Studies on the Disposition of Actinomycin D-3H in Virus-infected and Tumor-bearing Mice

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Summary

Actinomycin D-3H (80,000 cpn/µg) was administered to tumor-bearing mice and the disposition of the drug determined. Radioactivity was found in all peripheral tissues examined (including tumor), but none was found in the brain. No significant metabolism of actinomycin was observed, although binding of the drug altered its extraction characteristics. Differential centrifugation of liver homogenate showed that the drug was concentrated in the nuclear fraction.

Treatment with actinomycin significantly lowered the virus titer in mice infected with Rauscher virus. No significant binding of actinomycin D-H to the virus that was present was obtained after concentration of the plasma virus particles by a density gradient separation.

Introduction

Studies on the disposition of therapeutic doses of actinomycin have been limited because of the lack of sensitive assays to detect the small quantities of this antibiotic needed to elicit an in vivo response. For example, at a dose of 0.2 mg/kg it is possible to show marked antitumor activity in mice (5, 12).

Previous studies on the incorporation of amino acids into actinomycin by whole cells of Streptomyces antibioticus (7, 8) have made it possible to biosynthesize actinomycin D of high specific radioactivity (6). The availability of this radioactive compound has allowed quantitative studies to be conducted with 2 in vivo leukemic systems; the transplantable leukemia L1210 and the Rauscher virus-induced leukemia.

The leukemia L1210 system was employed for studying the disposition of actinomycin D-H in various tissues of the tumor-bearing mice. The Rauscher virus was used in experiments to determine whether extracellular virus recovered from the plasma of virus-infected animals treated with therapeutic doses of actinomycin D would be labeled either in the RNA or the lipid portion of the membrane. The latter studies were made possible by the development of technics which allow the concentration and isolation of virus particles from plasma (2, 9). During the preparation of this manuscript Ro and Busch (11) published their observations on the distribution of actinomycin D-3H in various rat tissues after i.v. administrations.

Materials and Methods

Radioactive Actinomycin. Actinomycin D-3H was prepared biosynthetically using methyl-labeled methionine as described previously (6). Its specific activity was 80,000 cpn/µg.

Leukemia L1210. C X DBA male mice (obtained from the NIH breeding colony), 8 weeks of age, weighing between 25 and 27 gm, were inoculated s.c. in the right inguinal area with 0.2 ml of a 1013 dilution of Rauscher virus. Fourteen days after virus infection, an intense viremia within 14 days after infection and as a result this virus can be easily concentrated. It was considered of interest to determine whether virus recovered from animals treated with therapeutic doses of actinomycin D-H would be
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been treated with actinomycin and those which had been treated was stored at -68°C. NT plasma1 measured 25 ml. T plasma measured 19.3 ml but was then diluted to 25 ml with saline. Both plasmas were separately centrifuged in Lusteroid tubes at 5000 rpm on the SW25 rotor in the Spinco model L preparative centrifuge for 10 min. A pellet resulted in each case. The supernatant solutions were separated and in each case recentrifuged under the same conditions for 20 min. Small pellets resulted in each case. The supernatants were again separated and then centrifuged at 22,500 rpm for 1 hr in Lusteroid tubes on the SW 25 rotor to pelletize the virus contents. A pellet was obtained in the tube containing the NT plasma and a small layer was observed at the liquid surface. The supernatant was removed, and the pellet was resuspended by gentle pipetting in successive aliquots to a total volume of 4 ml sodium citrate buffer (0.153 M, pH 6.7).

The tube containing the treated plasma showed only a slight pellet, and a minor lipid-like layer was present on the surface. The liquid contents of the tube were removed, and the bottom of the tube was carefully rinsed with a total volume of 4 ml of sodium citrate solution.

Each of the suspensions obtained by resuspending the pellets was centrifuged at 5000 rpm in Lusteroid tubes on the SW39L rotor. The NT concentrate gave a moderate pellet whereas the T concentrate gave none. The clarified supernatant was separated and centrifuged at 22,500 rpm in Lusteroid tubes on the SW 25 rotor to pelletize the virus contents. A pellet was obtained in each case. The supernatant was removed, and the pellet was resuspended by gentle pipetting in successive aliquots to a total volume of 4 ml of sodium citrate solution.

