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# Studies of a Methotrexate Binding Protein Fraction from L1210 Lymphocyte Plasma Membranes*

JOHN I. McCORMICK, SANDRA S. SUSTEN, JEANNE I. RADER and JAMES H. FREISHEIM†

*Department of Biological Chemistry, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267, U.S.A.*

**Abstract**—[$^{14}$C]-N-*ethylmaleimide has been used to label binding sites for methotrexate in the plasma membranes of L1210 cells previously 'protected' from reaction with unlabeled* N-*ethylmaleimide by the presence of methotrexate. A* [$^{14}$C]-N-*ethylmaleimide labeled protein of molecular weight* 56,000 *was subsequently identified by electrophoresis of crude plasma membranes in sodium dodecyl sulfate containing polyacrylamide gels.*

*Sepharose–methotrexate affinity chromatography was used to isolate an active methotrexate binding protein fraction from L1210 lymphoma cell plasma membranes. This protein fraction was isolated from crude plasma membrane preparations and from a detergent extract of intact cells. The latter method carried out in the presence of* 0.01% *TritonX-100 permits the rapid extraction of plasma membrane proteins from intact L1210 cells in the absence of extensive cellular destruction. The methotrexate binding capacity of the extracted protein material has been demonstrated. The protein fraction purified by affinity chromatography contains* 3 *major protein components of molecular weights* 67,000, 63,000 *and* 56,000 *as determined by sodium dodecyl sulfate gel electrophoresis.*

## INTRODUCTION

THE ANTI-FOLATE drug methotrexate (MTX) is a potent inhibitor of the enzyme dihydrofolate reductase [1], a property which has led to its widespread use in the treatment of a variety of malignant conditions [2]. The effectiveness of MTX *in vivo* ultimately depends on the transport of the drug across the plasma membrane of the tumor cell. Substantial information on the transport of MTX and folate compounds in general has been accumlated using L1210 lymphoma cells [3], although the protein components involved in the translocation of the compounds across the plasma membrane of this cell type have not been characterized.

This report describes the identification of plasma membrane-associated protein components that appear to play a role in MTX transport in L1210 lymphoma cells. The sensitivity of the transport system to *N*-ethylmaleimide (NEM) has been used to label MTX binding sites on the plasma membranes of L1210 lymphocytes in a manner similar to the substrate protection technique used by Fox and Kennedy [4] to label the 'M' protein of the β-galactoside transport system of *Escherichia coli*. In addition, affinity chromatography has been used to isolate an active MTX binding component from L1210 lymphoma cell plasma membranes.

## MATERIALS AND METHODS

### *Chemicals*

3′, 5′, 9(*n*)—[$^{3}$H]-MTX (Sp. act. 5.3 Ci/mmole and 13.5 Ci/mmole) was obtained from Amersham/Searle. For uptake studies [$^{3}$H]-MTX (5.3 Ci/mmole) was mixed with unlabeled MTX (Lederle Drug Company) and purified by thin layer chromatography on

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†To whom correspondence should be addressed.

an Eastman Chromagram Sheet (13255 cellulose) in 0.2 M ammonium acetate. The purified MTX was eluted from the cellulose sheet with 50 mM potassium phosphate buffer pH 7.5 and stored at −20°C. [$^3$H]-MTX (13.5 Ci/mmole) was purified in the same way without addition of unlabelled MTX. This high specific activity substrate was used for binding studies. Triton X-100 was obtained from Sigma Chemicals. Materials for gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemicals were of the best grade commercially available.

*Cell culture*

Murine leukemia L1210 cells were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 5 or 10% fetal calf serum, penicillin and streptomycin.

*Uptake studies*

[$^3$H]-MTX uptake studies were performed with L1210 lymphocytes using methods already described [5].

