SOME BIOCHEMICAL PROPERTIES OF CHINESE HAMSTER CELLS SENSITIVE AND RESISTANT TO ACTINOMYCIN D

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ABSTRACT

A graded series of drug-resistant Chinese hamster sublines has been examined for biochemical changes accompanying resistance to actinomycin D. The most highly resistant subline, DC-3F/AD X, is maintained at 10 μg/ml of the antibiotic. It was shown that over 250 times more actinomycin D is required to inhibit RNA synthesis in this subline than in the parental DC-3F line. The DC-3F/AD X subline was also shown to have a somewhat reduced capacity to transport uridine as compared to parental cells. Sensitive cells took up over 50 times more tritiated antibiotic than the most resistant cells, as determined in a 1-h assay. Uptake of actinomycin D was shown to be temperature-dependent in both resistant and sensitive cells and was not influenced by various metabolic inhibitors. Resistance could not be explained by a rapid uptake and release of the antibiotic, as demonstrated in efflux experiments, or by its metabolism. In addition, highly resistant cells which are cross-resistant to puromycin were shown to have a reduced capacity to take up labeled puromycin. These studies provide further evidence indicating that the mechanism of resistance to actinomycin D is reduced permeability to drug and suggesting that cell membrane alteration accounts for resistance to both actinomycin D and puromycin.

Cellular resistance to actinomycin D has been developed in a variety of mammalian cells in vitro (1, 2, 3, 4). Although the mechanism of action of actinomycin D has been elucidated (5), various parameters of natural or acquired resistance, mode of entry, and factors which regulate sensitivity to the antibiotic still remain obscure. The surface properties of the resistant Chinese hamster cells have been shown to be modified when compared to drug-sensitive parental cells (6, 7, 8). We have previously observed that striking morphological changes indicative of reversion to normal phenotype, as well as reduction or loss of oncogenic potential, accompany development of high levels of resistance to actinomycin D in Chinese hamster cells in culture (1). In the present study, some of the biochemical properties relevant to macromolecular biosynthesis and transport that accompany resistance to actinomycin D were investigated with the intent of gaining further insight into mechanisms of resistance.

MATERIALS AND METHODS

Cells and Methods of Culture

Details of the origin of the Chinese hamster cells and of the resistant sublines have been given previously (3). Monolayer cultures were maintained in Eagle's minimal medium (MEM) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). Resistant sublines were grown without drug for 10–15 days before experimental analysis. Exponentially growing cells were used throughout this study. Cells were

counted by means of a Coulter model F counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). Cells for experiments were grown in 25-cm^2 flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.), and in a standardized mixture of CO_2 and air. Routinely, after the culture medium was aspirated, the cells were washed several times with Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, and were detached from the polystyrene flasks with 0.02% (0.5 mM) sodium ethylenediamine tetraacetic acid (EDTA).

Development of actinomycin D-resistant sublines was described in a previous report (3). Development of the daunomycin-resistant subline DC-3F/DM XX, selected by direct exposure to daunomycin and maintained at 10 μg/ml, was also described previously (9). The vincristine subline DC-3F/VCRd was developed by similar methods, and is maintained with vincristine at a level of 10 μg/ml. Response of sensitive parental DC-3F cells and of resistant sublines to actinomycin D, daunomycin, vincristine, or puromycin was determined in a 3-day assay system detailed elsewhere (3). The concentration of drug required to inhibit growth to 50% of control (no drug) is reported as the ED_{50} value. Resistance or cross-resistance to a chemical agent is expressed as the ratio of ED_{50} values for resistance to parental line.

Isotopes and Chemicals

Actinomycin D was obtained from Merck, Sharp & Dohme, Inc., West Point, Pa. Tween 80 and tritiated actinomycin D (8.4 Ci/mmol) were obtained from Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N. Y. Puromycin dihydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. [5-^{3}H]Uridine (25.7 Ci/mmol), [*methyl*-^{3}H]thymidine (6.7 Ci/mmol), [8-^{14}C]adenine (6.53 mCi/mmol), [*methoxy*-^{3}H]puromycin dihydrochloride (838 Ci/mmol), and L-[4,5-^{3}H]leucine (5 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. All other reagents were the best grade commercially available.

