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The Relationship among Transport, Intracellular Binding, and Inhibition of RNA Synthesis by Actinomycin D in Ehrlich Ascites Tumor Cells *in Vitro*[1]

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SUMMARY

Uptake of actinomycin D (AD) was characterized in Ehrlich ascites tumor cells *in vitro*. The time course of uptake consists of an initial component that represents a unidirectional flux of AD into the cells, following which the net uptake rate falls to achieve a velocity that is constant over a 45-min interval of observation. The velocity of influx exceeds that of the late uptake phase by a factor of 2. Influx is 1st order over an AD concentration range of 0.02 to 14 μM. Influx is highly temperature sensitive (Q_{10} = 4.5); this is related only in part to the high temperature dependence (Q_{10} = 2) of the oil-aqueous partition coefficient for this agent. Within 4 min after exposure of cells to AD, a component of intracellular AD appears which exceeds the concentration of extracellular AD and rapidly leaves the cells ($t_{½} \simeq 3$ min) when the cells are resuspended into an AD-free medium. The level of this component of intracellular drug is unaffected by metabolic poisons, and the apparent chemical gradient is attributed to loose binding. While transport of AD does not appear to be energy-dependent, the data do not clarify whether translocation across the cell membrane is diffusional or carrier mediated. In addition to a loosely bound intracellular component, analysis of the unidirectional net efflux of AD indicates at least 3 other exit components; one with a $t_{½}$ of 35 min, a very tightly bound component, and another representing drug that is free within the intracellular water. The bulk of AD taken up is tightly bound to intracellular constituents, and the rate of accumulation of this cellular component accounts almost entirely for the net rate of uptake of AD during the late uptake phase.

Because influx exceeds the rate of tight binding within the cell by a factor of only 2, it is unlikely that osmotically active AD achieves thermodynamic equilibrium across the cell membrane over the interval of these experiments. Rather, prior to saturation of binding sites within the cell, the level of free AD achieved is probably below the extracellular concentration. Since the rate of binding should be influenced by the level of free intracellular AD, factors that increase influx and bring the intracellular AD level closer to equilibrium should also increase the rate of intracellular binding. Tween 80 augments both influx and AD binding within the cell, an effect that may be related to augmented transport alone, but an additional stimulatory effect on the binding process is also possible.

Association of AD with only a small number of the very-high-affinity intracellular binding sites, in the absence of free or loosely bound AD, results in ~35% inhibition of [2-^{14}C]uridine incorporation into RNA within 50 sec after exposure of cells to 1 μM AD. Within 10 min, 75% inhibition is achieved indicating that association of AD with only a small fraction of the total high-affinity sites is sufficient to suppress RNA synthesis.

INTRODUCTION

AD[4] is an anticancer agent with chemotherapeutic efficacy in the treatment of several malignant disorders (6, 7, 27, 32, 34). The agent achieves its lethal effect by association with double-stranded DNA leading to the cessation of DNA-dependent RNA synthesis (12, 22, 25). A number of studies have suggested that the cytotoxicity of AD may be determined by the transport of this agent across the plasma membrane of mammalian (19, 23, 24, 29) and bacterial (20, 33) cells, which limits the rate of association of AD with its intracellular target site(s). The observation that Tween 80 augments the net cellular uptake of AD and enhances the sensitivity of resistant cells to this agent has been interpreted as supporting the concept that transport is rate limiting to intracellular binding and is an important factor in cytotoxicity (29). However, the discrimination (experimentally) between factors that influence membrane transport of AD and intracellular binding has been difficult (15). Net uptake of AD is a function of both membrane transport and the number and affinity of binding site(s) within the intracellular compartment, and it is obvious from previous studies

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[4] The abbreviations used are: AD, actinomycin D; TCA, trichloroacetic acid.

that after brief intervals it is the latter event that accounts for the bulk of AD that is taken up within the cell (15). The objectives of the studies to be reported here were (*a*) to define the properties of the membrane transport process for AD in Ehrlich ascites tumor cells, (*b*) to delineate the relationship between the rate of translocation of AD across the cell membrane and the subsequent rate of intracellular binding to DNA; (*c*) to characterize the kinetics of AD binding to site(s) within the intact cell; (*d*) to relate the extent of intracellular binding to inhibition of RNA synthesis; and (*e*) to characterize the effects of Tween 80 on AD transport and binding within the cell.