*Preparation of plasma membranes*

Plasma membranes were prepared from L1210 cells by a procedure similar to that developed by Allen and Crumpton [6] for the isolation of a plasma membrane fraction from pig lymphocytes except that cells were disrupted by passage through a French Press at 200 psi. L1210 cells ($10^9$–$10^{10}$ cells) were suspended in 40 ml 10 mM Tris/HCl, 150 mM NaCl pH 7.4 and disrupted by passage through the French Press. The crude homogenate was subjected to centrifugation at 310 ***g*** (15 min), 7800 ***g*** (20 min) and 20,000 ***g*** (30 min). The fractions obtained were assayed for 5′-nucleotidase [7], $Na^+$-$K^+$-ATPase [8], succinic dehydrogenase [9] and glucose-6-phosphatase [10]. Phosphate was estimated by the method of Lowry and Lopez [11] and protein measured by the Lowry method [12] using bovine serum albumin as the standard.

*Protection studies*

Washed L1210 cells ($5 \times 10^6$ cells/ml) were incubated for 5 min at 37°C with varying concentrations of MTX in phosphate buffered saline (PBS), pH 6.8. Following incubation, *N*-ethylmaleimide (NEM) (100μM) was added and incubation continued for a further 10 min at which time β-mercaptoethanol (10 mM) was added and the cells harvested by centrifugation and washed in PBS, pH 7.4. [$^3$H]-MTX uptake in the cells was then measured over a 10 min period. Cell viability was checked by dye exclusion throughout the experiment.

*Differential labeling with* [$^{14}$C]-*NEM*

Two cell suspensions ($5 \times 10^6$ cells/ml) were prepared in PBS, pH 6.8 and designated 'protected' and 'unprotected' fractions. The 'protected' fraction was incubated for 5 min at 37°C with MTX (48 μM). NEM (100 μM) was then added and following a 10 min incubation, β-mercaptoethanol (10mM) was added and the incubation continued for a further 5 min. The cells were harvested, washed and resuspended in PBS, pH 6.8, to their original volume. [$^{14}$C]-NEM (100 μM, sp. act. 9.5–10mCi/mole, or 21,340 counts/min/mmole) (New England Nuclear) was added and the suspension incubated for 10 min at 37°C. The cells were harvested and a crude plasma membrane pellet was prepared.

In the "unprotected" fraction, pretreatment with MTX was omitted and the cell suspension incubated for 5 min prior to the addition of NEM (100 μM, 10 min) and β-mercaptoethanol (10 mM, 5 min). The cells were harvested, washed and incubated with [$^{14}$C]-NEM as described above, and a crude plasma membrane pellet prepared.

*Sodium dodecyl sulfate gel electrophoresis*

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on 100 μg samples of "protected" and "unprotected" fractions on separate gels by the method of Laemmli [13] as modified by Le Sturgeon and Rusch [14]. Membrane fractions were solubilized in 20 mM sodium phosphate pH 7.2, 280 mM β-mercaptoethanol, 0.8% SDS, 500 mM sucrose, and heated at 100°C for 5 min before addition of bromophenol blue (final concentration 0.002%). Samples were then briefly centrifuged and applied to 8.75% SDS gels. Following electrophoresis, the gels were fixed (50% methanol, 7.5% acetic acid: 16 hr, 23°C) and either stained (0.25% Coomassie brilliant blue, 50% methanol, 10% acetic acid: 3 hr, 23°C) or sliced for determination of radioactivity. For radioactivity determinations, 1 mm slices were placed in scintillation vials containing 0.5 ml of a 95% Soluene-100:$H_2O$ solution. The vials were incubated for 18 hr at 37°C and counted after the addition of 10ml scintillation fluid [5]. Stained gels were destained in a solution of

15% methanol and 7.5% acetic acid. Standard proteins employed in the SDS gel system were bovine serum albumin, ovalbumin, chymotrypsinogen. lysozyme and myoglobin.

*Effect of Triton X-100 on the transport of MTX into L1210 cells*

Washed L1210 cells were suspended ($5 \times 10^6$ cells/ml) in PBS, pH 7.4, containing varying amounts of Triton X-100. [$^3$H]-MTX was added to a final concentration of 1.2 $\mu$M and uptake was followed at 37°C for 14 min [5].

