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COOPERATIVE SEQUESTRATION OF m-AMSA IN L1210 CELLS

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Abstract—The anticancer drug 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) is known to bind to DNA by intercalation and to produce protein-associated DNA strand breaks in cells. Previous work [Zwelling *et al.*, *Biochemistry* **20**, 6553 (1981)] had shown that m-AMSA is in rapid equilibrium between extracellular and intracelluar compartments, and that the DNA strand breaks exist in a steady state of rapid formation and resealing. The current work reports an unusual uptake phenomenon of m-AMSA by mouse leukemia L1210 cells that occurs at higher drug concentrations than previously studied. The new uptake phenomenon was characterized by cooperativity, hysteresis, irreversibility, saturability, slowness and temperature dependence. It is concluded that m-AMSA concentrations above a critical value can initiate the irreversible sequestration of m-AMSA into a new phase, probably in an extranuclear compartment of the cell, from which the drug has no access to the nuclear DNA and probably does not contribute to cytotoxicity.

4′-(9-Acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA)†, a 9-aminoacridine derivative developed by Cain and Atwell [1], is currently undergoing clinical trials to determine its possible usefulness as an anticancer drug. It has been found to have activity in the treatment of adult myeloid leukemia in patients whose disease has become refractory to therapy with anthracyclines [2].

m-AMSA binds to DNA by intercalation [3] and, like other intercalating agents, produces in mammalian cells DNA strand breaks that are associated in some specific manner with covalently bound protein [4]. We have reported previously, using mouse leukemia L1210 cells, that the uptake and egress of m-AMSA at 37° are completed within minutes [4]. The protein-associated DNA strand breaks, which may be enzymatically induced, appear and disappear at rates comparable to the rates of uptake and egress. However, the formation or resealing of the DNA breaks is completely blocked at 4°, whereas drug uptake and egress are unchanged in extent, although slowed in rate. Under the conditions of the previous work, extracellular and intracellular m-AMSA were in rapid equilibrium with each other (at 37°) and with the protein-associated DNA strand breaks.

In the current work, we report an unusual uptake phenomenon which occurred when amounts of m-AMSA that were larger than used in the previous studies were added to the cell suspension. m-AMSA uptake was markedly enhanced due to sequestration of the drug in an extranuclear phase.

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† Abbreviations: m-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; RPMI 1630, Roswell Park Memorial Institute medium 1630; and HBSS, Hanks' balanced salt solution.

MATERIALS AND METHODS

Cells and radioactive labeling. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium plus 15% fetal calf serum plus penicillin and streptomycin. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures. Cultures utilized to assess drug effects were in exponential growth phase with a doubling time of 13–15 hr.

Most transport studies were performed with cells grown as above and then concentrated to approximately 10^7 cells/ml by centrifugation and resuspension in fresh medium. For assessment of DNA strand breakage by alkaline elution or for studies comparing drug transport in cells and nuclei, cellular DNA was radioactively labeled with either [2-^{14}C]thymidine (0.01 μCi/ml, sp. act. 58 mCi/mmole) or [methyl-^{3}H]thymidine (0.01 μCi/ml, 10^{-6} M unlabeled thymidine added, sp. act. 20 Ci/mmole) by overnight incubation. Radioactivity was removed by centrifugation prior to drug treatment.

Drugs and drug treatment. m-AMSA base (NSC 249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, Bethesda, MD. o-AMSA base (SN 12735) was a gift from Dr. Bruce F. Cain, Aukland Division of the Cancer Society of New Zealand. Both compounds were dissolved in dimethyl sulfoxide at 0.01 M and stored frozen. Adriamycin (NSC 123127) was dissolved just prior to use in sterile, glass-distilled water at 1 mg/ml. Ellipticine (NSC 71795) was dissolved in sterile 0.1 N HCl at 0.01 M and used immediately.

4′-(9-Acridinyl-[9-^{14}C]-amino)methanesulfon-*m*-anisidide ([^{14}C]m-AMSA) (19.6 mCi/mmole) was synthesized by SRI International, Menlo Park, CA, and was obtained through the Chemical Resources Section, NCI. This drug was also dissolved in dimethyl sulfoxide and stored frozen.

Transport studies. Cells were concentrated to approximately 10^7 cells/ml and drug treated ([^{14}C]m-AMSA) at 37°. A 1-ml aliquot was removed and gently layered above 0.6 ml of Versilube F-50 silicone oil in a microcentrifuge tube. The cells were pelleted through the oil by centrifugation at 12,000 *g* for 1 min. The tube bottom was cut off and placed in a scintillation vial. The cell buttom was dispersed in 1.5 ml of phosphate-buffered saline and the pellet was solubilized by the addition of 1.5 ml of 0.4 M NaOH and overnight incubation at 37°. The following morning 3 ml of water followed by 10 ml of Aquassure (New England Nuclear Corp., Boston, MA; 30 ml glacial acetic acid added per 4 liters) was added, and radioactivity was quantified by liquid scintillation spectroscopy. The amount of extracellular fluid trapped in the cell pellet was estimated by the use of [^{14}C]inulin (New England Nuclear Corp.) and was approximately 1.4 μl/10^7 cells. Based on this estimate, small corrections were applied to the measured radioactivity in the pellet. In studies assessing the actual drug concentration in cells, the uptake of [^{14}C]m-AMSA was compared with that of tritiated water (New England Nuclear Corp.). These techniques are adaptations of those of Vistica [5], whose help in setting up these methods is gratefully acknowledged.

