MOLECULAR PHARMACOLOGY, 17, 382–387

The Interaction of Carrier-Bound Methotrexate with L1210 Cells

BARBARA C. F. CHU[1] AND JOHN M. WHITELEY[2]

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received August 17, 1979; Accepted December 19, 1979

SUMMARY

CHU, B. C. F., AND J. M. WHITELEY. The interaction of carrier-bound methotrexate with L1210 cells. *Mol. Pharmacol.* **17**: 382–387 (1980).

Methotrexate (MTX), covalently linked to two randomly chosen proteins (α-chymotrypsinogen and bovine IgG), was as effective as free MTX and MTX bound to bovine serum albumin (MTX–BSA) in prolonging the life span of BDF_1 mice bearing the L1210 tumor, suggesting that MTX can act as an effective chemotherapeutic agent when covalently bound to a variety of proteins. MTX was more toxic than MTX–BSA to L1210 cells grown *in vitro* at 37°C, an observation which correlated with the relative internal drug concentrations recorded after incubation for 1 h with 4 μM free or derivatized MTX. Further *in vitro* experiments employing [^{3}H]MTX–BSA as a prototype of a drug–protein carrier indicated that MTX–BSA was only partially dependent on the same transport mechanism for cellular uptake as MTX. For example, MTX transport into cells was almost zero at 0°C, whereas MTX–BSA entry was only inhibited 50%; additionally MTX–BSA uptake was much less inhibited by *p*-chloromercuribenzenesulfonate and 5-formyltetrahydrofolate than the parent compound. Experiments using [^{3}H]MTX–BSA or MTX–^{125}I-BSA indicated that the MTX–BSA complex was not degraded on the external surface of the cell membrane, as 60–65% of both radioactive labels was found inside the cell lysate fraction with much of the iodinated material degraded to low-molecular-weight fragments. In contrast the ^{125}I marker remaining bound to the cell membrane was still present as a nondialyzable high-molecular-weight molecule. Experiments employing MTX bound to Dextran T70, an ineffective antitumor agent, indicated only limited cellular interaction and low drug uptake.

INTRODUCTION

The concept of covalently attaching drugs to long-chain molecules or entrapping them within the confines of polymeric residues in the hopes of making them more effective in chemotherapy has recently received wide attention. Carrier-bound drug systems which have been investigated include: DNA [adriamycin (1) and daunomycin (2)]; lipid vesicles [actinomycin D (3) and cytosine arabinoside (4)]; antibodies [chlorambucil (5) and *N,N*-bis(2-chloroethyl)-*p*-phenylenediamine (6)]; bovine serum albumin and high-molecular-weight dextrans [MTX[3] (7)]. Proteins have been of special interest because of the possibility that a tumor-specific antibody might be isolated which could act as a carrier and target its drug directly to the tumor site. However, the formidable obstacles of producing specific antibodies against the different antigens of each human tumor have not yet made this a feasible prospect. It has been found, however, that even a nonspecific protein, BSA, when linked to MTX, is as successful as the free drug in prolonging the lives of mice bearing the L1210 tumor (7) and can be more successfully used than the drug itself against the Lewis lung carcinoma (8). It appeared of interest, therefore, to (a) determine if proteins represent a general class of effective carriers *in vivo*, and (b) study the mechanism of action of MTX–BSA *in vitro* not only to define how this particular drug carrier acts, but also to create a reference point for any future more specifically targeted protein. MTX has therefore been linked to two additional proteins, α-chymotrypsinogen (MW 28,000) and a nonspecific antibody, bovine IgG (MW 120,000–150,000) and their therapeutic effects against the L1210 tumor have been established.

This investigation was supported by USPHS grants from the National Cancer Institute and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, CA-11778 and AM-07097, respectively, and by a grant from the American Cancer Society, CH-31.

[1] Present address: T006 Department of Medicine, University of California, San Diego, Calif. 92093.

[2] Recipient of a USPHS Research Career Development Award (CA-00106) from The National Cancer Institute.

[3] Abbreviations used: MTX, methotrexate; BSA, bovine serum albumin; pCMS, *p*-chloromercuribenzenesulfonate; 5-formylTHF, 5-formyl-5,6,7,8-tetrahydrofolic acid; UdR, 2′-deoxyuridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

0026-895X/80/030382-06$02.00/0

Previous experiments have shown that MTX linked to high-molecular-weight dextrans was ineffective as a chemotherapeutic agent (7); this indicated that the choice of a carrier has significant bearing on whether the drug can perform its cytotoxic functions. We have, therefore, used both MTX-[^{3}H]Dextran T70 and MTX-^{125}I-BSA to determine the fate of the carrier when incubated with L1210 cells *in vitro* and to see whether these results can give some indication why proteins appear to be more effective than dextrans for drug delivery. The results of these *in vitro* carrier-bound drug uptake experiments cannot totally reflect the events occurring *in vivo* since they take no account of the drug's pharmacological properties. They nevertheless provide information which, when considered together with the *in vivo* observations, could lead to a more complete analysis of the overall mode of drug action.