*Extraction of protein from intact L1210 lymphoma cells*

Washed cells were suspended ($5 \times 10^6$ cells/ml) in PBS, pH 7.4, containing 0.01% Triton X-100 (w/v), 14 mM $\beta$-mercaptoethanol and incubated at 37°C for 15 min. Following incubation, the cells were removed by centrifugation and the supernatant stored at 4°C. For the measurement of extracted [$^3$H]-MTX binding activity, the extraction procedure was carried out in the presence of $10^{-7}$M [$^3$H]-MTX (Sp. act. 13.5 Ci/mmole). Following removal of the cells by centrifugation, a 1 ml aliquot of the supernatant was removed and applied to a Sephadex G-25 column ($1.5 \times 21.5$ cm) previously equilibrated in 50 mM potassium phosphate pH 7.4, 0.01% Triton X-100 (w/v), 14 mM $\beta$-mercaptoethanol. One millilitre fractions were collected and the radioactivity present was determined by counting 250 $\mu$l aliquots in 10 ml ACS scintillation fluid (Amersham/Searle) containing 250 $\mu$l $H_2O$. Protein content was determined by measurements of the absorbance at 280 nm. The void volume of the column ($V_0$) was determined using bovine serum albumin.

[$^3$H]-MTX binding was also studied by addition of the labeled drug to the detergent-solubilized material following the extraction procedure. For this purpose, washed cells were suspended ($5 \times 10^6$ cells/ml) in PBS, pH 7.4 containing 0.01% Triton X-100 (w/v), 14 mM $\beta$-mercaptoethanol and incubated at 37°C for 15 min. Following incubation, the cells were removed by centrifugation and a 1 ml aliquot of the supernatant incubated for 15 min at 37°C with $10^{-7}$M [$^3$H]-MTX (Sp. act. 13.5 Ci/mmole). The material was then loaded onto a Sephadex G-25 column ($1.5 \times 21.5$ cm) and processed as described above.

*Affinity chromatography*

MTX-affinity resins were prepared by methods already described [15]. Before use, packed columns were extensively washed [15] and finally equilibrated in 50 mM potassium phosphate pH 7.5, 0.01% Triton X-100 (w/v), 14 mM $\beta$-mercaptoethanol. Protein was extracted from intact L1210 cells as described above and loaded onto a Sepharose-MTX affinity column ($1.5 \times 8$ cm) at a flow rate of approximately 20 ml/hr. Following loading, the column was washed with the equilibration buffer until the absorbance at 280 nm of the eluting material had returned to zero. The column was then loaded with a small volume (20–30 ml) of the equilibration buffer containing 0.12 mM 5-formyl-tetrahydrofolate (5-formyl-$FAH_4$) or 0.13 mM MTX. Following a 12–14 hr incubation at 4°C, the column was washed with the equilibration buffer. Fractions eluted were monitored at 280 nm. The protein fraction eluting with 5-formyl-$FAH_4$ or MTX was dialyzed against $H_2O$, lyophilized, dissolved in 20 mM sodium phosphate pH7.5, 0.2% SDS, 280 mM $\beta$-mercaptoethanol, 500 mM sucrose and characterized on SDS gels by the methods described above.

In order to demonstrate the binding capabilities of the material purified by affinity chromatography, the experimental procedure was slighty modified. The membrane proteins were solubilized as described above and loaded onto the MTX affinty column. After column washing, retained proteins were eluted with 15 ml of the equilibration buffer containing $10^{-7}$M [$^3$H]-MTX (Sp. act. 13.5 Ci/mmole). A volume of 1 ml of the eluted material was removed and applied to the Sephadex G-25 column described previously.

Affinity chromatography of a solubilized plasma membrane pellet was achieved in a similar manner. The 20,000 ***g*** membrane pellet was partially solubilized in PBS, pH 7.4, 0.01% Triton X-100, 14 mM $\beta$-mercaptoethanol, centrifuged at 20,000 ***g*** (30 min) and the supernatant loaded onto the MTX-affinity resin. Retained proteins were eluted from the column as described above.

