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RIBOFLAVIN–METHOTREXATE INTERACTIONS

PHOTOCHEMICAL REACTION AND COMPETITION FOR TRANSPORT IN THE L1210 MOUSE LEUKEMIA CELL

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Abstract—Methotrexate transport in the L1210 mouse leukemia cell is a carrier-mediated process. Riboflavin competitively inhibits the unidirectional influx of methotrexate and possibly utilizes this carrier, at least in part, for its own transport. The apparent affinity of riboflavin for this transport mechanism is, however, much lower than that of methotrexate.

In addition to their competition at the cell membrane for the transport mechanism, riboflavin and methotrexate undergo a time-dependent, photochemical reaction, which results in the formation of methotrexate derivatives that are separable by column chromatography. The compounds were not identified, but some of their properties were characterized. The labeled methotrexate derivatives do not significantly bind to the intracellular enzyme, dihydrofolate reductase. Their transport occurs by a route different from that for methotrexate, since uptake is more rapid, temperature sensitivity is markedly different, and inhibitors of methotrexate uptake are without effect on these derivatives.

Other flavin–folate interactions were demonstrated by the reaction between riboflavin and the parent compound, folic acid, and by the reaction between methotrexate and ethanol lumiflavin, a congener of riboflavin.

THE TRANSPORT of methotrexate (MTX) (4-amino-10-methyl-4-deoxy-pteroylglutamic acid) in the L1210 mouse leukemia cell has been shown to be a carrier-mediated, energy-dependent process.[1, 2] Initial studies from this laboratory suggested that riboflavin-5′-phosphate (RF) enhanced the unidirectional influx of ^{3}H-MTX,[1] while Hakala,[3] who used enzymatic assay techniques, reported inhibition of MTX uptake by riboflavin in Sarcoma 180 cells. In an attempt to resolve this apparent discrepancy and to characterize the MTX–RF interaction, the present study was undertaken. The data to be presented indicate that the interaction between MTX and RF consists of two basically different phenomena: (1) RF competitively inhibits MTX influx by the carrier transport mechanism; (2) MTX and RF undergo a time-dependent, photochemical reaction, which results in the formation of decomposition products that are taken up by the cells more rapidly than MTX. Whether RF will inhibit or enhance the uptake of isotope depends upon the relative magnitude of these processes.

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METHODS AND MATERIALS

All experiments were performed on L1210 mouse leukemia cells grown in cell culture. The methods of culture and the experimental procedures have been described in detail previously.[1] The buffer employed in these experiments consisted of 124 mM NaCl, 5·3 mM KCl, 1·1 mM NaH_2PO_4, 16 mM $NaHCO_3$, 1·9 mM $CaCl_2$ and 1 mM $MgCl_2$. The pH was maintained at 7·3 by passing 95% O_2–5% CO_2 over the cell suspension.

The method for measurement of unidirectional influx rates for MTX is based on the high affinity of MTX for the intracellular enzyme, dihydrofolate reductase.[1, 4, 5] During uptake, free intracellular MTX does not exist prior to saturation of these binding sites, and the net appearance of MTX within the cell is therefore equal to the true unidirectional influx velocity. For unidirectional influx measurements, incubation times were chosen to ensure that uptake did not exceed the enzyme binding capacity. For steady state conditions, where free intracellular MTX exists, cells were incubated until the dihydrofolate reductase binding sites were saturated and the free intracellular MTX concentration was constant.