Alkaline elution. The methodology of alkaline elution has been described in great detail in past publications [4, 6, 7]. The technique used in this manuscript is the low sensitivity assay for quantitating single-strand breaks as recently described by Zwelling *et al.* [4].

Isolation of nuclei. L1210 mouse leukemia cells with their DNA labeled with [^{3}H]thymidine as described above were centrifuged and resuspended in an equal volume of nuclei buffer (150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, pH 6.4). These cells were again centrifuged and resuspended in 1/10 volume iced nuclei buffer. Then 9/10 volume of nuclei buffer plus 0.3% Triton X-100 was added and the mixture was incubated for 10 min at 4°. The nuclei were then pelleted by centrifugation and resuspended in nuclei buffer. Nuclei were observed microscopically as trypan blue positive.

RESULTS

Uptake and egress. When m-AMSA at a concentration of 0.5 μM was added to L1210 cells at 37°, the drug was taken up rapidly and reached a steady-state level within 5 min (Fig. 1) [4]. When the cells were subsequently washed and resuspended in fresh medium, drug egress was prompt and nearly complete. Reducing the temperature to 23° had little effect, but at 4° uptake was slowed (Fig. 1). Even at 4°, however, the drug uptake achieved at the end of 60 min was at least as great as at 37°. (In this experiment, the 60-min uptake actually was higher at reduced temperature than at 37°, but this difference was not reproduced in other experiments.) Drug egress at reduced temperatures was slowed slightly and was not as complete as at 37° (Fig. 1).

At m-AMSA concentrations below 7.5 μM, uptake (measured at 60 min) was proportional to

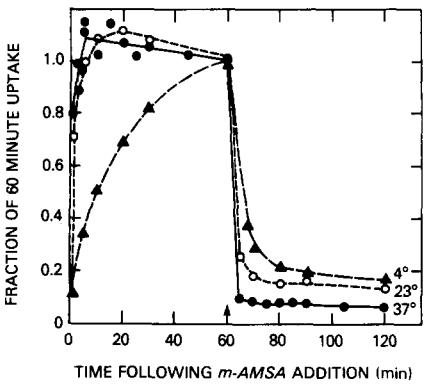

Fig. 1. Uptake and egress of 0.5 μM [^{14}C]m-AMSA. Uptake was followed during incubation of L1210 cells (10^7/ml) with drug over a 60-min period at 37° (●), 23° (○), or 4° (▲). Egress was followed in cells that had been incubated with drug for 60 min at 37° and then transferred to drug-free medium (arrow) at 37° (●) or 4° (▲). Egress at 23° (○) was measured following treatment at 23°. The ordinate was normalized by setting the uptake at 60 min equal to 1.0. The actual 60-min uptakes were: 2671 dpm/10^7 cells = 4.0×10^6 molecules/cell (37°); 3682 dpm/10^7 cells = 5.4×10^6 molecules/cell (23°); and 4886 dpm/10^7 cells = 7.2×10^6 molecules/cell (4°).

concentration of drug added to the medium and was similar at 4° and 37° (Fig. 2). At higher drug concentrations, however, uptake was markedly dependent on temperature. At 4°, uptake continued to rise in proportion to drug added, but at 37° an anomaly appeared. The anomalous uptake was observed to begin at a threshold concentration of approximately 10 μM m-AMSA added. Above this concentration, uptake was much greater than proportional to drug concentration, suggesting the onset of a cooperative process. The cooperative process was limited, in that uptake levelled off at concentrations of added m-AMSA above 20 μM. Thus, the cooperative uptake appeared to be saturable.

The possibility that m-AMSA was associated with extracellular precipitable material rather than cells was excluded by control experiments in which cells were omitted.

Cells incubated with high m-AMSA concentrations at 4° and then shifted to 37° exhibited increased uptake. Thus, low temperature did not permanently inactivate the cooperative uptake mechanism.

Quantitative aspects of m-AMSA uptake are summarized in Table 1. It is evident that the drug was strongly concentrated or accumulated in or on the cells.

In contrast to the rapid in and out transport of m-AMSA at low concentrations, uptake and egress under conditions of cooperativity exhibited entirely different characteristics. Uptake at 13 μM m-AMSA, which was within the cooperative range at 37°, was much slower than at 0.5 μM, which was in the proportional range (Fig. 3A). At 4°, at which both concentrations exhibited proportional uptake, the uptake kinetics for the two concentrations were the same (Fig. 3B).