MATERIALS AND METHODS

Materials. The following were obtained from commercial sources: bovine chymotrypsinogen, pCMS, 5-formylTHF, folic acid, and crystalline BSA (Sigma Chemical Co.); MTX (B.L.I. Biochemicals); bovine IgG (Schwarz/Mann Chemical Co.); Dextran T70 and Ficoll-Hypaque (Pharmacia Chemical Co.); [3′,5′-^{3}H]MTX (7.5 Ci/mmol) and [^{3}H]Dextran T70 (21 mCi/g, average MW 82,000) (Amersham/Searle); 6-[^{3}H]UdR (242 Ci/mmol) (New England Nuclear). MTX-BSA, [^{3}H]MTX-BSA, [^{3}H]MTX-Dextran T70, MTX-[^{3}H]Dextran T70, folate-BSA, MTX-chymotrypsinogen, and MTX-IgG (containing 30–80 mg of bound ligand/g of carrier) were synthesized according to a previously published procedure (7), in which MTX is coupled, via one of its terminal carboxyl groups, in a carbodiimide-promoted reaction to either lysine ε-amino groups contained in the protein or to aminoethyl groups chemically inserted into the dextran. MTX-^{125}I-BSA was obtained from MTX-BSA using the procedure of McConahey and Dixon (9). BDF_1 and DBA_2 female mice (19–22 g) were obtained from Simonsen Laboratories, Gilroy, California.

Methods. MTX and its derivatives were tested for antitumor activity in mice using the protocol recommended by the Drug Evaluation Branch, National Cancer Institute (10). Animals were inoculated with 10^6 L1210 cells on Day 0, and the drugs were injected intraperitoneally[4] on Day 1. Analytical chromatography was carried out with MeOH:NH_4OH:H_2O (7:1:2) on Whatman No. 1 paper, radioactivities of tritiated and iodinated materials were measured with Beckman LS-233 liquid scintillation and Nuclear Chicago 1185 gamma counters, respectively, and SDS-polyacrylamide gel electrophoreses were performed employing the procedures of Weber and Osborn (11).

L1210 and L1210 R6 cells were grown in RPMI 1640 medium (Flow Labs, Inglewood, Calif.) supplemented with 5% fetal calf serum and 1% penicillin-streptomycin, starting with a concentration of 0.4–0.5 × 10^5 cells/ml. Aliquots of the culture were removed 24, 48, and 72 h after start of growth and counted in a hemocytometer. Dihydrofolate reductase inhibition experiments were kindly performed by Dr. Chinan Fan of this department using the enzyme isolated from the MTX-resistant L1210 R6 strain of ascites tumor cells (12). The assays were performed in a system that contained, in 1.0 ml, 56 μM dihydrofolate, 150 μM NADPH 0.1 M K-phosphate, 0.1 M K-Hepes, and 0.5 M KCl, pH 7. Initial velocities (at 37°C) were determined via absorbance changes at 340 nm after a 10-min, 25°C preincubation period of the enzyme with MTX or the MTX derivative (13).

[^{3}H]UdR uptake experiments. L1210 cells were removed from DBA_2 mice 5–6 days after tumor transplantation and were diluted 1:10 with PBS [NaCl, 8.0; KCl, 0.2; $NaHPO_4$, 1.15; KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; anhydrous $CaCl_2$, 0.1 g/liter (14)], pH 7.5. The lymphocytes were then separated from red cells with a Ficoll-Hypaque gradient, washed with PBS, suspended in RPMI medium (containing no fetal calf serum) at a concentration of 1–2 × 10^6 cells/ml, and incubated with 10 μl PBS or drug at 37°C for 1 h. Two microcuries of [^{3}H]UdR (sp act 242 Ci/mmol) was then added to each sample. After 15 min the reaction was stopped by the addition of 10 ml cold PBS. The cell suspension was centrifuged, and the pellet was washed with a further 10 ml cold PBS and then resuspended in 1 ml PBS. The number of cells in each sample was determined with a hemocytometer and 0.3-ml aliquots of each sample were precipitated with 2 ml 10% TCA. After 30 min the precipitates were filtered on GFC filters (Whatman) and the radioactivity bound to the filters was measured in an Omnifluor scintillant (15).

