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THE CONCENTRATION OF ACTINOMYCIN D IN HEPATOCYTE NUCLEI AS RELATED TO INHIBITION OF RIBONUCLEIC ACID SYNTHESIS*

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Abstract—The administration of actinomycin D (Act D) to rats produces a transient inhibition of ribonucleic acid (RNA) synthesis. A method for the isolation of hepatocyte nuclei permitted simultaneous examination of intranuclear antibiotic concentration and RNA synthetic capacity. More than 95 per cent of the nuclei were from hepatocytes, permitting identification of the liver cell studied. A dose of approximately 25–30 μg/100 g of body weight (LD 10–20%, 5 days) produced a significant inhibition of nucleotide incorporation for 6–12 hr. The intranuclear concentration of Act D declined rapidly through the first 12 hr from a maximum at 1 hr. From 15 hr on, despite evidence of residual Act D, no inhibition of nucleotide incorporation into RNA was detected. No metabolic product of Act D could be demonstrated, nor could its intranuclear distribution explain the inactivity of this residual fraction. It is apparent that the normal hepatocyte possesses mechanisms for rapidly eliminating administered Act D.

ACTINOMYCIN D (Act D) is used to inhibit ribonucleic acid (RNA) synthesis in a variety of normal and malignant mammalian tissues.[1–3] Although it appears most effective in inhibiting "stimulated" RNA production, higher doses can also diminish basal RNA synthesis.[4] It has been suggested that it acts by interfering with the interaction of deoxyribonucleic acid (DNA) with RNA polymerase.[1] Act D has been demonstrated to complex with nuclear DNA after parenteral administration.[5, 6]

The duration of action of Act D has been less well studied, but its effect in the liver has been shown to be dependent on the presence of significant levels associated with the nucleus.[5–9]

The recent development of a technique for isolating large numbers of hepatocyte nuclei which display vigorous RNA synthesis offered a means by which intranuclear Act D concentration and activity could be measured directly. The purity of the hepatocyte nuclei permitted an accurate identification of the cell under study.[10]

The present study was aimed at correlating the concentration of Act D in nuclei with their RNA synthetic capacity. A dose of the antibiotic was chosen which had previously been demonstrated to reduce RNA synthesis significantly, but which was well below the lethal doses often utilized.[7] A second phase of the study consisted of examining the distribution of Act D within the nuclei and a search for evidence of metabolic alteration of the antibiotic.

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MATERIALS AND METHODS

Albino, male rats (Charles River Farms) weighing 175 ± 25 g were used throughout the experiments. [^{14}C]-Act D (New England Nuclear Corp.), with a specific activity of 11·4 c/m-mole and a specified radiochemical purity of 99 per cent was utilized. Unlabeled Act D (obtained through the generosity of Merck, Sharpe & Dohme) and [^{14}C]-Act D were diluted in sterile saline and administered at a total dose of 27·5 μg/100 g of body weight. Both intraperitoneal (i.p.) and intravenous (femoral, i.v.) doses were tested. Of the total dose, approximately 2·75 μg/100 g of body weight was labeled.

Nuclei. The method of preparing hepatocyte nuclei has been published previously in detail.[10] After homogenization of a liver mince in buffered, cation-containing sucrose, low speed centrifugation separated the nuclei from the cellular debris. Less than 5/100 hepatocytes were unbroken. Final nuclear purification was achieved by high speed centrifugation on a discontinuous, dense sucrose gradient. The nuclei of the resultant pellet were more than 95 per cent of hepatocyte-type and represented all cell ploidies, and few nuclei were lost due to breakage. Electron microscopy demonstrated that the nuclei were enveloped by an intact double membrane and possessed normal appearing nucleoli. Under electron microscopic examination, very little contamination of the nuclear preparation was noted.

Nucleoli. The pure nuclear pellet described was the source of the nucleoli. The nuclear pellet was suspended in 0·24 M sucrose–0·15 M NaCl and the nuclear suspension was sonicated (Branson Sonifier) at 150 W for 1 min. The sonicate was layered above an equal volume of 0·88 M sucrose–0·15 M NaCl and spun at 3000 rpm for 20 min. The nucleoli (pellet) were resuspended in 20 ml of the sonicating fluid and the layering and centrifugation repeated.[11] The resulting nucleolar pellet was free of nuclei or fragments as determined by methyl green pyronin stains[12] and by comparison of 260:280 spectroscopic examination compared to Burton[13] reaction determinations.