The difference between the proportional and the cooperative uptake processes was even more striking

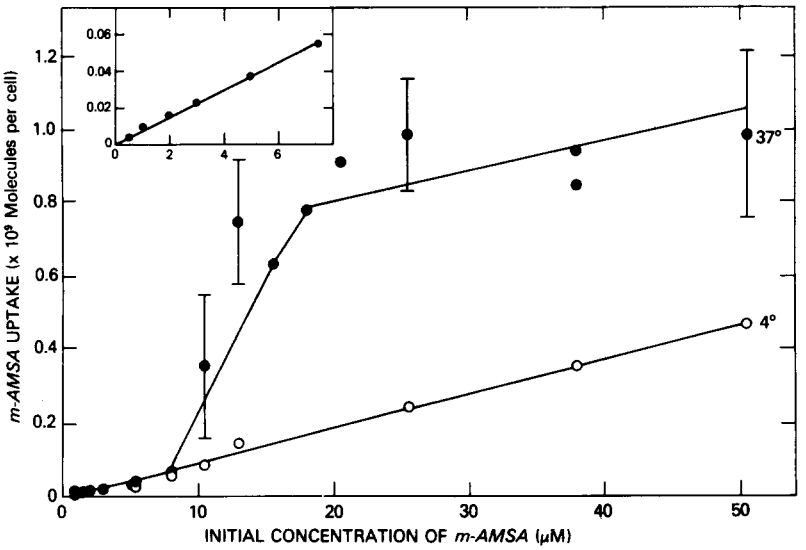

Fig. 2. Concentration dependence of m-AMSA uptake. Approximately 10^7 cells were incubated for 1 hr at 37° (●) or 4° (○) in 1 ml of medium containing various concentrations of m-AMSA, including 0.5 μM [^{14}C]m-AMSA. Inset shows proportionality of uptake at low m-AMSA concentrations.

in regard to drug egress (Fig. 4A). When cells had been loaded with drug under conditions of proportional uptake, drug efflux was rapid and nearly complete. By contrast, cells that had been loaded under conditions of cooperative uptake retained most of the drug irreversibly (Fig. 4A). The reversibility of drug uptake is shown in greater detail in Fig. 4B as a function of loading concentration. Below 10 μM, drug uptake was low but almost competely reversible. Between 13 and 20 μM, uptake rose sharply and irreversibility peaked at 85–95%. At higher concentrations, uptake leveled off or perhaps declined somewhat and the amount or fraction of drug in the reversible uptake compartment increased gradually. These results suggest that the cell-associated m-AMSA existed in two compartments: a proportional uptake compartment which was reversible and a cooperative uptake compartment which was irreversible. Drug in the latter compartment can be described as sequestered.

Another unusual characteristic of the cooperative uptake was hysteresis (Fig. 5). This was seen in experiments in which uptake was measured after 60 min, by which time most of the cooperative uptake had gone to completion. As the quantity of m-AMSA added to the system was increased, the quantity associated with the cells and the extracellular concentration at first increased proportionately. At a critical point, however, further increase in the quantity of drug in the system actually resulted in a sharp decrease in extracellular concentration as large quantities of drug were absorbed by the cells. Apparently there was a sudden collapse of a barrier for entry of drug into a previously inaccessible or non-existent compartment. Further added drug then went almost entirely into this new compartment with little or no

Table 1. Uptake per cell at various drug concentrations*

Drug added		Average cell volume	Drug uptake	
(μM)	(10^{-15} moles/cell)	(10^{-12} l/cell)	(μmoles/liter of cell water)	(10^{-15} moles/cell)
0.5	0.05	1.07	5.7	0.0061
5.5	0.55	1.62	45.3	0.0734
13.0	1.30	1.42	518.0	0.736
50.5	5.05	1.44	1339.0	1.93

* L1210 cells were incubated for 30 min at 37° with 0.5 μM [^{14}C]m-AMSA with or without added unlabeled m-AMSA and with 2.5 μl of tritiated H_2O (1 mCi/ml). Samples were taken as described in Materials and Methods. The volume of the cell pellet (corrected for extracellular fluid) was calculated as the dpm of ^3H_2O in the pellet divided by the dpm in the pellet + supernatant fraction of each 1 cc sample and corrected to volume/10^7 cells. The m-AMSA concentration was calculated by the formula:

$$\left(\frac{\text{corrected [}^{14}\text{C]m-AMSA dpm/}10^7\text{ cells}}{\text{volume of }10^7\text{ cells in ml}}\right)(1\ \mu\text{Ci}/2.22\times10^6\text{ dpm})(0.43\text{ mg}/20\ \mu\text{Ci})\times(10^6\ \mu\text{M}/393.5\text{ mg/ml})$$