Analytical

Uptake of tritiated actinomycin was determined essentially as described previously (2). Briefly, cells were exposed to tritiated actinomycin D (2.0 μCi/ml, 2.0 μg/ml [1.6×10^{-6} M], total external concentration) for 1 h. The labeled medium was aspirated and cells were washed five times with PBS without calcium and magnesium. The cells were then detached from the flasks with 0.02% EDTA. Flasks were rinsed twice with 2-ml portions of saline, and cells were collected by centrifugation. The pellet was taken up in 1 ml water and counted in a Triton-toluene scintillation fluid. Counts are expressed as counts per minute per 10^6 cells unless otherwise stated. All samples were counted in a Packard liquid scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.).

RNA, DNA, and protein were measured, as described before (10), by determining the amount of appropriate precursors incorporated into acid-insoluble 5% trichloroacetic acid (TCA). The precipitates were filtered through Millipore filters (0.45 μm pore size, Millipore Corp., Bedford, Mass.), washed with several washes of 5% TCA, dried, and counted in Omnifluor (New England Nuclear). Aliquots of the TCA-soluble material were counted directly in a Triton-toluene scintillation fluid.

RESULTS

Morphological Characteristics of Actinomycin D Resistance

The actinomycin D-sensitive parental line, DC-3F (Fig. 1 *a*), is a spontaneously transformed Chinese hamster line initially derived from normal lung fibroblast populations (3). The growth properties of this cell line reflect that of a malignant cell type, with an apparent lack of density-dependent inhibition of growth. These cells have previously been shown to be tumorigenic in a heterologous transplantation system (1). Resistance to actinomycin D in Chinese hamster cells is accompanied by striking morphological changes. The morphological manifestations of resistance are increased cellular adhesiveness with apparently stronger cell-to-cell interaction and cell-to-substratum adherence. The graded series of actinomycin D-resistant sublines (Fig. 1 *b–d*) tend to grow as flattened, oriented fibroblast-like populations with apparently more adhesive membranes, commensurate with increase in resistance to actinomycin D. The most resistant cell line, DC-3F/AD X, is nontumorigenic (1) and manifests density-dependent inhibition of growth (Fig. 1 *d*).

Uptake of tritiated antibiotic by DC-3F and drug-resistant sublines was measured. The sensitive parental DC-3F line takes up about 50 times more actinomycin D than the highly resistant DC-3F/AD X within a 1-h time period. The sublines with intermediate resistance, DC-3F/AD II and DC-3F/AD IV, take up correspondingly less labeled actinomycin D than do parental cells. Two other Chinese hamster lines, which were selected for resistance to vincristine and daunomycin, DC-3F/VCRd and DC-3F/DM XX (9), and which are cross-resistant to actinomycin D, also showed reduced uptake of tritiated antibiotic. Previous data (3, 9) based on biological assays demonstrated resistance to actinomycin D as well as cross-resistance to a variety of other chemical agents.