MATERIALS AND METHODS

Cells and Media. Ehrlich ascites tumor cells were obtained from CF-1 mice (Carworth Farms, New City, N.Y.) that were inoculated i.p. weekly with 0.2 ml of undiluted ascitic fluid. Mice were sacrificed 6 to 12 days after inoculation, the ascitic fluid was suspended into 0° buffer, and contaminating erythrocytes were removed from the tumor cells by aspiration of the erythrocyte-containing supernatant fraction after centrifugation for 1 min at 250 × *g*.

The buffer consisted of 135 mM NaCl, 16 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 4.4 mM KCl, 1.9 mM $CaCl_2$, and 1.0 mM $MgCl_2$; osmolality was 290 mOsm/liter. A modified minimal essential medium was used for studies of uridine incorporation into RNA (16). The cell suspension was stirred continuously with a motor-driven Teflon paddle as previously described (18). A pH of 7.4 was maintained by passing warmed and humidified 95% O_2/5% CO_2 over the cell suspension; the temperature was 37° unless otherwise indicated. Cell volume percents (cytocrits) were 3% and <1%, respectively, during uptake or efflux experiments.

Measurement of the Time Course of AD Uptake. A suspension of Ehrlich ascites tumor cells was exposed to [^{3}H]AD following which 1-ml portions of the cell suspension were injected into 10 ml of 0° buffered 0.9% NaCl solution (pH 7.4) and centrifuged at 2000 × *g* for 30 sec. The cell fraction was washed twice with 0°-buffered NaCl then aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare, and dried overnight at 60°. The dried cells were weighed on a Cahn G-2 electrobalance (Beckman Instruments, Fullerton, Calif.) and then placed in 20-ml liquid scintillation vials and digested in 0.1 ml of 1 N KOH for 1 hr at 60°. After cooling, 10 ml of scintillation fluor (18) were added and radioactivity was determined on a Beckman Model LS-133 liquid scintillation spectrometer. Counting efficiency for ^{3}H was 25%.

Measurement of AD Efflux. For determination of the efflux characteristics of AD, cells incubated with [^{3}H]AD were separated by centrifugation, washed twice with 0° buffer, and resuspended into a large volume of AD-free buffer at 37°. Portions of the cell suspension were sampled at appropriate intervals and injected into 10 ml of the 0° buffered 0.9% NaCl solution. The samples were then processed as described above.

Measurement of Intracellular Water. Using methodology developed in this laboratory and described previously in detail (18), intracellular water was determined from the difference between wet and dry weight of a cell pellet less the [^{14}C]inulin space. Counting efficiency for ^{14}C was ~80%.

Measurement of Uridine Incorporation into RNA. Uridine incorporation into RNA was determined by measuring ^{14}C in the TCA-insoluble cell fraction after exposure of cells to [2-^{14}C]uridine. The cells were washed twice with the 0° buffered 0.9% NaCl solution, and then the cell pellet was dispersed in 0.5 ml of this solution followed by addition of 0.5 ml 10% TCA at 0°. After 10 min at 0°, the precipitate was separated by centrifugation, washed twice with 5% TCA at 0°, and then transferred to polyethylene sheets and processed as described for the whole cells.

Chemicals. [^{3}H]AD (3.7 to 12 Ci/mmole) was obtained from Amersham/Searle, Arlington Heights, Ill., and nonlabeled AD was from Merck, Sharpe & Dohme, Rahway, N. J. Purity (95% or greater) was established by thin-layer silica gel chromatography in benzene:ethyl acetate:methanol (10:2.5:1). Nonlabeled AD was dissolved in 10% ethanol (1.72 M) and stored in the dark at −20°. The final ethanol concentration after addition to the cell suspensions did not exceed 124 mM and was usually less than 2 mM. In 7 experiments, concentrations of ethanol up to 124 mM did not affect AD influx into Ehrlich ascites tumor cells ($p > 0.2$ in control *versus* alcohol-exposed cells).