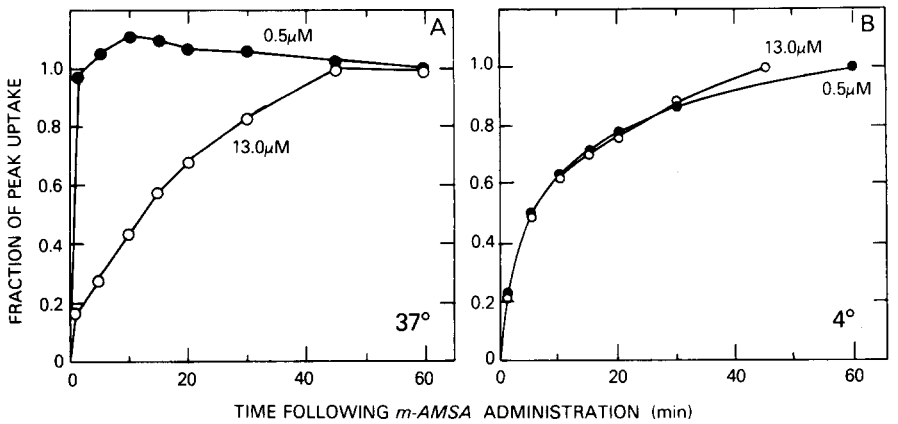

Fig. 3. Uptake kinetics of m-AMSA at 0.5 μM (●) or 13.0 μM (○), at 37° (A) or 4° (B). Actual uptakes at 45–60 min were: 3032 dpm/10^7 cells = 4.5 × 10^6 molecules/cell (0.5 μM, 37°); 508,560 dpm/10^7 cells = 7.5 × 10^8 molecules/cell (13 μM, 37°); 4142 dpm/10^7 cells = 6.1 × 10^6 molecules/cell (0.5 μM, 4°); and 96,096 dpm/10^7 cells = 1.4 × 10^8 molecules/cell (13 μM, 4°).

change in extracellular concentration. These phenomena, thus far, were akin to crystallization from a supersaturated solution. However, contrary to a simple crystallization process, the amount of drug that could be accommodated in the new compartment was limited. When the amount of cell-associated drug exceeded approximately 10^9 molecules/cell, entry into the cooperative compartment ceased, and further addition of drug led to large increases in extracellular concentration with little change in intracellular content.

The cooperative entry of large quantities of drug was not accompanied by any obvious morphologic changes when the cells were examined by phase microscopy or electron microscopy (performed by Drs. Ronald Gordon and Jerome I. Kleinerman of the Department of Pathology, Mt. Sinai School of Medicine, New York). Pre-loading the cells with 12.5 μM m-AMSA for various periods of time did not affect the uptake of subsequently added [^{14}C]m-AMSA (measured after a 2- to 4-min uptake period). Hence, the sequestered m-AMSA did not affect the membrane transport of the drug.

The possible involvement of energy-requiring processes in m-AMSA uptake or efflux was explored by examining the effect of 10 mM sodium azide, which should inhibit such processes. Sodium azide reduced m-AMSA uptake in some experients (Fig. 6), but these changes did not occur consistently and were too small for a crucial energy-dependent step to have been present in m-AMSA uptake. Hence, neither the proportional nor the cooperative uptake process was energy-dependent.

Azide had no effect on m-AMSA efflux (Fig. 6 and Table 2), indicating that efflux also was independent of major energy sources.

The data in Table 2, as well as that of other experiments, indicates that m-AMSA uptake was

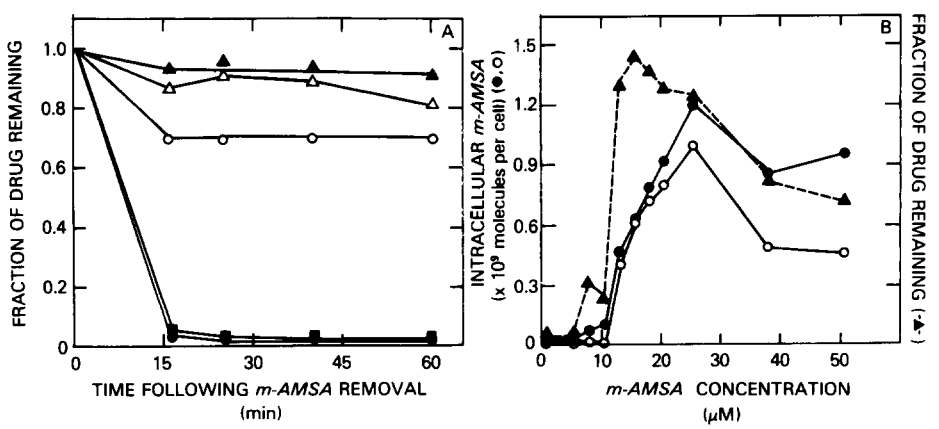

Fig. 4. Reversibility of m-AMSA uptake after initial incubation of cells with [^{14}C]m-AMSA at various m-AMSA concentrations. (A) Efflux following initial incubation for 60 min at the following m-AMSA concentrations (μM): 0.5 (●), 5.5 (■), 10.5 (△), 13.0 (▲), and 25.5 (○). Each incubation contained 0.5 μM [^{14}C]m-AMSA and 10^7 cells/ml. (B) Dependence of reversibility on the m-AMSA concentration during the initial incubation. Key: (●) initial uptake following 60-min incubations; (○) retention after 60 min of further incubation after washing the cells; and (▲) fraction of the initial uptake retained after 60-min incubation in fresh medium.