In vitro transport of [^{3}H]MTX, [^{3}H]MTX-BSA, and [^{3}H]MTX-Dextran T70. L1210 cell samples were removed from BDF_1 mice bearing 5- to 6-day-old tumors and each diluted into a 10-ml buffer solution, pH 7.5, which contained 0.1 M NaCl, 0.005 M KCl, 0.03 M $NaHCO_3$, 0.002 M $CaCl_2$, 0.001 M $MgCl_2$, 0.01 M glucose, 0.01 M Tris-HCl (16). The total number of cells harvested was measured by counting an aliquot in a hemocytometer and the remainder were centrifuged at 800*g* for 5 min. Contaminating erythrocytes were lysed by suspension in 0.2% NaCl for 30 s and the cell suspension was returned to isotonicity by the addition of concentrated NaCl. The leukocytes were isolated by centrifugation at 800*g* for 5 min and resuspended in the same buffer. Aliquots (2 ml) of this cell suspension containing 2–4 × 10^7 cells/ml were then incubated in medium which contained either 4 μM [^{3}H]MTX, [^{3}H]MTX-BSA (4.7 μCi/mg MTX), or [^{3}H]-MTX-Dextran T70 (2.3 μCi/mg MTX), for 1 h at 0°C in an ice bath, or 37°C in a CO_2 incubator; 95% of the cells remained viable in each case when tested by trypan blue dye exclusion. The incubation was stopped by dilution with 10 ml ice cold 0.02 M phosphate buffer, pH 7.5, containing 0.14 M NaCl, and the cells were isolated by centrifugation at 800*g* for 5 min. The pellet was repeatedly washed with buffer and an aliquot of each sample was then measured for retained radioactivity. The cells were lysed by freezing and thawing and the lysate separated by a 5-min centrifugation at 800*g* was dialyzed against two 30-ml volumes of H_2O for 18 and 8 h to separate free MTX. Aliquots of both dialyzate and sac contents were measured for radioactivity.

[4] Unless stated otherwise, all dosages reported for the high-molecular-weight derivations of folate or MTX refer to the quantity of ligand contained by the complex per kilogram weight of mouse.

In vitro transport of ^{125}I-BSA and MTX-^{125}I-BSA. A procedure similar to that outlined above was followed using labeled carrier—either 20 μg/ml ^{125}I-BSA or MTX-^{125}I-BSA (17 μCi/mg). In experiments where the lysate and combined membrane fractions of cells incubated with MTX-^{125}I-BSA were examined, duplicate control experiments were carried out to determine the amount of MTX-BSA degraded by proteolytic enzymes upon cell lysis. In these controls, the cells were incubated without added radioactive materials, but prior to the freeze-thaw procedure an amount of MTX-^{125}I-BSA equivalent to that found in the experimental samples was added. After lysis, the lysate and membrane fractions were separated by centrifugation at 800*g*. Aliquots of the cell supernatant, lysate, and membrane fractions were dialyzed and analyzed for low-molecular-weight fragments as described above. Samples were also examined by SDS-polyacrylamide gel electrophoresis; gels were sliced into 1-mm sections and each segment was measured for radioactivity.

RESULTS

The increase in survival times of mice bearing L1210 tumors after treatment with MTX-protein derivatives. The survival times of mice treated with 15 mg/kg MTX, MTX-chymotrypsinogen, MTX-IgG or an equivalent concentration of folate-BSA 1 day after the implantation of 10^6 L1210 cells are shown in Table 1. The MTX-protein derivatives give increases in survival times comparable to those observed with free MTX and to those previously reported for MTX-BSA (7), suggesting that proteins in general are efficient agents for transmitting MTX to L1210 cells *in vivo*. Moreover, mice treated with folate-BSA do not survive longer than the control mice, which counteracts the possibility that the protein carrier alone may possess therapeutic cytotoxic properties.

Inhibition of dihydrofolate reductase by MTX, MTX-BSA, and MTX-Dextran T70. Table 2 shows the concentration of free or carrier-bound MTX necessary to achieve 50% inhibition of dihydrofolate reductase at an enzyme concentration of 2 μM. It can be seen that both MTX-BSA and MTX-Dextran T70 have approximately one-tenth the inhibitory capacity of the free drug. This observation is consistent with the relative inhibitory powers observed when aminopterin or aminopterin bound to aminoethyl starch interacts with the chicken liver reductase (17), and when MTX or MTX-BSA interacts with the enzyme from *Lactobacillus casei* (unpublished observation).