Tween 80 and sodium azide were supplied by Fisher Scientific Co., Pittsburgh, Pa., iodoacetic acid was from Eastman Organic Chemicals, Rochester, N. Y., and [2-^{14}C]uridine (55.5 mCi/mmole) was obtained from New England Nuclear, Boston, Mass.

Identification of Intracellular Radiolabel. For determination of whether AD was metabolized during the longest experimental procedure, in 4 experiments cells were incubated with 0.21 μM AD for 2 hr. The cell fraction was then separated by centrifugation, washed twice with 0° buffer, and resuspended into 5 ml of 0° buffer. The tumor cells were disrupted by sonic oscillation and then centrifuged at 18,000 × *g* for 1 hr at 0°. The supernatant was lyophilized, redissolved in absolute ethanol, and then chromatographed on silica gel as noted above. By this technique no AD metabolites were identified, an observation compatible with studies by other workers (31, 35).

RESULTS

The Time Course of AD Uptake. Chart 1 illustrates the time course of AD uptake into Ehrlich ascites tumor cells at an extracellular AD concentration of 0.26 μM. The uptake sequence for AD is characterized by an initial rapid-uptake component which is linear for at least 4 min, following which the net uptake rate falls to a level that remains essentially constant over the 45-min interval of observation. The velocity of the initial uptake phase exceeds that of the late linear phase by a factor of 2.03 ± 0.23 (7 experiments).

To study the characteristics of AD transport across the cell membrane, the uptake process was evaluated over the 1st 100 sec (Chart 1, *inset*). The rise in cell AD was a linear function of time indicating that uptake over this interval

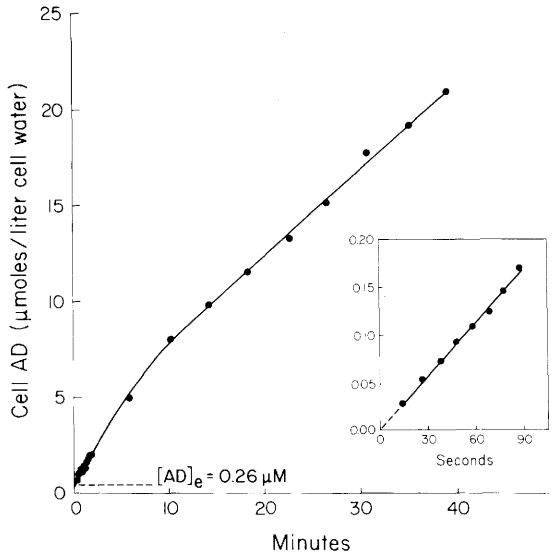

Chart 1. The time course of AD uptake into Ehrlich ascites tumor cells at 37°. The extracellular concentration of AD ([AD]$_e$) = 0.26 μM. *Inset*, uptake over 100 sec when [AD]$_e$ was 0.03 μM.

represents a unidirectional flux and reflects the properties of the membrane transport process unaffected by subsequent binding of AD to intracellular constituents. The interrupted line represents an extrapolation of the uptake slope to zero time. Since the line intercepts the ordinate close to the point of origin, this suggests that there is no significant surface adsorption or rapid association of AD with these cells.

Influx Kinetics for AD. The 100-sec unidirectional influx of AD was measured over an extracellular concentration range of 0.1 to 1.8 μM (Chart 2). Influx is a linear function of the extracellular AD concentration. The observation that the line extrapolates through the point of origin suggests that a high-affinity transport route was not already saturated at the lowest levels of AD used. The *inset* of Chart 2 is a logarithmic plot of influx as a function of the extracellular AD level over a greater concentration range (0.02 to 14 μM). The linearity of this relationship further supports a 1st-order (nonsaturable) influx process.