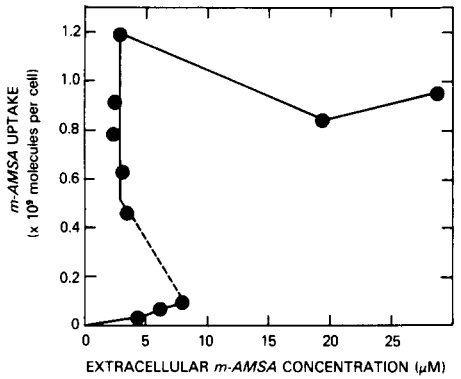

Fig. 5. Hysteresis in the uptake of m-AMSA by L1210 cells. Cells (10^7/ml) were incubated for 60 min at 37° in the presence of various concentrations of m-AMSA plus 0.5 μM [^{14}C]m-AMSA. Extracellular m-AMSA concentration was determined from the radioactivity in the supernatant fraction after pelleting the cells through oil as described under Materials and Methods.

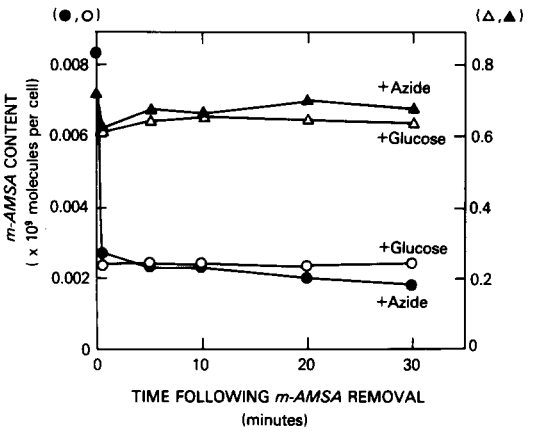

Fig. 6. Lack of effect of azide on efflux of m-AMSA. Cells (10^7/ml) were incubated for 10 min in HBSS plus 10 mM sodium azide. They were then treated with 0.5 μM (○, ●) or 13.0 μM (▲, △) m-AMSA (12.5 μM unlabeled + 0.5 μM [^{14}C]m-AMSA for 30 min. Zero-time samples were taken and cells were washed and resuspended in HBSS plus 10 mM sodium azide (●, ▲) or 10 mM glucose (○, △).

reduced in serum-containing medium. This was probably due to binding of m-AMSA to serum proteins [8].

The uptake of m-AMSA at 13 μM at 37° was found to have a striking pH dependence (Fig. 7). Between pH 6.7 and 7.1, the uptake was high and insensitive to pH. As pH was lowered from 6.7 to 6.0, however, uptake decreased to very low levels.

Since m-AMSA is known to bind to DNA by intercalation [3], several types of experiments were performed to determine whether DNA was involved in the sequestration phenomenon.

First, m-AMSA binding to isolated nuclei was examined (Table 3). Cells or nuclei were incubated for 30 min in the presence of 0.5 μM [^{14}C]m-AMSA with or without 12.5 μM unlabeled m-AMSA. Several modifications of the nuclei suspension medium were tried, including modification of pH, omission of ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) or EDTA, and replacement of NaCl by KCl. Under none of the conditions tested was the amount of [^{14}C]m-AMSA bound to the nuclei significantly affected by the total m-AMSA concentration. Hence, there was no cooperative binding of m-AMSA to isolated nuclei under the conditions tested.

Second, other DNA intercalators were tested to determine whether they would enhance m-AMSA uptake by cells. Neither adriamycin nor ellipticine had any effect on the uptake of a given amount of [^{14}C]m-AMSA (Table 4). The biologically inactive isomer, o-AMSA, did increase the uptake of [^{14}C]m-AMSA, but the magnitude of this enhancement was much less than that produced by m-AMSA itself.

Table 2. Effect of 10 mM sodium azide on m-AMSA uptake and its reversibility

m-AMSA added (μM)	Medium	m-AMSA content (10^8 molecules/cell) Uptake* −Azide	Uptake* +Azide	Retention† −Azide	Retention† +Azide
0.5	RPMI 1630 + FCS	0.0365	0.0561		
0.5	HBSS	0.0884	0.0676		
13.0	RPMI 1630 + FCS	3.57	2.57		
13.0	HBSS	8.85	6.40		
0.5	RPMI 1630 + FCS	0.0511		0.0076	0.0087
	HBSS			0.0118	0.0132
13.0	RPMI 1630 + FCS	3.20		1.55	1.59
	HBSS			2.21	2.21

* Cells (10^7/ml) were incubated for 10 min in fresh medium or HBSS with or without 10 mM sodium azide. m-AMSA was added to the concentrations indicated, including 0.5 μM [^{14}C]m-AMSA. Uptake time was 30 min at 37°.