## RESULTS

*Differential labeling with [$^{14}$C]-NEM*

The transport system in L1210 cells, shared by MTX, 5-methyl-tetrahydrofolate (5-methyl-$FAH_4$) and 5-formyl-$FAH_4$ is sensitive

to inhibition by NEM [16]. This effect is substantially reduced when cells are pretreated with MTX (Table 1). Partial protection of transport is observed after pretreatment of cell suspensions with 4.8 or 9.3 $\mu$M MTX and complete protection occurred after treatment with 48 $\mu$M MTX. These results formed the basis for the NEM labeling experiment.

*Table 1. Uptake of [$^3$H]-methotrexate by L1210 cells. Effect of treatment with methotrexate on inhibition by N-ethylmaleimide*

| Pretreatment* | | Uptake† | |
|---|---|---|---|
| MTX $\mu$M | NEM 100$\mu$M | pmole/$10^9$ cells | Per cent |
| None | − | 773 | 100 |
| | + | 372 | 48 |
| 4.8 | − | 181 | 100 |
| | + | 129 | 72 |
| 9.3 | − | 154 | 100 |
| | + | 129 | 84 |
| 24.0 | − | 139 | 100 |
| | + | 126 | 91 |
| 48.0 | − | 149 | 100 |
| | + | 164 | 110 |

*Pretreatment: methotrexate (MTX) 5 min; *N*-ethylmaleimide (NEM) 10 min.
†Uptake: 10 min; 1.7$\mu$M [$^3$H]-MTX.

NEM labeled proteins were analyzed on SDS gels after preparation of a crude plasma membrane pellet. The 20,000 ***g*** pellet isolated by the methods described was enriched approximately 4- and 3-fold for 5-nucleotidase and $Na^+$-$K^+$-ATPase, respectively and represents a crude plasma membrane preparation. The yield of membrance was approximately 20%. Problems with the method include the large amounts of 5′-nucleotidase and $Na^+$-$K^+$-ATPase activity remaining in the 20,000 ***g*** supernatant. Only a small amount (15–20%) of this activity sedimented after centrifugation at 100,000 ***g*** for 60 min. Similar problems with the preparation of plasma membranes from pig lymphocytes were observed by Allen and Crumpton [6]. The 20,000 ***g*** pellet obtained can be further purified by sucrose density gradient centrifugation [6]. However, this increased the time necessary for the preparation of the membranes and also further reduced the yield. In any case, the 20,000 ***g*** pellet was found to be a suitable source for the identification of NEM labeled proteins.

The NEM labeling procedure resulted in the incorporation of approximately 21% (11.3 $\times 10^6$ counts/min/$2 \times 10^9$ cells) of the [$^{14}$C]-NEM into cells previously "protected" by exposure to MTX. Incorporation of 16% (8.2 $\times 10^6$ counts/min/$2 \times 10^9$ cells) of the label was observed in the "unprotected" fraction. In a typical experiment, approximately 7% of the [$^{14}$C]-NEM was associated with the 20,000 ***g*** pellets, and the specific activities of labeling in terms of [$^{14}$C] NEM incorporated/mg protein from the 20,000 ***g*** pellet were 26,000 counts/min [$^{14}$C] NEM and 21,600 counts/min [$^{14}$C] NEM/mg for the "protected" and "unprotected" membrane preparations, respectively.

Crude plasma membrane pellets were prepared from the two fractions and samples run on separate SDS gels.

In both the "protected" and "unprotected" gels, most of the total radioactivity was associated with low mol. wt components (14,000–18,000 mol. wt, $R_f$ value + 1.0). In the "protected" gel, 7% of the applied radioactivity was associated with a protein component of mol. wt 56,000, while in the "unprotected" gel no radioactive peak was observed in this region. As the experiment was designed to locate components labeled by protection with MTX, the results are presented as a difference profile ("protected" − "unprotected", counts/min against $R_f$, see Fig. 1). The largest differential distribution of radioactivity occurs in the protein component with a minimum mol. wt of 56,000. Since most of the total radioactivity was associated with low mol. wt components in both "protected" and "unprotected" gels, this low mol. wt region becomes a minor peak at an $R_f$ value of 1.0 in the difference profile.