Temperature Sensitivity of AD Influx. Influx of AD in Ehrlich ascites tumor cells is temperature dependent. In 5 experiments, the average Q_{10} between 27 and 37° was 4.5 ± 0.5 and the energy of activation was 26.5 kcal/mole. The high temperature sensitivity of the influx process may be attributed in part to the tendency of AD to increase its partitioning into the lipid membrane as the temperature is increased. AD was dissolved in the usual buffer, then exposed to 1/100 the volume of corn oil, and the level of AD in the oil phase was measured after 30 min. The average increase in the partitioning of AD into corn oil as the temperature was increased from 27 to 37° was 2.13 ± 0.08 (from 4 experiments).

Exchangeability of Intracellular AD. Cells were loaded with AD for 200, 300, and 500 sec; the intracellular AD level was measured; then the remaining cells were separated by centrifugation, resuspended into AD-free medium at 37°, and the efflux of AD was monitored (Chart 3). Under all conditions, there is an initial rapid loss of intracellular AD after which efflux becomes negligible, indicating that a major component of intracellular AD is tightly bound within the cell. After a 200-sec incubation with this agent, a component of intracellular AD rapidly leaves the cells with a $t_{1/2} \simeq 3$ min (to be referred to as exchangeable intracellular AD; it will be shown that the exchangeable component must represent loosely bound as well as free intracellular

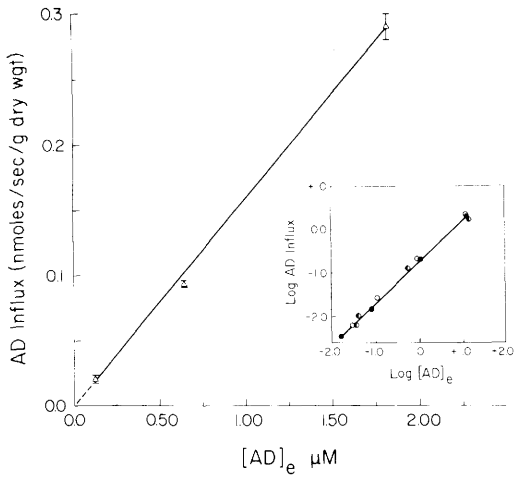

Chart 2. The relationship between the influx and extracellular concentration of AD ([AD]$_e$). Influx rates were obtained from the least-squares slope of 8 to 10 uptake points from the initial 100-sec uptake of AD (Chart 1, *inset*). Each *point* represents the mean ± S.E. of 3 experiments. *Inset*, influx kinetics for AD over a concentration range of 0.02 to 14 μM. Three experiments (○, ●, ○) are illustrated as a log-log plot of AD influx as a function of [AD]$_e$.

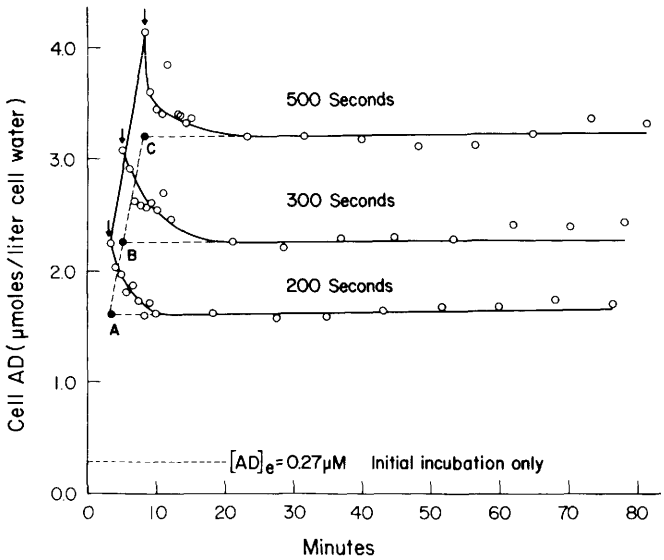

Chart 3. Exchangeability of cell AD. Cells were loaded with AD for 200, 300, 500 sec; then at the *arrows*, portions of the cell suspension were separated by centrifugation, washed with 0° buffer, and resuspended into large volumes of AD-free media. *A*, *B*, and *C*, the extrapolation of the nonexchangeable AD levels (*horizontal interrupted lines*) to the time when the cells were separated from their parent suspensions. *Diagonal interrupted line*, the rate of binding of AD to high-affinity sites within the cell. Extracellular AD concentration ([AD]$_e$) during the loading procedure was 0.27 μM.