TABLE 1

Survival times of L1210 tumor-bearing BDF$_1$ mice treated with MTX or high-molecular-weight MTX or folate derivatives

Mice were treated with drug samples 24 h after tumor transplant (10^6 cells). Results are expressed ± SD.

Drug	Dose	Number of mice used	Mean survival time
	(mg/kg)		*(days)*
Control	0	22	8.0 ± 1.5
MTX	15	11	15.2 ± 1.9
MTX-BSA	15	22	15.1 ± 2.3
MTX-chymotrypsinogen	15	12	17.3 ± 3.2
MTX-IgG	15	8	20.8 ± 3.7
Folate-BSA	15	6	7.8 ± 0.8

TABLE 2

Inhibition of L1210 R6 dihydrofolate reductase with MTX, MTX-BSA, and MTX-Dextran T70

The concentration of drug necessary to cause 50% inhibition of activity of dihydrofolate reductase (2 μM, specific activity 185 μmol/min/mg protein), I_{50}, was measured according to the procedure outlined under Materials and Methods.

Inhibitor	I_{50}
	(μM)
MTX	1.25
MTX-BSA	7.5
MTX-Dextran T70	10.0

L1210 cells grown in vitro with MTX, MTX-BSA, and MTX-Dextran T70. Figure 1 shows the growth curves of L1210 cells grown in RPMI media in the presence of MTX, MTX-BSA, or MTX-Dextran T70, starting with a cell concentration of 0.3×10^5 cells/ml. In the presence of 10^{-7} M MTX cell growth is almost completely inhibited, whereas growth in the presence of 10^{-7} M MTX-BSA or 10^{-6} M MTX-Dextran T70 is comparable to that of the untreated control cultures. At 10^{-6} M, MTX-BSA begins to exert some growth-inhibiting effects and after 72 h the cell concentration is 10-fold lower than that observed in the control cultures; cells grown in the presence of MTX-Dextran T70 are not severely inhibited until a concentration of 10^{-5} M is used.

Inhibition of [^{3}H]UdR incorporation by MTX and MTX-BSA. The inhibition of [^{3}H]UdR uptake into L1210 cellular DNA by 10^{-7} or 10^{-6} M MTX and 10^{-5} or 10^{-4} M MTX-BSA, expressed as a percentage of a control, where no antimetabolite is present, is shown in Table 3. It can be seen that incorporation of radioactivity is almost completely inhibited by 10^{-6} M MTX and 10^{-4}

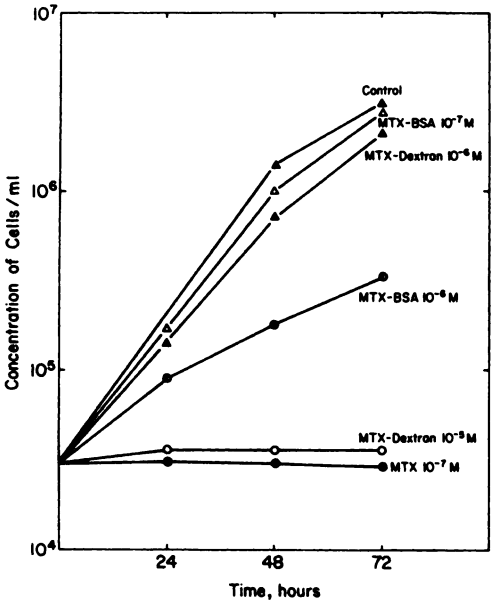

FIG. 1. *Comparative inhibition of growth of L1210 cells in RPMI 1640 medium which occurred in the presence of MTX, MTX-BSA, and MTX-Dextran T70 at the concentrations indicated*

TABLE 3

The percentage inhibition by MTX and MTX-BSA of [^{3}H]UdR incorporation into L1210 cells grown in vitro

Cells (1–2 × 10^6/ml) in RPMI 1640 medium containing no fetal calf serum were incubated with the two drugs at the indicated concentrations for 1 h at 37°C. 2 μCi [^{3}H]UdR (specific activity 242 Ci/mmol) was added to each sample and incubation was continued for a further 15 min before processing and counting. The results are expressed as a percentage of a control experiment in which the uptake of radioactivity was measured/10^6 cells (9250 cpm).

Drug	Concentration	Percentage of control
	(M)	(%)
Control	0	100
MTX	10^{-7}	100
MTX	10^{-6}	10
MTX-BSA	10^{-5}	100
MTX-BSA	10^{-4}	15

MTX-BSA. The 100-fold higher concentration of carrier-bound, compared to free, MTX necessary to inhibit incorporation of radioactivity is similar to the concentration difference required to give comparable inhibition of L1210 cell growth in culture shown in Fig. 1. This observation suggests that MTX, whether in its free or its bound form, remains metabolically active.

