carotenoid, melanin and porphyrin pigments. Most of the pigmentation occurs in the integument, but in some cases certain of the internal organs are also coloured.

Carotenoids are particularly abundant in the integument of asteroids. In echinoids, carotenoids are principally restricted to the gonads. There has been insufficient investigation of the holothuroidea[2] and so far as we are aware, there are no published reports on the isolation of carotenoid pigments from *Holothuroidea*.

Ovaries (500 g) containing mature eggs of *H. leucospilota* collected on the sea-shore of Kushimoto in Wakayama prefecture in July 1968 were dehydrated by treating with ethyl alcohol for several days at room temperature. The solvent was then removed by decantation and the ovaries were extracted with successive portions of acetone in the dark until the final extract was colourless. The combined reddish-orange coloured extracts were concentrated under reduced pressure under nitrogen below 40 °C. The deep-red residue was dissolved in petroleum ether. A strong blue colour was obtained with antimony trichloride reagent, indicating the presence of polyene nature of the pigments. The petroleum ether solution exhibited the very characteristic intense single banded absorption spectrum (λ_{max} 472 nm) suggesting the presence of ketocarotenoid. The petroleum ether solution was washed with water to remove the acetone and the reddish petroleum ether solution was then separated into hypophasic and epiphasic carotenoids by shaking the solution with 90% methanol. The pigment was entirely extracted by the methanol. This experiment clearly confirms that the principal carotenoid pigment is unesterified xanthophyll.

From the experimental results obtained by thin-layer chromatography of original petroleum ether extract solution and partitioned methanolic solution, it was shown that the principal pigment (about 70% of total pigments) transferred to methanolic solution by the partition procedure. The methanolic solutions were concentrated under reduced pressure. The deep-red residue was dissolved in a small amount of methanol, covered with petroleum ether, and crystallized by the addition of a little water. After 2 recrystallizations of the black-violet pigment from aqueous pyridine, 1.1 mg of glistening plates were obtained. Its properties indicate that it is unesterified astaxanthin. This was confirmed by mixed melting-point determination with an authentic specimen of astaxanthin obtained from the integument of starfish (*Asterina pectinifera* Müller and Troschel) according to the method of TSUMAKI et al.[3].

Astaxanthin was further characterized by its reaction with potassium hydroxide under nitrogen to form a blueish-purple enolic salt which in the presence of air almost instantaneously autoxidized into red coloured astacene, which was thin-layer chromatographically and spectroscopically identical with astacene produced in a similar way from the authentic starfish astaxanthin. From the testes (300 g) of the sea-cucumber, 0.2 mg of astaxanthin was obtained by the same procedure described above in the case of ovaries.

Furthermore, no differentiation in the pattern of carotenoid pigments in gonads of both male and female has been noted by thin-layer chromatography using different solvent systems.

Zusammenfassung. Aus den Geschlechtsdrüsen von *Holothuria leucospilota* Brandt wurde Astaxanthin extrahiert.

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Kyoto (Japan), 7 July 1969.

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