† Cells were loaded by incubation with m-AMSA as above in RPMI 1630 medium + fetal calf serum (FCS), 30 min, 37°. Aliquots of washed cells were further incubated in the absence of drug and in the presence or absence of 10 mM sodium azide for 30 min.

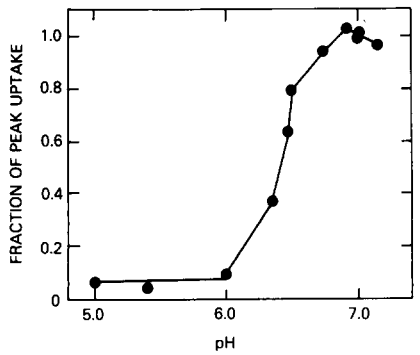

Fig. 7. Effect of pH of the medium on the uptake of m-AMSA at 13 μM. Cells were incubated in RPMI 1630 medium containing 15% fetal calf serum and m-AMSA for 1 hr at 37°.

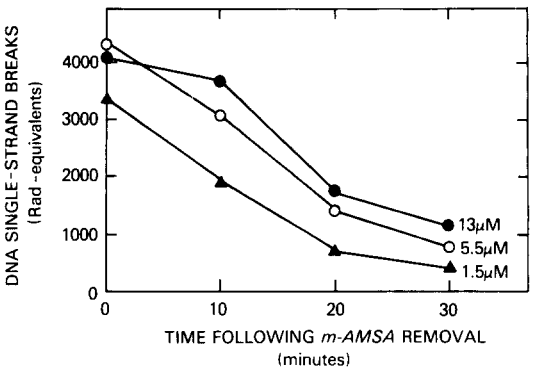

Fig. 8. Reversal of DNA single-strand breaks with time following a 30-min treatment of cells with 13.0 (●), 5.5 (○), or 1.5 (▲) μM m-AMSA. L1210 cells were treated for 30 min, and drug was then removed by centrifugation. Single-strand breaks were quantified by the alkaline elution method using proteinase and are expressed in terms of the dose of X-rays that produces an equal break frequency.

Third, cells were X-rayed in order to produce DNA strand breaks that could allow changes in chromatin conformation to take place. X-ray had little or no effect on the cooperative uptake of m-AMSA (Table 4).

Finally, the question of whether m-AMSA in the sequestered compartment had access to the nuclear DNA was addressed using the alkaline elution technique [4, 6, 7]. Using this technique, we had found previously that low concentrations of m-AMSA produce protein-associated DNA single-strand breaks which, upon washing the cells with fresh medium, are reversed within a few minutes [4]. Since m-AMSA in the sequestered compartment did not wash out of the cells, the question was posed as to whether the continued presence of m-AMSA in this compartment would prevent the reversal of the DNA strand breaks. Figure 8 shows that the DNA strand breaks formed after treatment with 13 μM m-AMSA for 30 min reversed as quickly as breaks formed after treatment with 1.5 μM. Also, these two concentrations produced similar frequencies of strand breaks. Hence, the m-AMSA in the sequestered compartment did not have access to the nuclear DNA.

The lack of accessibility of nuclear DNA to sequestered m-AMSA raises the question of whether the sequestered drug affects cell survival. The survival curves (Fig. 9) are seen to level off above 10 μM of added m-AMSA, as would be expected if sequestered drug did not contribute to cell killing. This is not a firm conclusion, however, because the survival above 10 μM was only 1% or less and may have represented a resistant cell population. However, any existing resistant cell population did not remain totally resistant under conditions of more prolonged exposure to lower m-AMSA concentrations (Fig. 9B). This is further noted in that an increase in drug exposure time from 20 to 30 min lowered the survival more than could be achieved by raising the drug concentration. This result is most easily explained on the assumption that only the non-sequestered drug, which is in equilibrium with extracellular drug, contributes to cell killing.

DISCUSSION

The uptake of m-ASMA by L1210 cells may be described according to the diagram in Fig. 10. The diagram has been simplified by omission of the rapid reversible binding that may occur between free m-AMSA and protein or lipid components in the cells or in the extracellular medium [8]. This omission does not alter the essential aspects of the sequestration phenomenon that we are reporting. The diagram also omits possible covalent reactions of m-AMSA, such as with serum proteins in the medium [9, 10].