Effect of temperature and inhibitors on MTX and MTX-BSA transport. The amounts of drug found in L1210 cells grown *in vitro* in the presence of 4 × 10^{-6} M [^{3}H]MTX, [^{3}H]MTX-BSA, or [^{3}H]MTX-Dextran T70 at 37°C are indicated in Table 4. The growth-inhibiting properties of the various drugs illustrated in Fig. 1 appear to follow the intracellular concentrations achieved with each agent. For example, after a 1-h incubation at 37°C, the internal drug concentrations of cells incubated with 4 μM MTX, MTX-BSA, and MTX-Dextran T70 are 2.2, 1.12, and 0.42 nmol/10^9 cells, respectively. The differing intracellular levels of MTX and MTX-BSA observed when cells are incubated in the presence of each drug at 0°C instead of 37°C, and in the presence of two known inhibitors of MTX transport, pCMS (18) and 5-formylTHF (19), are also shown in the table. At 0°C MTX transport is almost completely inhibited, whereas that of MTX-BSA is only reduced by 46%. In the presence of 100 μM pCMS at 37°C, MTX transport is inhibited by 65% in contrast to MTX-BSA where drug transport is only reduced by 32%; moreover, at 0°C 100 μM pCMS has little effect on MTX-BSA transport. When the medium contains 10:1 excess 5-formylTHF over MTX, again the transport of MTX alone is inhibited to a greater extent (55%) than that of MTX-BSA (22%). When cells are incubated with [^{3}H]MTX and a 10-fold excess of nonradioactive MTX-BSA, or with [^{3}H]MTX-BSA and a 10-fold excess of nonradioactive MTX, 12% inhibition is observed in each case.

Table 4 also shows that when the lysate and membrane fractions are separated, almost all the radioactivity of cells incubated with MTX is found in the lysate, and that when this fraction was dialyzed, 68% of the radioactivity passed out of the sac, leaving a residual 0.7 nmol, possibly that bound to dihydrofolate reductase. With MTX-BSA, only 34% of the lysate radioactivity was lost by dialysis, leaving a similar quantity, 0.6 nmol, of drug within the dialysis membrane.

In order to determine if hydrolysis of MTX-BSA had occurred on the external surface of the cell membrane, the supernatant fractions of cells incubated at 37°C with [^{3}H]MTX-BSA, a 1:10 ratio of [^{3}H]MTX-BSA:5-formylTHF, and an unincubated sample of [^{3}H]MTX-BSA were analyzed by paper chromatography. Excess 5-formylTHF was included in one case to restrict the cellular uptake of any MTX that appeared in the medium should MTX-BSA be hydrolyzed on the outer membrane surface. In each case 2–3% of the label appeared as MTX. The lack of a significant increase in MTX in the medium when cells were incubated with [^{3}H]MTX-BSA suggests

TABLE 4

Intracellular drug concentrations of L1210 cells incubated with [^{3}H]MTX, [^{3}H]MTX-BSA, and [^{3}H]MTX-Dextran T70 at 0 and 37°C

L1210 cells (2 × 10^7) were incubated for 1 h with either 4 × 10^{-6} M drug alone, or with the addition of the transport inhibitor or competitor indicated. After lysis, lysate and membrane fractions were separated by centrifugation and the lysate was dialyzed against water. Results are expressed ± SD.

Drug	Concn	Inhibitor	Concn	Intracellular drug concn		Inhibition	Percentage of total radioactivity in lysate	Percentage of lysate radioactivity remaining after dialysis
				37°C	0°C			
	(μM)		(μM)	(nmol MTX/10^9 cells)		(%)	(%)	(%)
MTX	4			2.24 ± 0.51			94–100	32
					<0.1	~100		
		pCMS	10^2	0.78 ± 0.13		65		
		5-FormylTHF	40	1.00 ± 0.20		55		
		MTX-BSA	40	1.98 ± 0.23		12		
MTX-BSA	4			1.12 ± 0.33			75–85	66
					0.60 ± 0.13	46		
		pCMS	10^2	0.76 ± 0.19		32		
					0.52 ± 0.07	54	64–75	
		5-FormylTHF	40	0.84 ± 0.12		22		
		MTX	40	0.98 ± 0.29		12		
MTX-Dextran T70	4			0.42 ± 0.02	<0.05			

TABLE 5

Quantity of radioactively labeled carrier which binds to L1210 cells after incubation with ^{125}I-BSA, MTX-^{125}I-BSA and MTX-[^{3}H]-Dextran T70

Suspensions (2 ml) of L1210 cells were incubated for 1 h with 20 μg/ml carrier-bound drug or carrier alone at 37 and 0°C. Results are expressed ± SD.