AD, see "Effect of Metabolic Inhibitors"). The level of this intracellular component exceeds the AD concentration in the initial incubation medium. When the level of the tightly bound fraction is extrapolated (*interrupted horizontal line*) back to the point at which the cells were separated from the AD-containing suspension (*closed circles*), it is observed that the rate of uptake of this component (*interrupted diagonal line*) is similar to the net rate of accumulation of total AD within the cell (*diagonal continuous line*) suggesting that the bulk of uptake can be accounted for on the basis of tight binding of AD within the cell. The observation that tightly bound intracellular AD increases with time when exchangeable intracellular AD is already present suggests that, early in the uptake sequence, transport and accumulation of exchangeable AD is more rapid than the rate of tight binding of AD within the cell.

Chart 4 contrasts the unidirectional efflux characteristics when cells are loaded with AD for 40 min as compared to 3 min. As described above, intracellular AD consists of at least 2 components after a 3-min exposure to AD (*inset*). The exchangeable component rapidly leaves the cells and the other component is tightly bound. After the cells are loaded with AD for 40 min, another slow exit component is distinguished with a half-time of 35 min followed by the efflux of a more tightly bound fraction. The rapid-exiting AD component observed after a 3-min incubation with AD is not distinguished after a 40-min exposure because this level of AD represents only a trivial part of the total amount of AD taken up into the cells over the longer interval.

Effects of Metabolic Inhibitors. As indicated in Chart 3, the level of exchangeable intracellular AD is greater than the extracellular AD level. If all the exchangeable intracellular AD is osmotically active in the intracellular water, this implies a concentration gradient for AD across the cell membrane, a process that would require the expenditure of metabolic energy and should be depressed by metabolic poisons. However, neither the exchangeable nor the tightly bound components of intracellular AD are influenced by exposure of cells to sodium azide and iodoacetic acid (Chart 5), suggesting that the apparent chemical gradient for AD is related to loose binding of this agent within the cell.

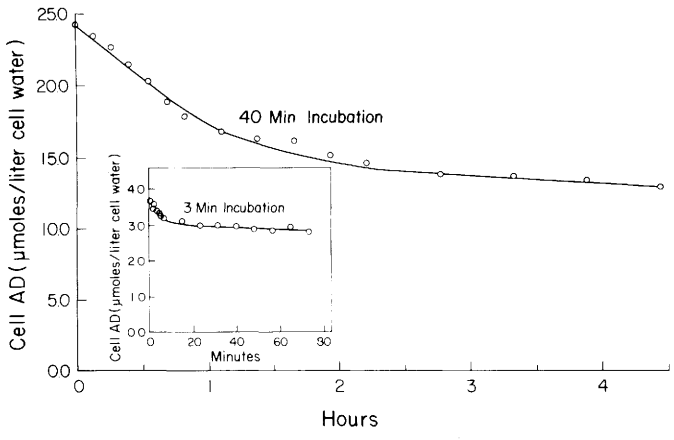

Chart 4. Exhangeability of cell AD over longer loading intervals. Cells preloaded with AD at a concentration of 0.26 μM for 40 min were separated by centrifugation, washed with 0° buffer, and resuspended in a large volume of AD-free medium at 37°, and the efflux of radiolabel was monitored. *Inset*, the efflux of AD in cells loaded with this agent for 3 min.

Augmentation of AD Uptake by Tween 80. Exposure of tumor cells to 0.01% Tween 80 prior to the addition of AD resulted in an increase in AD influx and the late uptake rate (Chart 6). In 4 such experiments, the initial uptake velocity and the late linear uptake phase were increased by factors of 3.0 ± 0.2 and 2.3 ± 0.1, respectively.