According to our previous work with L1210 cells [4], the main feature of m-AMSA uptake at low

Table 3. Uptake of m-AMSA in isolated nuclei compared with whole cells (30-min treatment at 37°)

Medium	m-AMSA added (μM)	[^{14}C]m-AMSA Cells (10^3 dpm)	Nuclei (10^3 dpm)
RPMI 1630 + 15% FCS	0.5	3.69	
	13.0*	17.6	
Nuclei buffer	0.5		1.30, 1.34
(pH 7.1)	13.0*		1.20, 1.06
Nuclei buffer	0.5	3.53	1.66, 1.64
(pH 6.4)	13.0*	14.5	1.44, 1.64

* [^{14}C]m-AMSA (0.5 μM) plus unlabeled m-AMSA (12.5 μM).

Table 4. Effect of other intercalating agents or X-radiation on the uptake of m-AMSA*

Expt. No.	Intercalator or X-rays	Conc. (μM)	Cellular uptake (dpm/10^7 cells $\times 10^3$)
1	None		2.26
	Adriamycin	51.7	2.35
		25.9	2.57
		17.2	2.32
		8.6	2.49
	o-AMSA	100	2.08
		75	2.39
		50	3.18
2	None		2.29
	Ellipticine	80	2.43
		60	2.36
		40	2.63
		20	2.06
		10	2.02
	o-AMSA	50	2.89
		25	4.07
		12.5	2.95
		10	2.63
		5	2.38
3	None		2.08
	o-AMSA	150	3.93
		37.5	4.23
		25	3.12
		20	2.71
		17.5	2.52
		15	2.56
		12.5	2.32
		10	2.14
		5	2.14
4†	None		2.79
	X-Rays	3000R	3.24
5‡	None		2.85
	m-AMSA (30 min)	12.5	14.01
	X-Rays	20,000R	3.05
	m-AMSA (30 min) plus X-Rays	12.5 20,000R	11.38

* Cells were treated with 0.5 μM [^{14}C]m-AMSA for 60 min (unless otherwise indicated) in the absence or presence of the indicated unlabeled compound.

† Cells were chilled, X-radiated and then treated at 4° with 0.5 μM [^{14}C]m-AMSA.

‡ Cells were chilled, X-radiated, and treated with 0.5 μM [^{14}C]m-AMSA in the presence or absence of 12.5 μM unlabeled m-AMSA at 37° for 30 min.

concentration is rapid equilibration of free m-AMSA outside and inside the cell. The half-time for equilibration is approximately 30 sec, 1 min, and 10 min at 37°, 23°, and 4° respectively. Under these conditions, no more than a few percent of the drug is irreversibly retained by the cells. A steady state develops between opening and closing of protein-associated DNA strand breaks, indicating that m-AMSA has ready and reversible access to the nuclear DNA. The opening and closing of strand breaks can be prevented by reducing the temperature, whereas drug entry into and exit from the cells are slowed but not prevented. The DNA effects possibly are mediated by an enzyme such as a topoisomerase [11].

The current work shows that when larger amounts

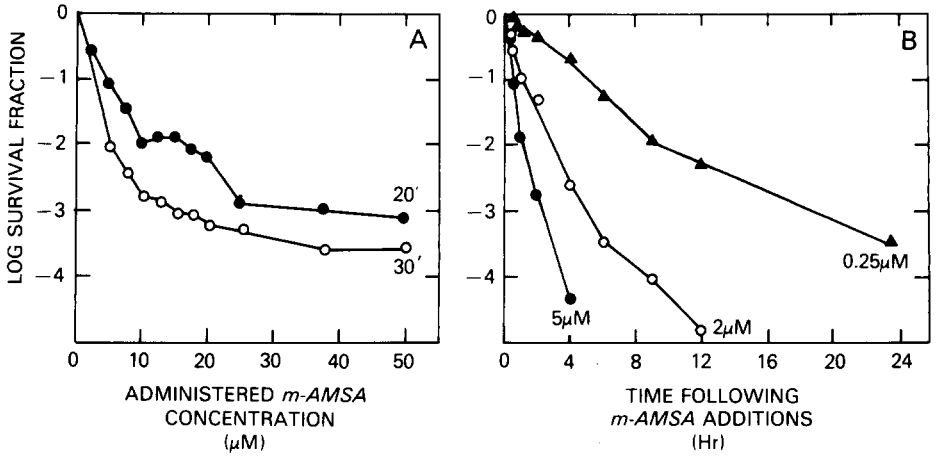

Fig. 9. Survival of L1210 cells treated with various concentrations and for various durations with m-AMSA. (A) Cells were treated for 20 (●) or 30 (○) min with various concentrations of m-AMSA and the survival was measured by soft agar colony formation. (B) Cells were treated with 5 μM (●), 2 μM (○), or 0.25 μM (▲) m-AMSA and aliquots were removed at various times following drug addition.

of m-AMSA were added, a new uptake process appeared. Several characteristics of this process were defined.