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The tubes were then illuminated from the top and photographed side by side. The tube containing the NT concentrate showed a strong opaque band at the 1.17 density position, characteristic of the virus (9).

Distribution of Actinomycin D-3H Extraction of Radioactive Compounds

NEUTRAL EXTRACTION. One ml of the tissue homogenate or diluted plasma was extracted with 3 ml of ethyl acetate. Two ml of the ethyl acetate phase were then assayed for radioactivity using a naphthalenedioxane mixture in a Packard Tri-Carb scintillation counter. Unbound actinomycin is quantitatively extracted under these conditions. All radioactive samples were corrected for quenching, due to colored substances extracted from the tissues, by the addition of an external standard to the counting sample. Results are expressed as cpm/gm of tissue or cpm/ml of plasma.

ACID EXTRACTION. One ml of the tissue homogenate was acidified by the addition of 0.1 ml of 2 N HCl. The acidified homogenate was extracted as described above and an aliquot of the ethyl acetate phase assayed for radioactivity. This acidic extraction procedure has been employed as a measure of total metabolites and represents free actinomycin, acid-extractable metabolites and acid-extractable-bound actinomycin (see below).

IN VITRO CONVERSION OF ACTINOMYCIN TO AN ACID-EXTRACTABLE FORM. Actinomycin D-3H (0.43 µg, 34,000 cpm) was incubated with 0.05 ml of normal mouse liver homogenate (1 gm + 3 volumes of 0.1 M of potassium phosphate buffer, pH 7.4), 0.8 ml of 0.1 M potassium PO4 buffer, pH 7.4, in a total volume of 1 ml. At various time intervals 0.2 ml of the incubation was removed and diluted to 1 ml with water. The free actinomycin was extracted with 3 ml of ethyl acetate. Two ml of the ethyl acetate layer were removed for radioactive assay and the remaining organic layer carefully discarded. The aqueous layer was acidified by the addition of 0.2 ml of 2 N HCl and extracted with 3 ml of ethyl acetate. Two ml of the organic phase were then assayed for radioactivity. All free actinomycin was removed by the neutral extraction.

IDENTIFICATION OF ACTINOMYCIN. The radioactivity in the extracts was identified as actinomycin by silicic acid chromatography, thin layer chromatography (6), and paper chromatography [methanol, butanol, benzene, and water (2:1:1:1), ethanol, butanol, and water (2:1:1)].

Actinomycin was readily eluted from silicic acid columns with 3% methanol in benzene. The sample was prepared by extraction of the tissue with ethyl acetate, evaporation of the ethyl acetate, and dissolving of the sample in 20 ml of 1% methanol in benzene. This was then applied to a silicic acid column (0.6 × 10 cm). One%, 2%, 3%, and 10% methanol in benzene (40 ml of each) were used as the eluting solutions.

DISTRIBUTION OF ACTINOMYCIN IN CELL FRACTIONS. Normal (20-gm) mice were given injections of 4 µg actinomycin D-3H i.p. After 1 hr the livers were removed, homogenized, and the nuclear fraction obtained using the procedure of Dingman and Sporn (3). Mitochondria and microsomes were obtained by centrifugation at 8000 × g and 100,000 × g, respectively. The various cell fractions were suspended in 0.25 M sucrose, and aliquots were extracted after acidification of the solution into 3 volumes of ethyl acetate. Two ml of the ethyl acetate layer were assayed for radioactivity.

LOCALIZATION OF ACTINOMYCIN IN THE TCA-INSOLUBLE FRACTIONS OF LIVER. Three ml of the liver homogenate were treated with 3 ml of 10% TCA. After centrifugation, the precipitate was washed 2 times with 5 ml of 2% TCA (intracellular pool). The precipitate was suspended in 6 ml of 4% TCA and heated at 100°C for 15 min to hydrolyze and solubilize the nucleic acids. The lipids were solubilized by extraction of the precipitate after hot TCA treatment with 4 ml of alcohol-ether (3:1). The remaining protein precipitate was solubilized using 0.1 ml NaOH. Aliquots of the cell pool, nucleic acid, and protein fractions were assayed for radioactivity.