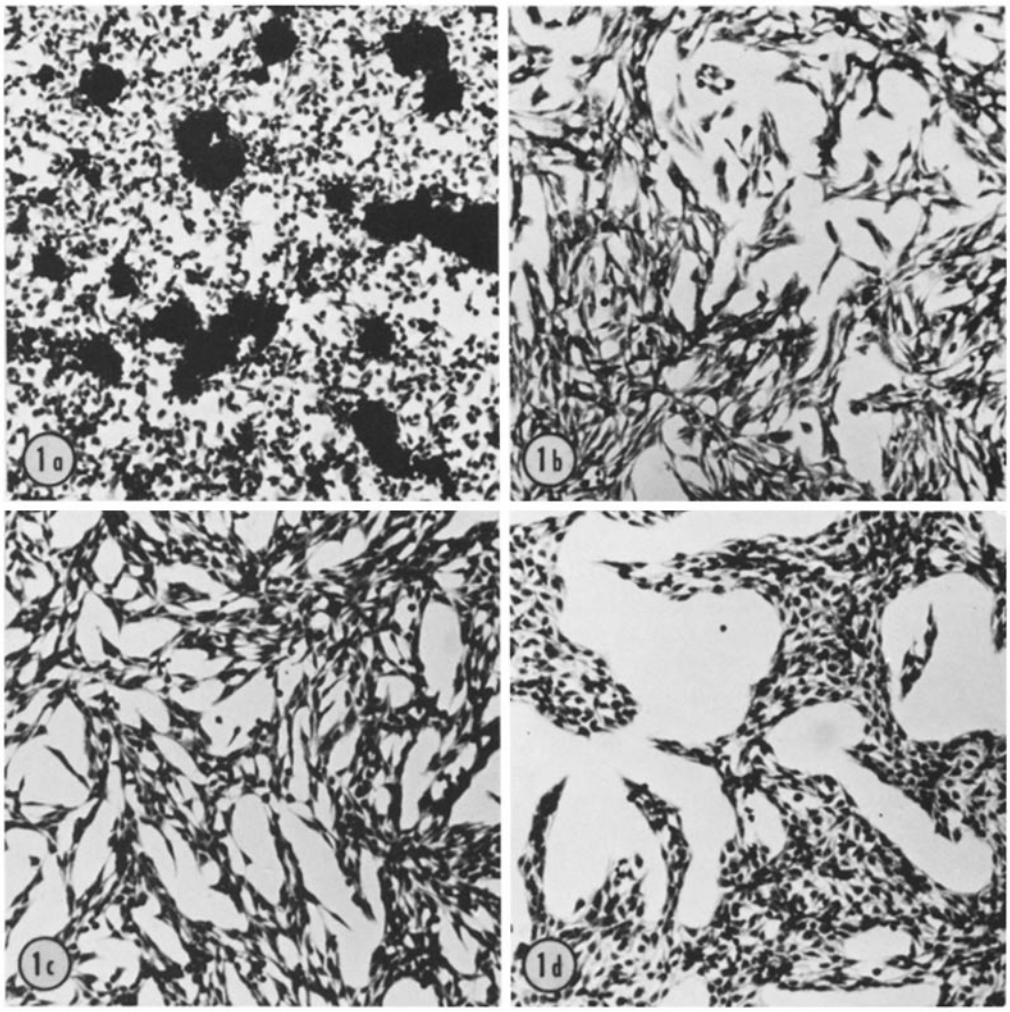

FIGURE 1 Fixed monolayer cultures: (*a*) DC-3F; (*b*) DC-3F/AD II; (*c*) DC-3F/AD IV; (*d*) DC-3F/ADX. Stained with May-Grünwald-Giemsa. × 75.

Dose Response of Inhibition of RNA Synthesis

The effect of actinomycin D on RNA synthesis is shown in Fig. 2. The parental DC-3F cell line is most sensitive to inhibition of RNA synthesis while the highly resistant subline, DC-3F/AD X, requires over 250 times more actinomycin D to inhibit uridine incorporation into RNA. At a concentration of 50 μg/ml of actinomycin D, RNA synthesis is inhibited only by about 80%. Under a similar set of experimental conditions, there was no inhibition of DNA synthesis as measured by thymidine incorporation (data not shown).

In order to assess whether the rate of RNA synthesis is different in the resistant cells, apparent rates of synthesis were measured by following uridine incorporation into RNA, in logarithmically growing cells (Fig. 3). It appears that the relative rate of RNA synthesis in the DC-3F/AD II subline is consistently slightly higher than in parental cells, while in the more resistant sublines it is somewhat lower. These differential rates of RNA synthesis cannot be adequately accounted for by differences in growth rate since the population doubling times are: DC-3F, 13 h; DC-3F/AD II, 15 h; DC-3F/AD IV, 14.5 h; and DC-3F/AD X, 15 h (3). The possibility was considered that the DC-3F/AD X subline might be a transport variant for uridine uptake and, thus, that uridine incorporation was not a reflection of the rate of RNA

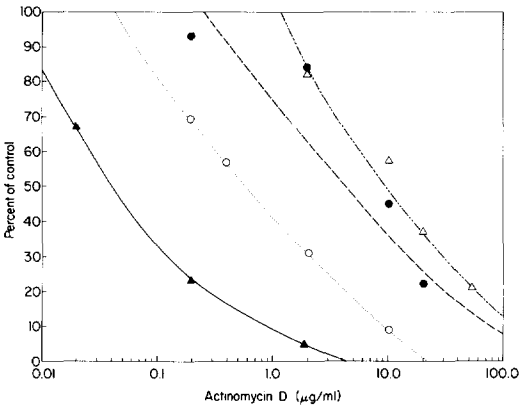

FIGURE 2 Concentration-dependent inhibition of RNA synthesis. Duplicate cultures were incubated with actinomycin D at the indicated concentrations for 30 min. Control cultures received no actinomycin D. To all cultures [5-^{3}H]uridine (0.1 μCi/ml; 25.7 Ci/mmol) was added and uridine incorporation into cold acid-insoluble material was determined after 1 h, as described in Materials and Methods. ▲, DC-3F; O, DC-3F/AD II; ●, DC-3F/AD IV; and Δ, DC-3F/AD X.