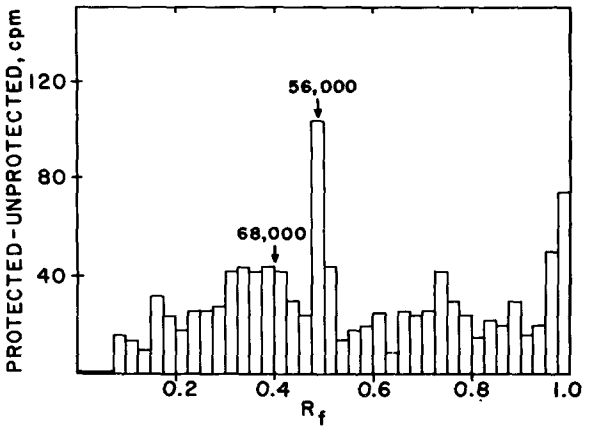


*Fig. 1. Difference profile following SDS gel electrophoresis of "protected" and "unprotected" crude plasma membrane fractions.*

*Interaction of Triton X-100 with the intact L1210 lymphoma cell*

In the presence of increasing concentrations of Triton X-100, the observed uptake of MTX by L1210 cells decreases (Fig.2). The maximum effect is at a concentration of

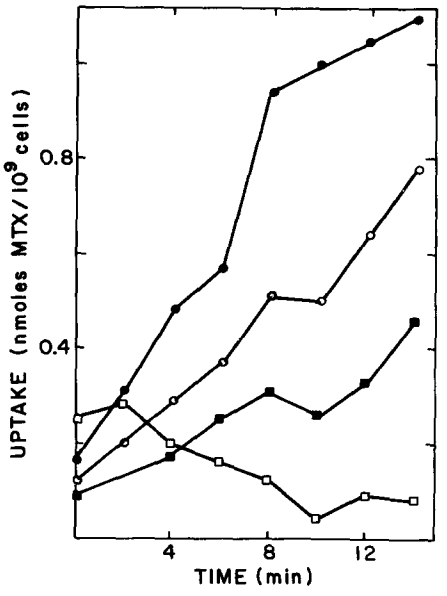


*Fig. 2. Effect of Triton X-100 on the uptake of* [$^3$H] *MTX by L1210 cells. Washed cells were suspended at* $5 \times 10^6$ cells/ml *in PBS buffer* pH 7.4 *and* [$^3$H] *MTX uptake was measured in the presence of* 0.0025% (○——○), 0.005% (■—■) *and* 0.01% (□—□) *Triton X-100 (w/v) at* 37°C. *Normal uptake* (●—●) *was measured in PBS,* pH 7.4, *at* 37°C.

0.01%. At concentrations above 0.01% Triton X-100, no transport was observed and complete cell lysis occured after a 15 min incubation.

The cessation of MTX uptake observed in the presence of 0.01% detergent is accompanied by a release of protein from the cell, a process which occurs in the absence of extensive cell destruction as determined by measurements of cell viability. Indications are that a substantial portion of the proteins released from the intact cell are derived from the plasma membrane. Thus, approximately 50% of the L1210 cell plasma membrane 5′-nucleotidase is released and in addition, SDS gels of the released or solubilized proteins are qualitatively similar to SDS gels of isolated crude plasma membranes. In contrast, the lack of succinic dehydrogenase activity in the intact cell solubilized material is indicative of some maintenance of cellular structure.

When solubilization is carried out in the presence of [$^3$H]-MTX, drug binding can be studied by application of an aliquot of the extracted material to a Sephadex G-25 column (Fig. 3). Preliminary investigations indicate that when the detergent extraction is