	Carrier associated with 10^8 cells		
	MTX-^{125}I-BSA	^{125}I-BSA	MTX-[^{3}H]Dextran T70
		(μg)	
37°C	0.42 ± 0.12	0.11 ± 0.03	0
0°C	0.22 ± 0.04	0.10 ± 0.02	

that hydrolysis of MTX from its carrier does not occur on the outer membrane, but that the originally synthesized sample of MTX-BSA might contain a small percentage of noncovalently bound MTX which resisted previous separation by column chromatography and dialysis. Alternatively it is possible that certain of the MTX-BSA amide linkages are more labile than others.

Transport of MTX-[^{3}H]Dextran T70, MTX-^{125}I-BSA, and ^{125}I-BSA into L1210 cells. The amount of carrier (dextran or BSA) found associated with L1210 cells after incubation with 20 μg/ml MTX-[^{3}H]Dextran T70, MTX-^{125}I-BSA or ^{125}I-BSA is shown in Table 5. MTX-^{125}I-BSA (0.42 μg) binds to 10^8 cells, and a significantly smaller amount, 0.11 μg, binds to cells which have been incubated with the unsubstituted carrier. At the same concentration, there are no counts associated with cells incubated with the MTX-[^{3}H]Dextran T70, and even when 50 μg/ml of the polysaccharide-bound MTX is used, only 0.05 μg is bound/10^8 cells.

The percentage of degraded MTX-^{125}I-BSA in the cell supernatant, lysate and membrane fractions was determined by dialysis experiments (Table 6). Seventy percent, of the radioactivity was found in the lysate fraction, and almost half of this label was present as low-molecular-weight fragments, compared with 12% in the control experiments, where the MTX-BSA was added to the cell suspension just prior to lysis. In contrast, the label remaining in the cell supernatant or found associated with the membrane fraction remained primarily as the high-molecular-weight derivative.

The distribution of radioactivity in the nondialyzable high-molecular-weight supernatant, lysate, and membrane fractions after SDS-polyacrylamide gel electrophoresis is shown in Fig. 2. In the lysate and cell supernatant fractions, large peaks of radioactivity are found with the same mobility as a MTX-BSA marker, with some residual radioactivity at the origin. In the membrane fraction the MTX-BSA peak is not visible; instead, virtually all the radioactivity is found at the origin.

DISCUSSION

The results described above and in previous reports from this laboratory indicate that when MTX is coupled to randomly chosen proteins of varying molecular weights and conformations such as BSA, bovine IgG, and chymotrypsinogen, products are formed which are equally effective as the parent compound in prolonging the survival time of mice bearing the L1210 ascitic tumor. These observations suggest that proteins constitute an effective class of carriers for MTX, and possibly for other chemotherapeutic agents, and that they may deliver the bound agent to the target cells through some common pathway that may first require binding to the external surface of the cell membrane. The poor response of the L1210-carrying mice to treatment with MTX-Dextran T70 coupled with the inability of MTX-[^{3}H]Dextran T70 to interact with the L1210 cell membranes suggests that the capacity of the complexes to bind to the membrane (Table 5) is indeed one requirement for an effective carrier.

Low temperature and agents such as pCMS and 5-formylTHF, whose presence inhibits MTX transport into L1210 cells, have much less effect on MTX-BSA transport, suggesting that the carrier-bound drug may be transported into the cell by a mechanism different from that for the parent compound. The active component of protein-bound drug remains undefined, however, so that the quantitative significance of the data remains unclear. Experiments employing [^{3}H]MTX-BSA or MTX-^{125}I-BSA strongly suggest that the hydrolysis of the drug from the carrier occurs inside the cell rather than on the external cell surface, since virtually no free [^{3}H]MTX or low-molecular-weight ^{125}I fragments were found in the external cell supernatant after a 1-h incubation with the radiolabeled compound at 37°C; a situation which contrasts with that of insulin, where low-molecular-weight fragments were found in the external cell media after only a 5-min incubation of the peptide with liver membranes (20). In addition, the dialysis and SDS-gel electrophoresis experiments demonstrate that whereas a significant percentage of both low-molecular-weight tritiated and iodinated fragments could be found in the cell lysate fraction, virtually no similar small fragments were found associated with the cell membrane fraction. Moreover, in separate experiments where either [^{3}H]MTX-BSA or MTX-^{125}I-BSA was incubated with L1210 cells, almost equivalent amounts of each of the two labels appeared in the lysate and in the membrane fractions;