synthesis but rather of a limited capacity of the cells to take up uridine. Therefore, adenine incorporation into DNA and RNA was measured. Again, it appeared that the apparent rate of RNA synthesis, as reflected by incorporation of adenine, is lower in the more resistant DC-3F/AD X subline as compared to sensitive parental cells. In order to clarify whether differences in apparent rates of RNA synthesis were due to decreased synthesis or reduced rate of uridine and, possibly, adenine transport, whole cell uptake and incorporation of both precursors into acid-soluble and insoluble fractions were measured. It was found that whole cell uptake and transport of uridine into the actinomycin D-resistant DC-3F/AD X cell line are markedly reduced on a per cell basis and per total cellular protein (data not shown). This may, in part, account for the apparent differences in rate of RNA synthesis. Reduced uptake of uridine is not due to density inhibition of growth in this experiment, as the cell concentration was 3×10^6 cells per flask, which is far below the saturation density of resistant cells under these conditions (unpublished observations). Further, to exclude the possible effects of density inhibition of uptake, sensitive and resistant cells were exposed to labeled uridine in suspension cultures and similar results were obtained (data not shown). Adenine uptake and incorporation are also reduced in the DC-3F/AD X subline but not to the same extent as uridine. These data suggest that the actinomycin resistant subline DC-3F/AD X has a reduced capacity to transport uridine. In another experiment it was found that the ribose content of both cell lines was similar on a per milligram protein basis (DC-3F, 0.299 mg RNA/mg protein and DC-3F/AD X, 0.240 mg RNA/mg protein). Leucine incorporation into protein in the resistant DC-3F/AD X subline was only about one-third the rate in the parental DC-3F cells. This is inconsistent with the growth rates of the two cell lines. In general, the DC-3F/AD X cells may have reduced capacity to accumulate small precursors as well as large molecules.

Factors Affecting Uptake of Actinomycin D

The concentration-dependent uptake of labeled actinomycin D is shown in Fig. 4. It appears that at 3 h uptake of labeled antibiotic in parental cells is approaching steady-state levels at concentrations of 2 and 5 μg/ml, while the resistant subline takes up an extremely small amount of actinomycin D even at a concentration as high as 20 μg/ml.

The effect of the surfactant Tween 80 was used to measure enhancement of uptake of actinomycin

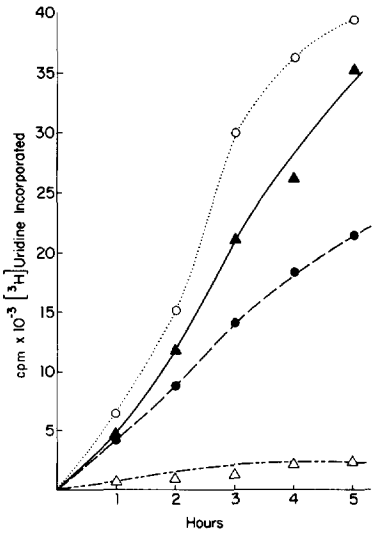

FIGURE 3 Rate of RNA synthesis in sensitive and resistant cell lines. Logarithmically growing cells were exposed to [5-^{3}H]uridine (0.1 μCi/ml; 1.0 μg/ml) at time zero. Duplicate flasks were harvested and uridine incorporation into RNA was determined as described previously (9). ▲, DC-3F; O, DC-3F/AD II; ●, DC-3F/AD IV; and Δ, DC-3F/AD X.

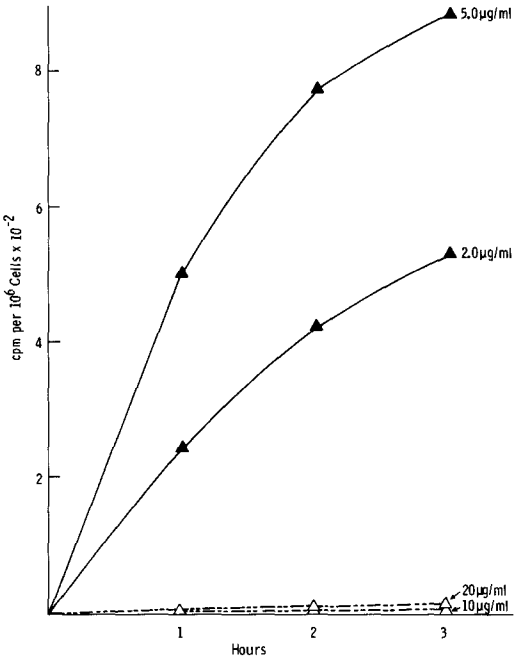

FIGURE 4 Concentration-dependent uptake of [^{3}H]actinomycin D in Chinese hamster cells sensitive and resistant to actinomycin D. Actinomycin D was added to DC-3F (1×10^{-2} μCi/μg) and to DC-3F/AD X (5×10^{-2} μCi/μg) at time zero. Uptake of actinomycin D was determined as described in Materials and Methods. ▲, DC-3F; and Δ, DC-3F/AD X.