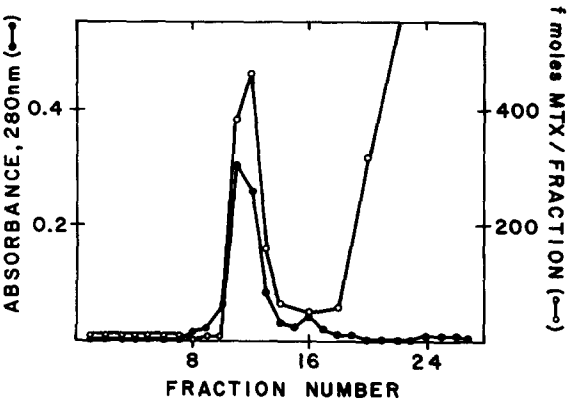


*Fig. 3. Sephadex G-25 chromatography of proteins extracted from the intact cell in the presence of* [$^3$H] *MTX. L1210 cells were incubated with PBS* pH 7.4, 0.01% *Triton X-100 (w/v)* 14 mM *β-mercaptoethanol at* 37°C *for* 15 min. *Following incubation, cells were removed by centrifugation and an aliquot of the supernatant loaded onto a Sephadex G-25 column* (1.5 × 21.5 cm). *Fractions* (1 ml) *were collected and analyzed as described in the text.*

carried out in the absence of [$^3$H]-MTX, and the labeled drug added to the extracted material following solubilization, binding is reduced by approximately 70%. This would appear to indicate that maximum binding is obtained only when the extraction is carried out in the presence of [$^3$H]-MTX.

Affinity chromatography of the intact cell detergent solubilized material results in the purification of a protein fraction containing three major proteins of mol. wts 67,000, 63,000 and 56,000 (Fig. 4). The binding capacity of the purified material can be demonstrated by the elution of the protein frac-

tion retained on the affinity column with high specific activity [$^3$H]-MTX and the subsequent filtration of an aliquot of the effluent material on a Sephadex G-25 column. As shown in Fig. 5, a peak of radioactivity is located at the column void volume. No radioactivity was located in this region when a similar quantity of [$^3$H]-MTX was applied to the column, in the absence of protein.

The protein fraction purified by affinity chromatography of solubilized crude plasma membranes is identical to that purified by affinity chromatography of the intact cell solubilized material, as judged by SDS gel electrophoresis. The major protein bands corresponding to mol. wts of 67,000, 63,000 and 56,000 are again present.

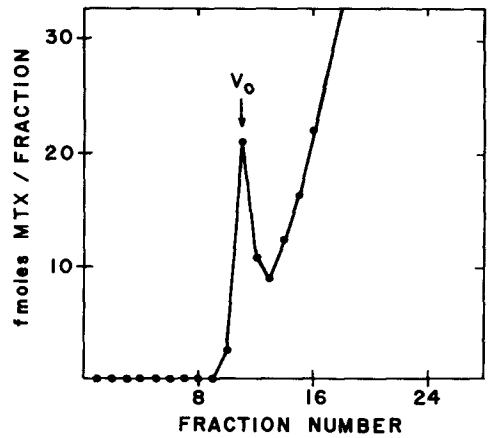


*Fig. 5. Binding of* [$^3$H] *MTX by the protein fraction purified by affinity chromatography. Proteins were extracted from intact cells by treatment with Triton X-100 and loaded onto a Sepharose–MTX affinity column. The retained protein fraction was eluted from the column with* $10^{-7}$M [$^3$H] *MTX* (Sp. act. 13.5 Ci/mmole). One millilitre *of the eluted material* (5% *of the total*) *was applied to a Sephadex G-25 column* (1.5 × 21.5 cm). *Fractions* (1 ml) *were collected and analyzed as described in the text. The column void volume* ($V_0$) *was determined by use of bovine serum albumin.*

## DISCUSSION

MTX, the inhibitory 2,4-diamino folate analog, shares the same transport system for 5-methyl-$FAH_4$ and 5-formyl-$FAH_4$ in L1210 cells [3, 16] and, therefore, serves as a useful model for investigations of this important system.