For assessment of the effect of Tween 80 on exchangeability of intracellular AD, cells were exposed to AD for 5 min in the presence and absence of Tween 80, separated by centrifugation, and then resuspended into a large volume of

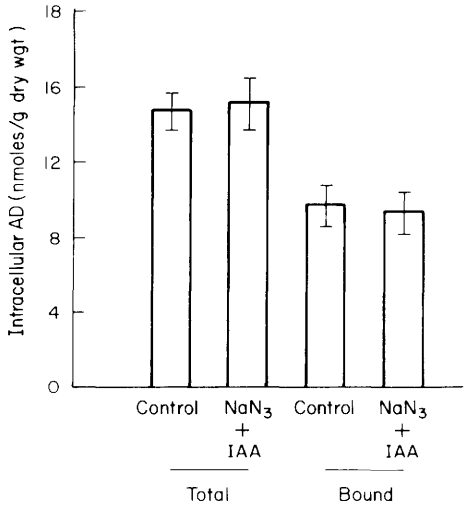

Chart 5. The effect of NaN_3 and iodoacetic acid (*IAA*) on tightly bound and total cellular AD levels. Cells were loaded with AD in the presence and absence of 10 mM NaN_3 plus 1 mM iodoacetic acid for 200 to 300 sec following which the total cell AD levels and the tightly bound fractions were determined from efflux studies (see Charts 3 and 4). *Bars*, mean ± S.E. of 5 experiments.

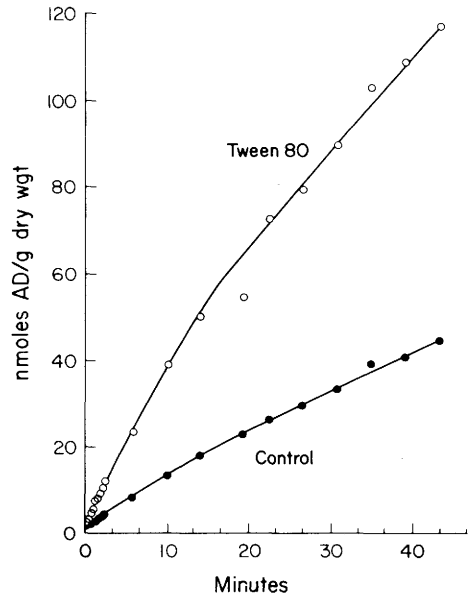

Chart 6. The effect of Tween 80 on AD uptake. Cells were exposed to 0.01% Tween 80 for 1 min prior to the addition of AD.

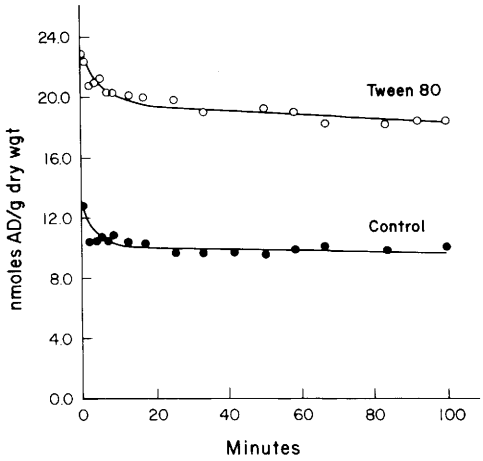

Chart 7. The effect of Tween 80 on the efflux components of AD. Cells were incubated with 0.2 μM AD in the absence and presence of 0.01% Tween 80 for 5 min, and then separated by centrifugation, washed with 0° buffer, and resuspended into AD- and Tween 80-free media.

AD- and Tween 80-free medium (Chart 7). The augmented net uptake for AD is associated with a large increase in the tightly bound intracellular AD component without a concomitant increase in the exchangeable drug fraction. In a paired t test analysis from 7 experiments, there was no significant difference ($p > 0.2$) between the exchangeable levels of AD in cells treated with Tween 80 as compared to control cells (ratio of Tween 80 to control was 1.08 ± 0.06).