D at levels which produce no obvious morphological changes (6). A concentration of 0.075% Tween 80 produced an approximately fivefold enhancement of uptake of actinomycin D in the highly resistant DC-3F/AD X cells.

To gain some insight into the process of actinomycin D accumulation in sensitive and resistant Chinese hamster cells, the effect of temperature was determined. Both sensitive cells and highly resistant cells selected by actinomycin D exhibit temperature-dependent uptake of antibiotic. Drug-sensitive DC-3F cells take up about 450 times more actinomycin D at 37°C than at 4°C. Resistant cells also exhibit temperature dependence even though uptake is less than for parental cells. That uptake of actinomycin D may not be an energy-dependent process is exemplified by the fact that no inhibition of uptake was observed in the presence of potassium cyanide or dinitrophenol.

Efflux experiments with cells prelabeled with actinomycin D indicate that the excretion rate in resistant cells is much less rapid than in sensitive cells (Fig. 5). It must be noted that resistant cells cannot be loaded with drug to the same extent as sensitive cells. However, resistance to actinomycin D is apparently not due to rapid uptake and release of antibiotic (see also Fig. 4).

Chinese hamster cells which have been selected for resistance to actinomycin D are also cross-resistant to a variety of other drugs within a certain molecular weight range, irrespective of their mode of action (3). The resistant line, DC-3F/AD X, was found to be cross-resistant to puromycin (3). In the present study, response of DC-3F/AD X to puromycin was determined in the assay system previously described (3). An ED_{50} value of 270 μg/ml was obtained; cross-resistance of DC-3F/AD X to puromycin is 116-fold. As shown in Fig. 6, cross-resistance may be attributed to a reduced capacity for uptake of puromycin. In another experiment, pretreatment of resistant cells with puromycin at a concentration of 500 μg/ml for 30 min followed by exposure to tritiated leucine for 1 h showed only 60% inhibition (data not shown).

In order to eliminate the possibility that resist-

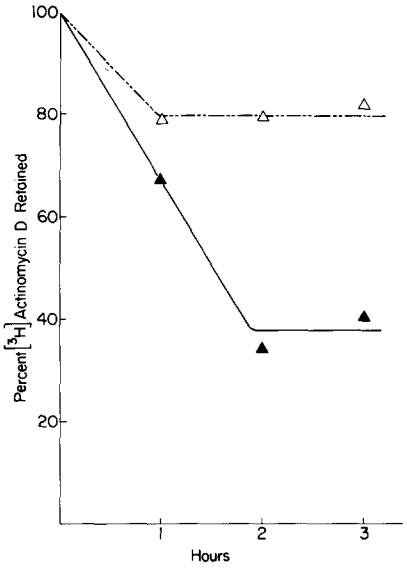

FIGURE 5 Rate of release of [^{3}H]actinomycin D. Cultures of DC-3F were treated with [^{3}H]actinomycin D (0.5 μCi/ml; 2 μg/ml) for 1 h and DC-3F/AD X (1 μCi/ml; 2 μg/ml) for 2 h. Medium containing labeled actinomycin D was removed and flasks were rinsed three times with fresh medium. Conditioned medium was then added back to the flasks and uptake of [^{3}H]actinomycin D was determined as described in Materials and Methods. ▲, DC-3F and Δ, DC-3F/AD X.