The experimental procedure for unidirectional influx measurements was as follows: ^{3}H-MTX and test substances were added to 13-ml centrifuge tubes and gassed with 95% O_2–5% CO_2; 1 ml of a cell suspension (1–3% cytocrit) was added and the tubes were vigorously shaken in a water bath at 37°. Influx was terminated by the addition of 10 ml of an 0·85% NaCl solution at 0°. The cells were separated by centrifugation and washed twice with the 0·85% NaCl solution at 0° to remove extracellular MTX. The cellular material was weighed, digested in 1 N KOH, and an aliquot was counted in a liquid scintillation spectrometer as previously described.[1]

Chemicals. ^{3}H-MTX and ^{3}H-folic acid (labeled in the 3′ and 5′ positions of the benzoic acid moiety) were obtained from Amersham/Searle, Des Plaines, Ill., and purified by fractionation on a diethylaminoethyl (DEAE) cellulose column as previously described.[1] Riboflavin-5′-phosphate was obtained from Mann Research Labs., New York, N. Y.; methotrexate and folinic acid (5-formyl-5,6,7,8-tetrahydropteroyl glutamic acid) from Lederle Laboratories, Pearl River, N. Y.; and lumichrome from Aldrich Chemical Company, Milwaukee, Wis. Ethanol lumiflavin was generously provided by Dr. Lin Tsai of the National Institutes of Health.

Solutions of MTX, RF and their congeners were always kept stored in the dark at 4°. During experiments they were exposed to fluorescent room light of average intensity for specified periods of time.

Spectrophotometric studies were performed with a Beckman DU spectrophotometer.

RESULTS

Competitive inhibition of MTX influx by RF. When MTX and RF are mixed together at the instant of exposure to L1210 cells, the unidirectional influx of MTX is inhibited. The time course of this effect is illustrated in Fig. 1. Although RF is light-sensitive, as will be discussed later, RF inhibition of MTX influx was not affected when RF was exposed to light for 1 hr prior to the experiment.

The depression of MTX influx as a function of RF concentration was studied in three experiments. In Fig. 2, as the extracellular RF concentration, $(RF)_e$, was increased with a constant extracellular MTX level, the unidirectional influx of MTX was

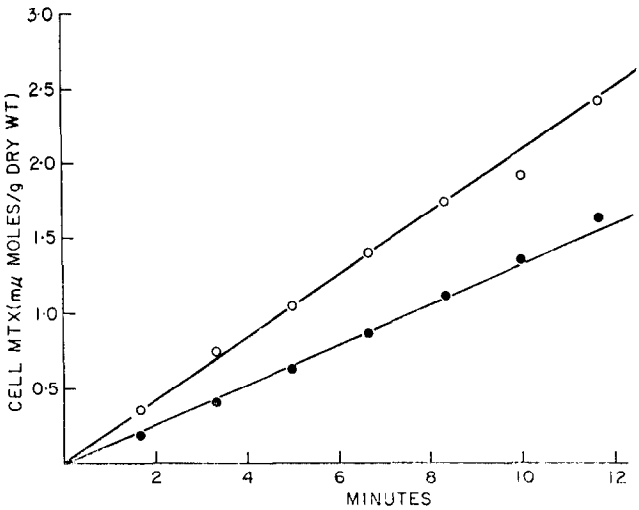

FIG. 1. Time course of uptake of 0·1 μM MTX in the presence (●) and absence (○) of 290 μM RF. Both compounds were mixed together at the instant of exposure to the cells.