Inhibition of RNA Synthesis by AD. The relationship between binding of AD within the cell and inhibition of RNA synthesis was assessed by an analysis of the effect of AD on [2-^{14}C]uridine incorporation into a 0° precipitate. After exposure of Ehrlich ascites tumor cells to 1 μM AD for <50 sec, 5 min, and 10 min (Chart 8), the cell fractions were separated by centrifugation and resuspended into large volumes of AD-free media. After sufficient time was allowed for the major portion of exchangeable intracellular AD to exit from the cells, [2-^{14}C]uridine was added. Incorporation rates were calculated over the interval of 2.5 to 5.5 min following addition of the radiolabel when the rates of ^{14}C incorporation were constant (Chart 8), suggesting that this process reflects an initial rate of RNA synthesis. It can be seen that, within 50 sec after exposure to AD, inhibition of RNA synthesis is depressed ~35%. After 5 and 10 min, [2-^{14}C]incorporation into the TCA precipitate is inhibited by 61.9 ± 2.8% and 74.8 ± 2.5%, respectively (from 3 experiments). As indicated from Charts 3 and 4, saturation of high-affinity binding sites with AD is far from complete over these intervals; hence, association of AD with only a small fraction of the total high-affinity intracellular binding sites is sufficient to inhibit RNA synthesis appreciably.

DISCUSSION

The uptake sequence for AD in Ehrlich ascites tumor cells is determined both by the membrane transport and by the intracellular binding of this drug. Over short intervals of exposure to AD, uptake is characterized by a rapid component following which the net uptake rate declines to a constant velocity which approximates the rate at which AD is tightly bound within the cell. The early departure from linearity is attributed to the rapid accumulation of osmotically active (free) AD within the cell, the early development of an efflux component, and a resultant reduction in the net uptake velocity. Because the late uptake phase proceeds at a constant velocity, it is assumed that the level of free AD within the cell during this uptake interval is also essentially constant. The observation that exchangeable intracellular AD has appeared within the cell (Chart 3) when the tightly bound component of AD is still increasing with time suggests that AD influx is rapid in comparison to the rate at which AD is tightly bound within the cell. However, since the free intracellular AD level must be determined by the net effects of influx less the rates of efflux and intracellular binding and since influx exceeds the rate of tight binding by a factor of only 2, it is unlikely that free intracellular AD reaches equilibrium with extracellular drug during the late linear uptake phase. Rather, during this interval the free intracellular AD level is probably less than the extracellular concentration. Since the rate of binding should be determined by the level of free AD, factors that increase AD transport and bring the intracellular AD level closer to equilibrium should augment the rate of intracellular binding. A time-dependent binding of AD to DNA within the cell might be based upon steric hinderance by the pentapeptide moieties during the mutual conformational adaptation of AD and DNA (3, 28). Consistent with this is the observation that actinomine, an analog of AD without the pentapeptides, binds to DNA more rapidly than AD (28).

Influx of AD was 1st order over a very broad concentration range (Chart 2). Although this excludes a high-affinity

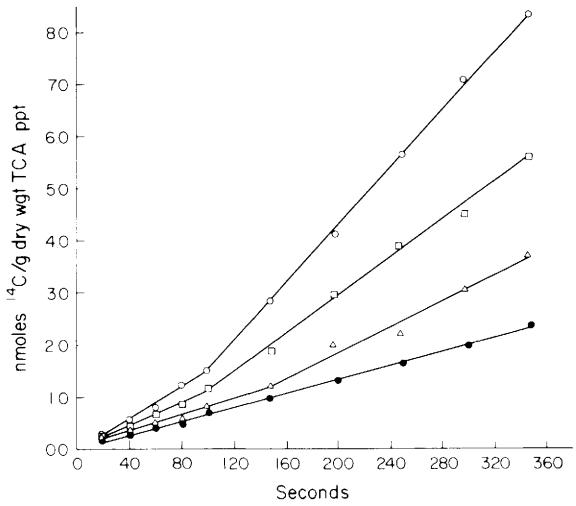

Chart 8. Inhibition of [2-^{14}C]uridine incorporation into RNA by AD. Cells were incubated with 1 μM AD for 44 sec, 5 min, or 10 min. The cells were washed twice with 0° buffer and resuspended into a large volume of AD-free medium. After a 10-min incubation at 37°, [2-^{14}C]uridine was added to achieve a level of 0.13 μM and the rate of accumulation of radiolabel in a 0° TCA precipitate was measured as described in "Materials and Methods." Control cells, ○; cells exposed to AD for 44 sec (□), 5 min (△), and 10 min (●).