RNA synthesis. Within 1–2 hr after preparation, the nuclear pellet was sampled and studied for RNA polymerase activity and Act D content. The details of the polymerase assay are published elsewhere.[14] The nuclei were incubated in a Tris-sucrose medium, pH 8·4, in the presence of Mg^{2+} with unlabeled nucleotides added in excess. The incorporation of [^{3}H]-CTP (Schwartz, Inc.), specific activity 1·4 c/m-mole, was determined in trichloroacetic acid precipitates. Counting was performed in Liquifluor in a Beckman LS-250 scintillation counter. Nuclear [^{14}C]-Act D levels were too small to produce a significant increase in background counts per minute.

When added *in vitro*, unlabeled Act D was suspended in Tris-sucrose buffer and used at a concentration of 10 μg/100 μg of DNA.

Extraction and study of Act D. The extraction of Act D was performed upon the crude nuclear pellets derived from the initial low speed sedimentation and on the pure hepatocyte nuclear pellet which resulted from high speed sedimentation. The nuclei were suspended in 9 vol. of distilled water and acidified with 1·0 ml of 2 N HCl. Three volumes of ethyl acetate were added and the mixture was stirred vigorously by a magnetic stirrer for 10 min. The sediment from a 1000 *g*, 15-min centrifugation was extracted twice with 3 vol. of ethyl acetate and the three supernatants were pooled. Extracts of the crude nuclear pellets were yellow and demonstrated high viscosity when concentrated. Those derived from pure nuclear pellets were colorless and of low viscosity. The supernatants of the low speed nuclear pellet, which was free of nuclei

on phase-contrast microscopy, were extracted in a similar manner. The efficacy of our extraction procedure was determined by adding [^{14}C]-Act D (76 μg, 67,000 dpm) to 6 g liver prior to homogenization. Extraction of Act D was performed upon aliquots of the whole homogenate, and on the nuclear pellet and the supernatant, after low speed centrifugation. Three extractions yielded more than 100 per cent of the total added Act D. Fifty-five per cent was found in the homogenate supernatant, the remaining Act D being associated with the nuclear pellet. It appears possible therefore that some nuclear associated Act D may result from redistribution of the antibiotic during nuclear preparation.

Chromatographic procedures. Thin-layer chromatographic plates were prepared from cellulose MN-300 with a cyclohexanane–absolute ethanol (5:1) solvent system. Plates (20 × 20 cm) were prepared by a spray technique using 10 g of absorbant and 75 ml water per plate. Identification of Act D was performed by comparing it to untreated material (unlabeled) added at the origin in parallel (R_f 0·57).

RESULTS

Intracellular Act D

Distribution and quantitation. An extremely rapid uptake of Act D into the nuclei was demonstrated after either i.p. or i.v. administration. Throughout the 24-hr period of observation after i.p. administration, the concentration of Act D in the liver nuclei was consistently higher, but the values were not significantly different. A significant nuclear level was demonstrated at 30 min (Fig. 1), and maximal concentration of the antibiotic was achieved at 1 hr (Figs. 1 and 2). Although still elevated at 6 hr, a 50 per cent elimination had already occurred when determined upon pure (high speed) nuclear pellets. The level of nuclear Act D slowly declined over the next 12 hr (to

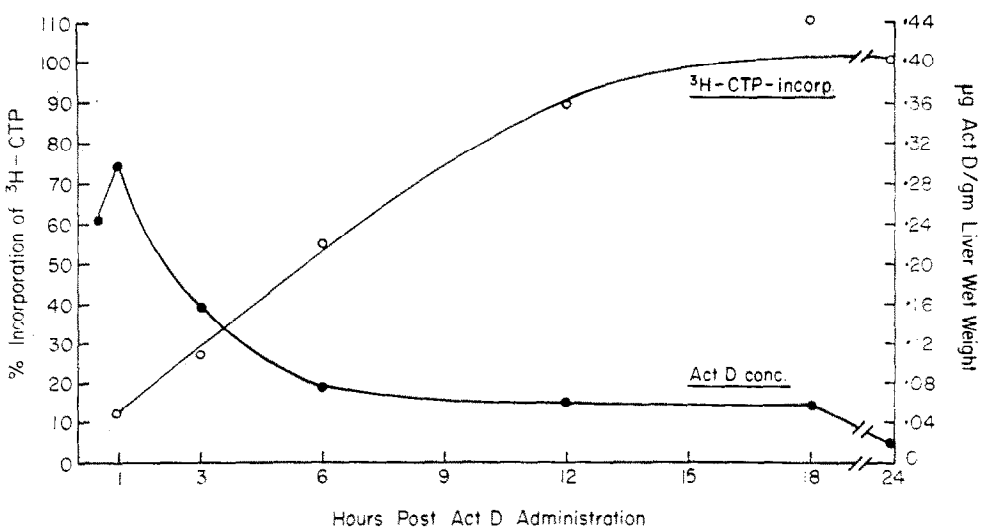

FIG. 1. Inhibition of nucleotide incorporation by hepatocyte nuclei as related to intranuclear Act D concentration. The nuclei were pooled samples (three to four rats). The values are in terms of micrograms of Act D per gram, wet liver weight; low speed nuclear pellets were used. The average incorporation of [^{3}H]-CTP by nuclei of control rats was 100 $\mu\mu$moles/300 μg DNA/10 min.