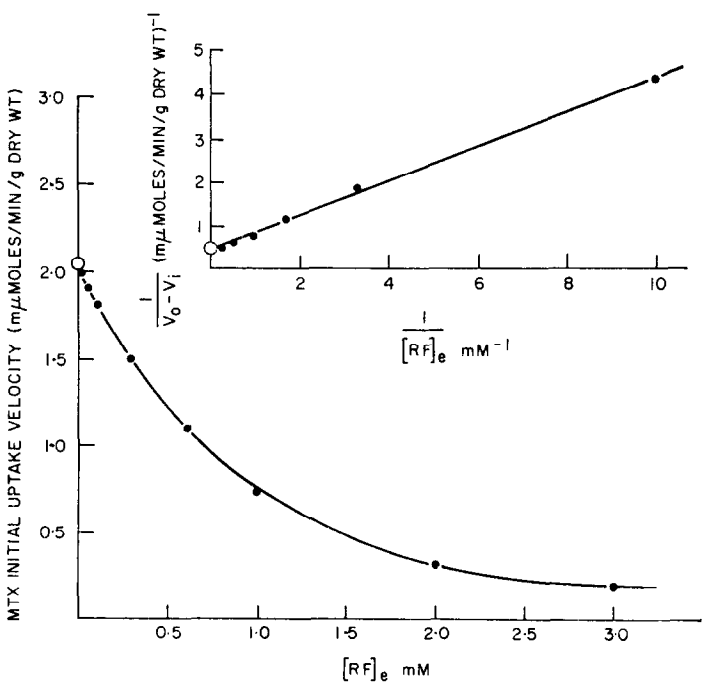

FIG. 2. Kinetics of MTX influx inhibition by RF. MTX (1·5 μM) and RF were mixed together at the instant of exposure to the cells. Influx was measured over 60 sec. Insert: Plot of the data according to the method of Inui and Christensen.[6] V_0 = MTX influx with no inhibitor present; V_i = MTX influx with inhibitor present at an extracellular concentration of $(RF)_e$. The line was drawn by the method of least squares. The large open circles indicate the measured uninhibited MTX influx rate.

progressively reduced. To determine the maximum inhibition of MTX influx by RF, the data were analyzed in the insert of Fig. 2 according to the method of Inui and Christensen.[6] The reciprocal of the difference between the uninhibited MTX influx (V_0) and the inhibited rate (V_i) was plotted as a function of the reciprocal of $(RF)_e$. In this kind of plot, if at infinite RF concentration inhibition of MTX is complete, the reciprocal of the ordinate intercept should equal the reciprocal of the MTX influx rate with no inhibitor present. This was found to be the case, as indicated by the correspondence of the ordinate intercept and $1/V_0$ (open circle).

The kinetics of RF inhibition of MTX influx were also studied by varying the MTX concentration while maintaining $(RF)_e$ constant. On a double reciprocal plot

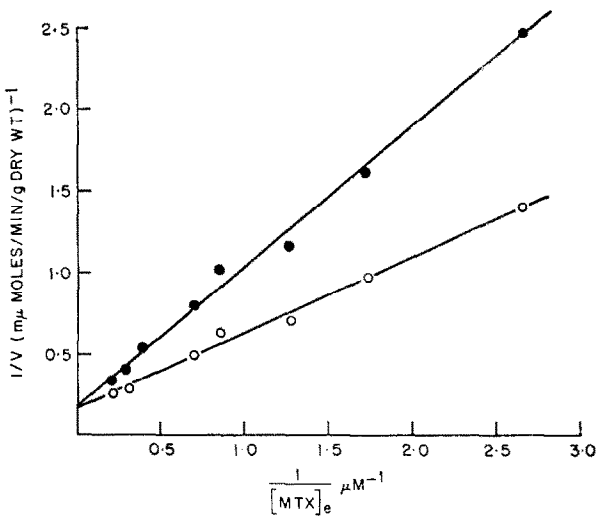

FIG. 3. Lineweaver–Burk plot of the relationship between MTX influx and the extracellular MTX concentration in the presence (●) and absence (○) of 240 μM RF. Influx was measured over 60 sec. Lines were drawn by the method of least squares. MTX K_t = 3 μM, V_{max} = 6·1 mμmoles/min/g dry wt. RF K_i = 340 μM.

(Fig. 3), the slope is increased in the presence of RF and the ordinate intercepts are the same, indicating that RF inhibition of MTX influx is competitive. However, the affinity of RF for this transport mechanism must be small, since from Fig. 3 it can be determined that the inhibition constant for RF on MTX unidirectional influx is 340 μM, whereas the MTX concentration at half-maximal influx velocity, K_t, is 3 μM.