transport carrier, the possibility of a very low-affinity saturable transport route remains, since the concentration of AD that could be achieved in these studies was limited by the solubility of this agent in aqueous solutions. Influx was more temperature sensitive than expected for a passive diffusional process (26), but this may be related in part to the high thermal energy required to break AD hydrogen bonds with water (21) to permit penetration of the lipid membrane. Although the temperature dependence of the partitioning of AD in corn oil can account for only one-half the Q_{10} for AD influx in the intact cell, the temperature sensitivity of the passive permeability of AD in the intact cell with a different membrane lipid composition might be even greater. While these data do not distinguish between a passive diffusional and equilibrating carrier-mediated transport process, the data suggest that transport of AD is not energy dependent. This is based upon the observation that net accumulation of exchangeable intracellular AD is unaffected by the presence of inhibitors of both anaerobic and aerobic energy metabolism, conditions that would markedly inhibit transmembrane gradients of actively transported substances [rapid alterations in energy-dependent transport after addition of metabolic inhibitors have been observed for folate compounds as well as in the model amino acid, α-aminoisobutyric acid, in other studies from this laboratory (9, 11, 13, 14)]. Thus, the apparent chemical gradient for exchangeable AD across the cell membrane must be accounted for on the basis of loose binding of AD within the cell. Likewise, the high cell-to-medium AD ratio observed in L1210 leukemia cells (24) is probably related to tight and loose intracellular binding rather than to a gradient for osmotically active AD.

Analysis of efflux kinetics for AD in Ehrlich ascites tumor cells is compatible with a variety of intracellular binding sites with different affinities for AD. This is an expected finding since AD binds to a variety of sites on double-stranded DNA with differing affinities for this agent (4, 5, 28). While the possibility cannot be excluded that the characteristics of the time course of the unidirectional fluxes of AD may be related, in part, to artifacts related to the experimental protocol or heterogeneity of the cell population, this is considered unlikely since other sensitive properties of membrane integrity and biochemical stability are intact in Ehrlich ascites tumor cells under these experimental conditions such as (*a*) cell volume (8, 11); (*b*) intracellular cation levels (10); (*c*) transmembrane gradients for α-aminoisobutyric acid and methotrexate (8, 9, 11, 13, 18); and (*d*) DNA synthesis (16, 17). The data suggest further that the critical element in the AD-cell interaction resulting in inhibition of RNA synthesis is related to the interaction between AD and a small number of intracellular binding sites with a high affinity for this agent. While recovery of RNA synthesis in 37 RC cells has been attributed to dissociation of AD from low-affinity binding sites (1, 2), it would be expected that recovery of RNA synthesis in Ehrlich ascites tumor cells, similar to that observed for the HeLa cell (1, 2), would be slower as a result of the critical role of high-affinity binding sites in these cell lines and the slow rate of dissociation of AD from these sites.

Tween 80, a membrane-active detergent, potentiates the cytotoxicity of AD, an effect attributed to stimulation of the membrane transport of AD (29). In the studies reported here, Tween 80 augments AD influx, reflecting a stimulation of the membrane transport process. In addition, however, Tween 80 also increases the later linear phase of AD uptake, the component of the uptake sequence that reflects the rate of tight binding of AD to intracellular constituents. Since the level of free intracellular AD should influence the rate of binding, the apparent effects of Tween 80 on the rate of AD binding could be accounted for on the basis of augmented influx and an increased free intracellular AD level closer to thermodynamic equilibrium. If this were the case, however, it would be expected that Tween 80 would increase the exchangeable AD fraction, but this was not observed (Chart 7). Of course, if the ratio of free to loosely bound intracellular AD is very low, small alterations in the former might be difficult to detect. Alternatively, in addition to stimulating the membrane transport of AD, Tween 80 may independently enhance the binding of AD to DNA; this might be related to a Tween 80-induced dissociation of histones from DNA, thereby modifying DNA acceptor sites so that they are more accessible to AD (30).

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