First, the uptake exhibited a sharp rise when the amount of m-AMSA added exceeded a critical value (Fig. 2). This signaled the onset of a cooperative process in which the drug stimulated its own uptake.

In addition to cooperativity, moreover, the process exhibited hysteresis, in that increasing the amount of m-AMSA beyond a critical value actually reduced the extracellular drug concentration that was finally achieved (Fig. 5).

Third, the cooperative drug uptake was irreversible. Contrary to free m-AMSA, the drug taken up by the cooperative process was not readily washed out of the cells and did not produce DNA strand breakage (Figs. 4 and 8).

Fourth, the cooperative uptake was relatively slow. Approximately 1 hr was required for the process to go to completion at 37°, whereas the reversible uptake was complete within 20 min (Figs. 1 and 3).

Fifth, the cooperative component of uptake was temperature-dependent and did not occur at 4° (Fig. 2).

Finally, the amount of drug that could be taken up by the cooperative process was limited, i.e. the cooperative process was saturable (Figs. 2 and 4).

These characteristics suggest that, when the concentration of m-AMSA exceeds a critical value, the drug sequesters at some site in or on the cells. Drug that is sequestered would no longer be removed upon washing the cells, and the persistence of sequestered drug would not intefere with the resealing of DNA strands breaks upon washing out the free drug.

Sequestration, however, would not always occur when it was thermodynamically favored, but would be blocked by a free energy barrier. In the cold, this barrier would prevent sequestration even at high concentrations of free drug. At 37° sequestration was not triggered until the free drug concentration

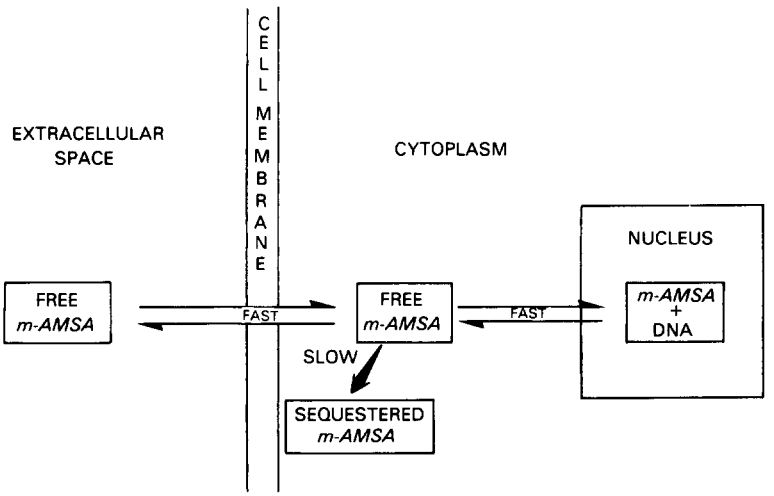

Fig. 10. Model for the uptake and sequestration of m-AMSA.

was about twice that which maintained the sequestration phenomenon once it had been initiated. This was demonstrated by the hysteresis effect shown in Fig. 5; at the critical point at which sequestration was initiated, the extracellular drug concentration fell by approximately a factor of 2 despite the addition of increasing amounts of drug to the system. Further increase in the amount of drug in the system then increased the amount of drug associated with the cells, but did not change the extracellular drug concentration. It is as if a new phase had been triggered to appear, which then absorbed drug until the free drug concentration reached a certain value corresponding to a saturation concentration relative to the new phase.

The sequestered phase, however, had a limited capacity to adsorb drug and saturated at approximately 10^9 molecules per cell. Therefore, the phenomenon was not a simple crystallization of m-AMSA but required a component contributed by the cells that was present in limited quantity.

We do not know the site or chemical nature of the sequestration. However, certain things can be said about it. It required the presence of cells and, hence, was not merely a precipitation of drug in the medium. It did not occur with isolated nuclei and, hence, probably occurred at an extranuclear site. It was not inhibited by azide and, hence, did not require metabolic energy. It was not accompanied by gross changes in cell morphology visible by phase microscopy or routine electron microscopy and, hence, was probably not due to gross coagulation of cell components. Finally, it was initiated at m-AMSA concentrations that were only a factor of 5–10 higher than those which permitted survival of colony-forming ability by 50% of the cells; hence, the sequestration phenomenon may not be irrelevant to biology.

The possibility must be considered that the chemistry of the sequestration event is related to the thiolytic cleavage reaction described by Cain *et al.* [9] and Wilson *et al.* [10]. The critical event could be a rearrangement of a cell constituent to make accessible a large but limited number of reactive thiol groups. By this reaction, only the acridine moiety of the m-AMSA molecule would become bound. Arguing against this mechanism (but not decisively) is the finding that sequestration was inhibited by low pH whereas thiolytic cleavage is stimulated at low pH.

In a recent abstract, Kessel *et al.* [12] independently reported an anomalous uptake of m-AMSA in an anthracycline-resistant line of P388 cells. It is possible that our finding and those of Kessel *et al.* have a common basis.

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