TABLE 6

Dialyzable radioactivity, expressed as a percentage of the total applied radioactivity, associated with the L1210 cell lysate, membrane, and supernatant fractions after prior incubation of the whole cells with MTX-^{125}I-BSA

L1210 cells incubated for 1 h with 20 μg/ml MTX-^{125}I-BSA were isolated from their supernatant by centrifugation and lysed by freezing and thawing. The lysate and membrane fractions were then separated by a further centrifugation and each fraction was dialyzed against H_2O. Control experiments contained equivalent amounts of MTX-^{125}I-BSA added to cells immediately before lysis (cf. methods section).

	Dialyzable radioactivity		
	Lysate[a]	Membrane	Supernatant
		(%)	
Cells incubated with MTX-^{125}I-BSA	47	13	5
Control	12	9	

[a] The lysate and membrane fractions contain 70 and 30% of the total cellular associated radioactivity, respectively.

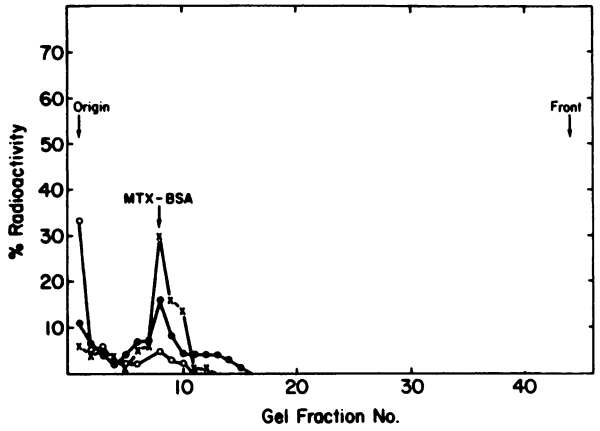

FIG. 2. *The distribution of radioactivity observed after SDS–polyacrylamide gel electrophoresis*

Previously dialyzed cell supernatant (×), membrane (○), and lysate (●) fractions were obtained from L1210 cells grown *in vitro* in the presence of MTX-^{125}I-BSA. The mobility of an MTX-^{125}I-BSA control is indicated.

an observation which again suggests that the drug and its carrier enter the cell as a single entity. Since BSA alone has been demonstrated to enter cells by pinocytosis (21), a similar mechanism of entry probably occurs with the MTX-BSA complex.

The *in vitro* growth experiments of L1210 cells with varying concentrations of MTX, MTX-BSA, and MTX-Dextran T70 would not predict MTX-BSA to be an effective chemotherapeutic agent *in vivo*. However, the differing pharmacokinetic parameters of the drug *in vivo*, in particular its longer half-life within the animal, are probably responsible for the observed effectiveness in therapy (7, 8). For example, it was determined that a 7.5-mg/kg dose of MTX injected into BDF_1 mice reached its maximum concentration in the serum (5–7 μg/ml) within 10–15 min and had almost left the circulation after 1 h. MTX-BSA at the same dose slowly reached a 5-fold higher peak serum concentration at 2–3 h which gradually declined to 10 μg/ml after 8 h and disappeared only after 24 h. Thus in the *in vivo* situation MTX-BSA maintains a 10- to 20-fold higher serum concentration during the 24 h after injection. The differing transport properties of MTX-BSA compared to MTX observed in the *in vitro* experiments (Table 4), coupled with the recent results obtained by Ryser and Shen with MTX-poly-L-lysine (22), suggest that high-molecular-weight derivatives may be particularly effective when used in the treatment of tumor cells which have developed resistance to MTX therapy because of an acquired transport deficiency; an abnormality for which much recent evidence has accumulated (12, 23). Although caution should be observed in applying *in vitro* observations to the *in vivo* situation, previous experiments have indicated that MTX-BSA is not extensively hydrolyzed *in vivo* in serum or in the ascitic fluid (7). It is possible, therefore, that the transport characteristics determined here *in vitro* for the L1210 cells may also apply *in vivo*.