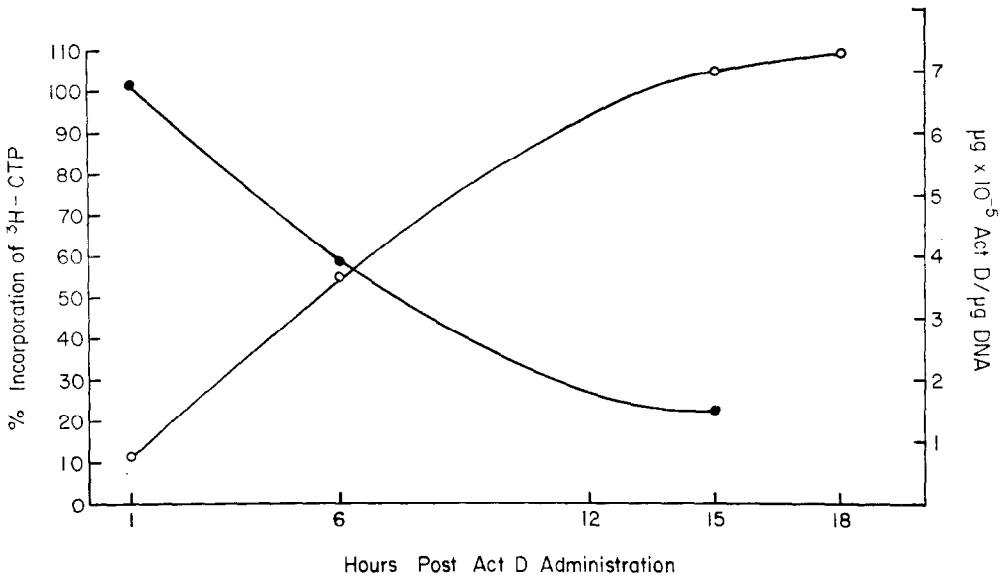

FIG. 2. Inhibition of nucleotide incorporation by hepatocyte nuclei as related to intranuclear Act D concentration. The nuclei were pooled samples (three rats). The values have been expressed in terms of micrograms of Act D per μg of DNA; high speed nuclear pellets were used.

hour 18 approximately 15 per cent) and then continued a small but significant decline to approximately 10 per cent of maximal at 24 hr. The total quantity of extractable Act D was consistently lower in the supernatants than in nuclei (approximately 15 per cent of total counts throughout these experiments). In a separate group of experiments which utilized crude (low speed) nuclear pellets, the decline in nuclear Act D content was similar in slope, although somewhat different quantitatively (Figs. 1 and 2).

The distribution of Act D was studied at 15 hr to determine if intranuclear redistribution could explain its loss of effectiveness. The concentration of Act D associated with extranucleolar and nucleolar DNA remained roughly equivalent ($2{\cdot}7 \times 10^{-5}$ μg Act D/μg nucleic acid:$3{\cdot}8 \times 10^{-5}$ μg Act D/μg nucleic acid).

Chromatographic procedure (qualitative studies). Chromatography of extracts from low speed nuclear pellets was consistently unsatisfactory, presumably due to the presence of viscous and highly colored contaminants. These apparently prevented normal migration of added Act D and made localization by colour or by radioactivity impossible. However, ethyl acetate extracts from high speed nuclear pellets (12 and 15 hr) were crystal clear and of low viscosity. A single narrow band of radioactivity (containing 93 per cent of the total extracted label) was found surrounding the added carrier Act D. No other peaks were present and the remaining radioactivity was demonstrated at the origin, suggesting that altered forms of Act D were not present.

RNA polymerase

Normal nuclei. The nucleotide-incorporating capacity of the hepatocyte nuclei of untreated rats (groups of four) remained consistent throughout all of the experiments, ranging from 25 to 75 $\mu\mu$moles [^{3}H]-CTP/100 μg of DNA/5 min. (The absence of the three unlabeled nucleotides reduced incorporation 90 per cent.) When Act D was added *in vitro*, incorporation was inhibited approximately 70–80 per cent.

Act D in vivo. Although the inhibitory effect of Act D administered intraperitoneally was consistently greater and more persistent than that of intravenous Act D, their activity was roughly comparable.