The uptake of MTX in L1210 lymphoma cells is inhibited by NEM [16] an observation that potentially permits the identification of binding and or transport proteins by use of a substrate protection technique. Such methods have been used successfully to label the 'M' protein of the β-galactoside transport system in *Escherichia coli* [4] and also to specifically label and purify a *D*-glucose binding protein from the membranes of rat kidney cortex brush border cells [17, 18]. The prerequisite for such experiments is that the substrate provide protection against inhibition. In the present system, the inhibitory effect of NEM is substantially reduced when cells are pretreated with MTX (Table 1). This result indicates that the presence of the substrate protects a transport component from chemical modification and this permits labeling of the transport component with [$^{14}$C]-NEM as described above. Subsequent analysis of the labeled proteins on SDS gels, after prepartion of a crude plasma membrane pellet, indicates that a number of proteins are labeled by the procedure (Fig.1). However, the largest differential distribution of radioactivity occurred in protein components with a minimum mol. wt of 56,000, indicating that this membrane component plays a central role in MTX binding. The high background level of radioactivity (Fig.1) may be due to the level of MTX used to achieve "protection". This level of MTX (48 μM) is about 25-fold higher than the *Km* value for the uptake of this compound by L1210 cells [3]. As a result, a non-specific protection of other sulfhydryl containing membrane proteins may occur, resulting in the high background radioactivity.

Following the protection and labeling experiment, the 56,000 mol. wt component in a 100 μg membrane sample had approximately 5 pmole [$^{14}$C] NEM associated with it, by estimations from the difference profile shown (Fig.1). However, the number of MTX-binding receptors per unit weight of membrane is unknown since the stoichiometry of the reaction of NEM with the receptor under these conditions is unknown. This procedure can only be considered as a qualitative method in terms of identification of a membrane-associated MTX binding receptor.

The procedure used for the isolation of membranes provided a rapid means for the preparation of a crude plasma membrane pellet, in a reasonable yield. The method is similar to that used by Allen and Crumpton [6] to isolate plasma membranes from pig lymphocytes except that cells were disrupted by use of a French Press. Plasma membranes sedimented in the 20,000 ***g*** pellet as evidenced by the enchancement of 5′-nucleotidase and $Na^+$-$K^+$-ATPase activity. Considerable amounts of 5′-nucleotidase and $Na^+$-$K^+$-ATPase remain in the 20,000 ***g*** supernatant and only a small amount of the activity

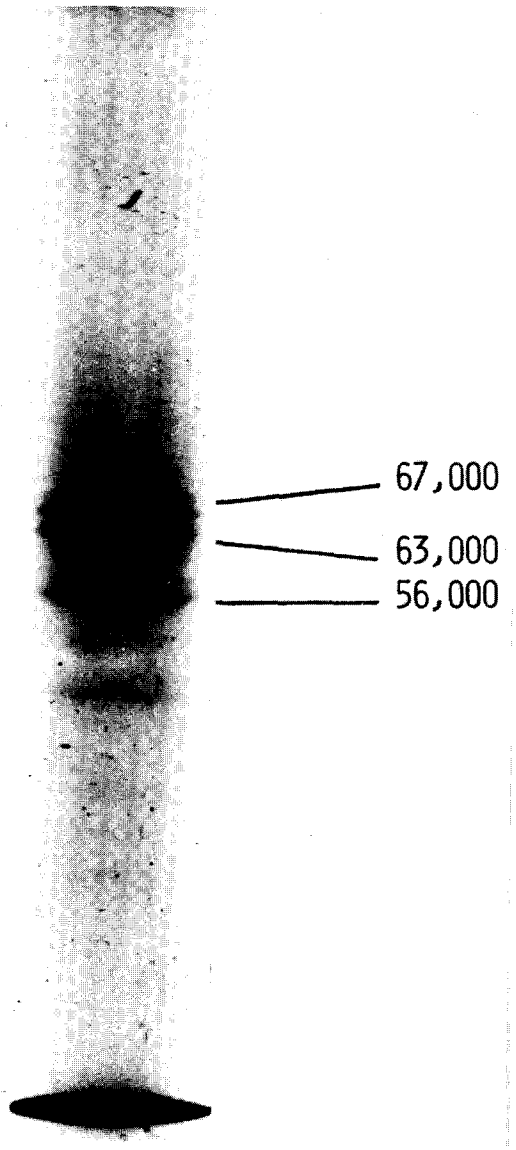


*Fig. 4. SDS gel electrophoresis of the protein fraction purified by Sepharose–MTX affinity chromatography.*

sediments at 100,000 ***g***. In their studies with pig lymphocytes, Allen and Crumpton [6] also found that substantial amounts of 5′-nucleotidase remained in the 20,000 ***g*** supernatant and that only a portion of this activity was sedimentable at 100,000 ***g***. The origin of this activity appears to be the formation of small plasma membrane vesicles during the cell breakage step.