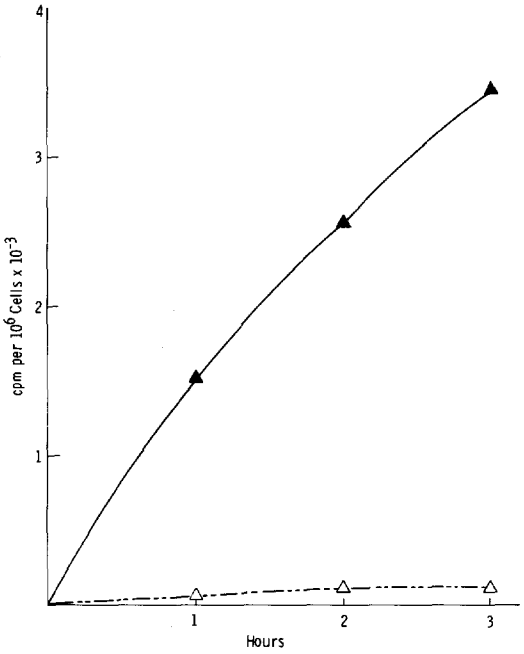

FIGURE 6 Uptake of [^{3}H]puromycin. Growing cultures were exposed to [*methoxy*-^{3}H]puromycin dihydrochloride (2 μCi/ml and 2 μg/ml unlabeled puromycin). Uptake of puromycin was measured as described for actinomycin D. ▲, DC-3F and △, DC-3F/AD X.

ance to actinomycin D is due to metabolism of the antibiotic, sensitive and resistant cell lines were exposed to labeled actinomycin D for 48 and 72 h, respectively. The actinomycin D was extracted from the cells and chromatographed with authentic standard labeled antibiotic. No differences could be seen with either the sensitive or resistant cell extracts (data not shown). Therefore, the resistant cells are not able to metabolize actinomycin D to a less toxic metabolite.

DISCUSSION

Chinese hamster cells in culture with acquired resistance to actinomycin D exhibit a trend toward normal growth properties as compared to the spontaneously transformed parental cell population (1). The morphological alterations coincident with resistance to actinomycin D are exemplified by a change from typical "malignant" cell types, to more flattened, oriented cells with concomitant reduction in tumorigenicity (1). In the present study some of the biochemical parameters accompanying resistance to actinomycin D were investigated. Recently, other cell lines have been developed which are resistant to actinomycin D (4, 11, 12) and which exhibit altered growth patterns when compared to parental cells. The sensitive parental DC-3F line takes up over 50 times more labeled antibiotic than the most resistant DC-3F/AD X subline. Similar results were reported earlier for HeLa cells by Goldstein et al. (2), and more recently for murine leukemia cells by Kessel and Bosmann (13).

The Chinese hamster cells most resistant to actinomycin D, DC-3F/AD X, require over 250 times more antibiotic to shut off RNA synthesis than do sensitive parental DC-3F cells and over 10 times more than do Vero cells (14). Preliminary experiments indicate that the highly resistant cells have a reduced capacity to transport uridine. Since it has been known for some time that contact-inhibited cells have a reduced capacity to incorporate uridine (15, 16, 17), the possibility of a density-dependent inhibitory effect on uridine transport was experimentally excluded. The fact that the growth rates and the total RNA content of the sensitive and parental cells are similar would suggest that the actual rate of RNA synthesis is also quite similar. This could be established by measuring pool sizes.

Results of efflux experiments suggest that resistance to actinomycin D in these Chinese hamster cells is not due to rapid uptake and release of drug. Sawicki and Godman (14) have shown that Vero cells have greater natural resistance to actinomycin D than either HeLa or L cells. However, when the cells are preloaded with actinomycin D, Vero cells excrete bound antibiotic about twice as fast as HeLa or L cells, independent of time of exposure or internal drug concentration.

Uptake of actinomycin D is temperature dependent in both sensitive and resistant Chinese hamster cells. Kessel and Wodinsky (18) reported a similar finding for L1210 cells. The observation that drug uptake was not inhibited in either sensitive or resistant cells in the presence of various metabolic inhibitors suggests that actinomycin D is not actively transported.

Resistance to actinomycin D might also be explained by metabolism and breakdown to a less toxic substance. However, we observed no evidence of breakdown after growth of resistant cells for 72 h in the presence of antibiotic.

Actinomycin D-resistant Chinese hamster cells were previously shown to be cross-resistant to puromycin, as well as to a variety of other

biologically active agents within a molecular weight range of approximately 350 to 1,300 (3). In the present study we show that cross-resistance to puromycin is due to reduced permeability of DC-3F/AD X cells to this antibiotic. Thus, cellular resistance to both actinomycin D and puromycin appears to be the consequence of an impaired mechanism of uptake of antibiotic into the cell. Increased adhesiveness and altered surface constituents of resistant cells may in some manner contribute to the permeability barrier at the level of the plasma membrane. Alterations of plasma membrane components in resistant cells are currently being investigated in our laboratory.

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