Maximal inhibition of nucleotide incorporation was achieved at 1 hr, at which time 5–15 $\mu\mu$moles CTP/100 μg of DNA/5 min represented approximately 10 per cent of normal (Figs. 1 and 2). Between the third and sixth hours, incorporation of nucleotide rose from 25 to 60 per cent of normal. Although the intranuclear level of Act D remained relatively constant between the sixth and eighteenth hours (at approximately 20–25 per cent of maximal), polymerase activity was still consistently, although minimally, depressed (90 and 95 per cent of control at 9 and 12 hr). Without significant change in Act D levels present at 15 and 18 hr, incorporation reached levels of 105 and 110 per cent. Further loss of nuclear Act D at 24 hr was associated with normal incorporation.

A plot of intranuclear Act D concentration against the inhibition of nucleotide incorporation demonstrated a direct proportionality (Fig. 3).

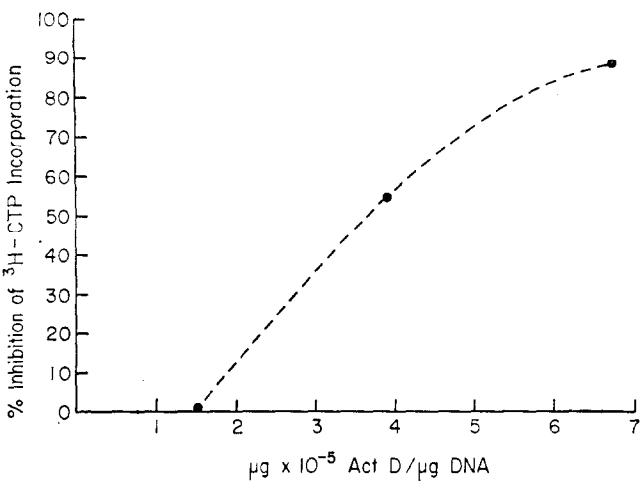

FIG. 3. Inhibition of nucleotide incorporation as related to intranuclear Act D concentration. Act D concentration is expressed in terms of micrograms of Act D per μg of DNA. The values were obtained from the results using high speed nuclear pools at 15, 6 and 1 hr respectively.

DISCUSSION

It has been suggested that the sensitivity of certain cells,[9] both normal and malignant, to the cytotoxic effect of Act D may result from their ineffective elimination of this agent.[7] However, the results of studies concerned with the distribution, fate, and effectiveness of Act D depend somewhat upon the dose administered. Doses exceeding the LD_{50} (which are frequently used) may have acute toxic properties which alter the capacity of cells to metabolize or eliminate the antibiotic or to do both.

The dose chosen for this study was well below the LD_{50} for rats and in our hands caused no deaths during the period of observation. However, this dose is active against RNA synthesis *in vivo*. It has been demonstrated to inhibit the induction of new protein synthesis and also to delay, for a considerable period, the biochemical alterations in

residual hepatocytes subsequent to 70 per cent hepatectomy.[15] The use of the nuclear RNA synthesizing system made it possible to identify the hepatocyte as the site of Act D localization. In addition, it was possible to demonstrate the extreme activity of this agent, since intranuclear levels as low as 0·04 μg Act D per mg of DNA produced significant inhibition of RNA synthesis. Levels of 20-fold greater were necessary to produce comparable inhibition when added *in vitro*. Alterations in membrane permeability, length of exposure, or other factors may account for these differences.

These experiments, in confirmation of previously published results, indicate that Act D enters the hepatocyte nucleus with great rapidity after parenteral administration. Maximal levels were reached within 30–60 min. Throughout the first 12 hr, suppression of nucleotide incorporation was proportional to the intranuclear concentration of Act D. The presence of Act D within the nuclei after 12 hr, without suppression of nucleotide incorporation, may reflect a threshold phenomenon or the inability of our testing methods to demonstrate its effect, i.e. on the synthesis of a specific RNA in small quantities. The return to baseline synthesis by 12 hr, seen in these studies, is more rapid than that recently reported for whole liver,[3] but is comparable to results of autoradiographic analysis.[9] This inactivity did not appear to result from a gross redistribution, since the antibiotic was still bound to nucleoli, but a shift to functionless template or activation of Act D free template may occur.

The mechanism by which the antibiotic was eliminated is unclear, since no metabolic products were demonstrated on chromatographic analysis. (Although this finding and the work of others have failed to demonstrate such by-products, the chromatographic methods utilized may not have been sufficiently sensitive to detect the minimal structural alterations which can cause inactivation.)

It is concluded that a single dose of Act D inhibits RNA synthesis in isolated nuclei in proportion to the intranuclear levels of the antibiotic.

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