Results

TISSUE LEVELS OF RADIOACTIVITY AFTER A SINGLE DOSE OF ACTINOMYCIN D-3H. Charts 1–5 show the distribution of radioactivity as a function of time in mouse kidney, liver, spleen, plasma, and tumor, after the administration of a single dose of radioactive actinomycin. Both the neutral and the acid-extractable radioactivity increased for a period of 30–60 min in the tis-

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1 The abbreviations used are: NT plasma, plasma from mice not treated with actinomycin; T plasma, plasma from mice treated with actinomycin; TCA, trichloroacetic acid.
Charts 1–5. Neutral and acid-extractable radioactivity in various mouse tissues after injection of actinomycin D-3H. The details of the experiments are described in the text. ○ — — ○, acid-extractable cpm; • — •, neutral extractable cpm.
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The binding of actinomycin D-3H to tissue fractions with subsequent disappearance of the neutral extractable radioactivity could also be shown in vitro. Incubation of a liver homogenate with radioactive actinomycin (see "Methods") at pH 7.4, either at 37°C or 0°C resulted in the disappearance of neutral extractable counts, with an increase in acid-extractable radioactivity. This is shown in Chart 6. Over a period of 30 min there was a marked loss of neutral extractable radioactivity with a concomitant increase in acid-extractable radioactivity. By the criteria (chromatographic behavior) used, the acid-extractable material in these experiments was again unchanged actinomycin. The in vitro reaction also occurred at 0°C although the rate was about \( \frac{1}{3} \) that at 37°C. It thus appeared from both the in vivo and in vitro experiments that there was a nonenzymatic binding of actinomycin to tissue fragments which prevented extraction of actinomycin from neutral solution. Upon acidification the actinomycin was released in a form extractable into ethyl acetate.

TABLE 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Recovered cpm</th>
</tr>
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<tbody>
<tr>
<td>Nuclear fraction</td>
<td>73</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>4</td>
</tr>
<tr>
<td>Microsomes</td>
<td>4</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>19</td>
</tr>
</tbody>
</table>

* TCA, trichloroacetic acid.
The data presented here indicate that if mild conditions are used to disrupt the cell the actinomycin present can be shown to be bound predominantly to the nuclear fraction. These in vivo results are in agreement with previous data which demonstrated that actinomycin can bind to DNA under conditions which do not grossly alter the DNA structure. Precipitation with TCA must release the actinomycin bound to the nucleic acid and, because of the lipophilic nature of the drug, it is bound (or dissolved) by the lipids in the cell. Thus only extraction of the TCA-insoluble material with organic solvents (Table 1) removes the bound actinomycin. The data in Charts 1–6 also suggest that the nuclear binding of actinomycin alters the extraction characteristics of the drug as evidenced by the fact that acidification to denature the DNA is necessary for complete extraction.

After a single injection of actinomycin D-3H (0.2 mg/kg) into mice infected with leukemia L1210 there is low but significant radioactivity in the tumor (Chart 4). Based on specific activity of the actinomycin D-3H it can be calculated that the tumor contains, after 2 hr, only 0.07 µg/gm of tissue, an extremely small quantity in terms of drug action, but consistent with the low levels of this compound known to affect biologic reaction.

Actinomycin D treatment of mice inoculated with leukemia L1210 has been reported (5, 12) to result in a greater than 36% increase in survival time. After Rauscher virus infection, treatment with actinomycin D-3H resulted in a significant reduction in extracellular virus and spleen weight, confirming results previously reported (1). In addition, this regimen of treatment resulted in a substantial increase in the survival time over untreated controls. The virus reduction detected by the bioassay method was further substantiated by a physical method, i.e., by density gradient. It is interesting to note that the bioassay method appeared to be more quantitative. It was shown by this procedure that 10^8 infectious units of virus still remained in plasma of treated animals, while the density gradient procedure showed only the slightest evidence of virus.

References


FIG. 1. Centrifugation of plasma concentrates from Rauscher virus-infected mice in potassium citrate gradients. Left tube: Plasma concentrate from mice not treated with actinomycin D. Right tube: Identically processed plasma concentrate from mice treated with actinomycin D. Apparent bottom band in each tube is an optical artifact resulting from internal reflections of illumination at bottom of tube.
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