When samples of the 20,000 ***g*** supernatant from "protected" and "unprotected" preparations are subjected to SDS gel electrophesis using procedures already described, the resulting difference profile is almost identical to that obtained for crude plasma membranes (Fig. 1). The similarities in the labeling patterns between the supernatants and crude membrane pellets confirms the observation that the plasma membranes are considerably fragmented by the procedure used to disrupt the cells and that membrane-associated proteins are found in the 20,000 ***g*** supernatant.

For the study of an active plasma membrane-associated MTX binding complex, a novel procedure was developed which permits the characterization of binding activity by solubilization from the intact cell, thus avoiding the preparation of plasma membranes. The methodology for this procedure evolved from observations of the effect of Triton X-100 on MTX uptake (Fig. 2). Drug uptake is very sensitive to the presence of detergent, and at 0.01% Triton X-100, virtually ceases. Inhibition by low concentrations of detergent has been observed in membrance-associated enzyme systems [19] and in the uptake of proline by *Escherichia coli* membrane vesicles [20]. However, inhibition in these cases occurs in the absence of membrane solubilization and is probably due to the binding of detergent to the protein while in association with the membrane [21]. In contrast, the inhibition of MTX uptake observed in the present study is accompanied by a release of protein from the cell, a process which occurs in the absence of extensive cellular destruction, as determined by measurements of cell viability. Indications are that a substantial portion of the proteins released are plasma membrane derived and probably not deeply imbedded in the membrane. Similar procedures have been developed for the analysis of plasma membrane proteins from fibroblasts [22] and pig lymphocytes [23].

When the solubilization is carried out in the presence of [$^3$H]-MTX, drug binding can be studied by the use of Sephandex G-25 chromatography (Fig.3). Maximum binding is achieved only when the extraction is carried out in the presence of labeled drug, perhaps implying that the binding complex is stable only when solubilization occurs while MTX is bound. In a similar manner, a folic acid (FA) binding protein can be purified from *Lactobacillus casei* membranes, when solubilization takes place in the presence of [$^3$H]-FA [24]. However, in this case, the affinity of FA for the binding protein is such that it permits the purification of the complex, using conventional methods, by following the radioactivity. In L1210 cells, Sepharose-MTX affinity chromatography has been used to purify a protein fraction, consisting of three major protein species of mol. wts 67,000, 63,000 and 56,000 (Fig.4), which has a MTX binding activity (Fig. 5). The same protein fraction can be be purified by affinity chromatography of either the material extracted from intact cells or from a solubilized crude plasma membrane pellet, emphasizing the fact that the intact cell solubilization procedure does insure the extraction of mainly plasma membrane proteins from the intact cell.

By summation of the [$^3$H]-MTX present in the column void volume (Fig. 5) and correction for the total number of cells used in a typical experiment, approximate estimations of total binding can be made. These give a figure of $10^{-12}$ mole for the concentration of binding protein complex extractable from $10^9$ cells, assuming that one molecule of MTX binds to one binding receptor molecule. Preliminary studies of this purified protein fraction on Sephadex G-100 indicate that the proteins of mol. wts 67,000, 63,000 and 56,000 are subunits of a protein complex, the mol. wt of which is in excess of 100,000. The NEM labeling experiment further confirms the role of a 56,000 mol. wt protein in the binding of MTX.

In conclusion, a protein complex with a subunit composition of 3 polypeptides with mol. wts of 67,000, 63,000 and 56,000 has been implicated in MTX binding at the level of the plasma membrane. Differential labeling with NEM implies that the 56,000 mol. wt polypeptide contains a reactive thiol group intimately involved in MTX binding. The polypeptides of mol. wt 67,000 and 63,000 may play a structural role in the association of the protein complex with the plasma membrane.

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