REFERENCES

1. Trouet, A., D. Deprez-de Campeneere and C. DeDuve. Chemotherapy through lysosomes with a DNA-daunorubicin complex. *Nature New Biol.* **239**: 110–112 (1972).
2. Ghose, T., M. R. C. Path and S. P. Nigam. Antibody as a carrier of chlorambucil. *Cancer* **29**: 1396–1400 (1972).
3. Mayhew, E., D. Papahadjopoulos, Y. M. Rustum and C. Dave. Inhibition of tumor cell growth *in vitro* and *in vivo* by 1-β-D-arabinofuranosylcytosine entrapped within phospholipid vesicles. *Cancer Res.* **36**: 4406–4411 (1976).
4. Gregoriadis, G. and E. D. Neerunjun. Treatment of tumour-bearing mice with liposome-entrapped actinomycin D prolongs their survival. *Res. Commun. Chem. Pathol. Pharmacol.* **10**: 351–362 (1975).
5. Atassi, G., M. Duarte-Karim and H. J. Tagnon. Comparison of adriamycin with DNA-adriamycin complex in the chemotherapy of experimental tumours and metastases. *Eur. J. Cancer* **11**: 309–316 (1975).
6. Rowland, G. F., G. J. O'Neill and D. A. L. Davies. Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage. *Nature (Lond.)* **255**: 487–488 (1975).
7. Chu, B. C. F. and J. M. Whiteley. High molecular weight derivatives of methotrexate as chemotherapeutic agents. *Mol. Pharmacol.* **13**: 80–88 (1977).
8. Chu, B. C. F. and J. M. Whiteley. Control of solid tumor metastases with a high-molecular-weight derivative of methotrexate. *J. Natl. Cancer Inst.* **62**: 79–82 (1979).
9. McConahey, P. J. and F. J. Dixon. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy* **29**: 185–189 (1966).
10. Geran, R. I., N. H. Greenberg, M. M. MacDonald, A. M. Schumacher and B. J. Abbott. Protocol 1:1000. Lymphoid leukemia L1210. *Cancer Chemother. Rep. Part 3* **3**: 7 (1972).
11. Weber, K. and M. Osborn. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406–4412 (1969).
12. Jackson, R. C., D. Niethammer and F. M. Huennekens. Enzymic and transport mechanisms of amethopterin resistance in L1210 mouse leukemia cells. *Cancer Biochem. Biophys.* **1**: 151–155 (1975).
13. Fan, C., G. Henderson, K. Vitols and F. M. Huennekens. Molecular targets for methotrexate, in *Antimetabolites in Biochemistry, Biology and Medicine* (J. Skoda and P. Langen, Eds.). Pergamon, Oxford, England/Elmsford, New York, 313–326 (1979).
14. Dulbecco, R. and M. Vogt. Plaque formation and isolation of pure lines with poliomyelitis virus. *J. Exp. Med.* **99**: 167 (1954).
15. Krishan, A., K. D. Paika and E. Frei III. Cell cycle synchronization of human lymphoid cells *in vitro* by 2,3-dihydro-1H-imidazol(1,2-b)-pyrazole. *Cancer Res.* **36**: 138–142 (1976).
16. Sirotnak, F. M. and R. C. Donsback. Stereochemical characteristics of the folate-antifolate transport mechanism in L1210 leukemia cells. *Cancer Res.* **34**: 371–377 (1974).
17. Whiteley, J. M., R. C. Jackson, G. P. Mell, J. H. Drais and F. M. Huennekens. Folate antagonists covalently linked to carbohydrates: synthesis, properties, and use in the purification of dihydrofolate reductases. *Arch. Biochem. Biophys.* **150**: 15–22 (1972).
18. Rader, J. L., D. Niethammer and F. M. Huennekens. Effects of sulfhydryl inhibitors upon transport of folate compounds into L1210 cells. *Biochem. Pharmacol.* **23**: 2057–2069 (1974).
19. Nahas, A., P. F. Nixon and J. R. Bertino. Uptake and metabolism of N^5-formyltetrahydrofolate by L1210 leukemia cells. *Cancer Res.* **32**: 1416–1421 (1972).
20. Freychet, P., R. Kahn, J. Roth and D. M. Neville, Jr. Insulin interactions with liver plasma membranes. *J. Biol. Chem.* **247**: 3953–3961 (1972).
21. Shen, W. C. and H. J.-P. Ryser. Conjugation of poly-L-lysine to albumin and horseradish peroxidase: A novel method of enhancing the cellular uptake of proteins. *Proc. Natl. Acad. Sci. USA* **75**: 1872–1876 (1978).
22. Ryser, H. J.-P. and W. C. Shen. Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cultured cells. *Proc. Natl. Acad. Sci. USA* **75**: 3867–3870 (1978).
23. Sirotnak, F. M., S. Kurita and D. J. Hutchison. On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. *Cancer Res.* **28**: 75–80 (1968).

Send reprint requests to: Dr. John M. Whiteley, Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.