Evaluation of exchange phenomena between MTX and RF. Exchange phenomena between MTX and RF were explored by evaluation of possible unidirectional influx changes for MTX in cells loaded with RF. Cells were incubated for 30 min in the presence and absence of 1·2 mM RF, then recovered by centrifugation, washed twice with RF-free buffer at 0°, and resuspended in buffer containing MTX. In six experiments the unidirectional influx of MTX was not significantly different in control cells and in cells preincubated with RF.

To determine whether RF could cause countertransport of MTX, cells were brought to equilibrium with 2 μM MTX, after which RF was added to an aliquot to obtain a

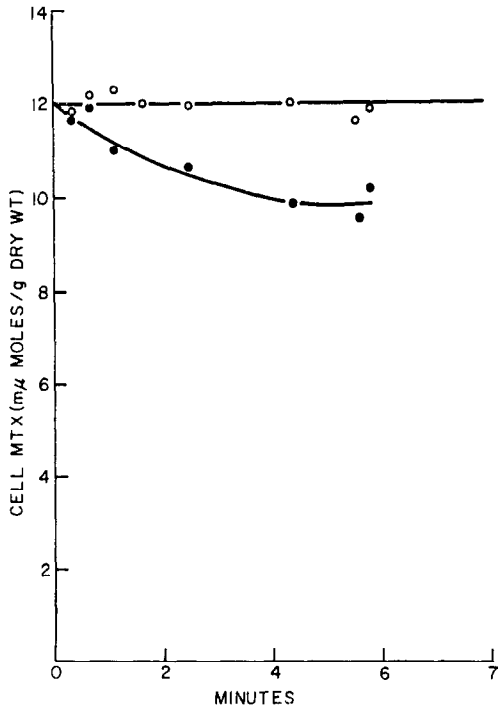

FIG. 4. Effect of RF on steady state levels of MTX. Cells were brought to equilibrium with 2 μM MTX in 45 min. At zero time, RF was added to an aliquot of cells to obtain a final concentration of 1·2 mM (●). Control cells are indicated by (○).

final concentration of 1·2 mM. As seen in Fig. 4, upon addition of RF there was a net fall in intracellular MTX. In two other experiments it was determined that the net efflux of MTX continued until the free intracellular MTX concentration was less than $\frac{1}{10}$ the extracellular level, suggesting that RF induced an efflux of MTX against an electrochemical potential gradient.

Photochemical interaction between RF and MTX. The possibility of a chemical interaction between MTX and RF was evaluated spectrophotometrically. Since both compounds absorb strongly in a similar ultraviolet range, changes in the MTX spectrum were detected by the difference between an MTX–RF solution and a reference solution of RF at the same concentration. Specifically, 20 μM RF in 0·1 M NH_4HCO_3 (pH 8·3) placed in two matched cuvettes gave a zero baseline throughout the u.v. range. When MTX was added (to a final concentration of 20 μM) to one cuvette, the spectrum for MTX was obtained. After 1 hr in the dark, the MTX spectrum remained unchanged. After both cuvettes were exposed to light for an additional hour, however, a definite spectral shift was observed (Fig. 5, upper half). Under similar conditions during this interval of exposure to light, MTX alone exhibited no change in spectrum.

In the lower half of Fig. 5, the converse experiment is illustrated: 20 μM MTX in 0·1 M NH_4HCO_3 (pH 8·3) was placed in two matched cuvettes and again gave a constant zero baseline. When RF, at a final concentration of 20 μM, was added to one

cuvette the spectrum for RF was obtained and was not altered after 1 hr in the dark. After both cuvettes were exposed to light for 1 hr, there was a shift in the RF spectrum; this same shift occurred when RF alone was exposed to light. Since MTX does not influence the light-induced change in RF, when RF is present in both cuvettes and MTX in one (Fig. 5, upper half), the alterations in RF are balanced and equal in the two cuvettes; therefore, the observed change in the spectrum must be due to an RF–MTX interaction resulting in a shift in the MTX spectrum.

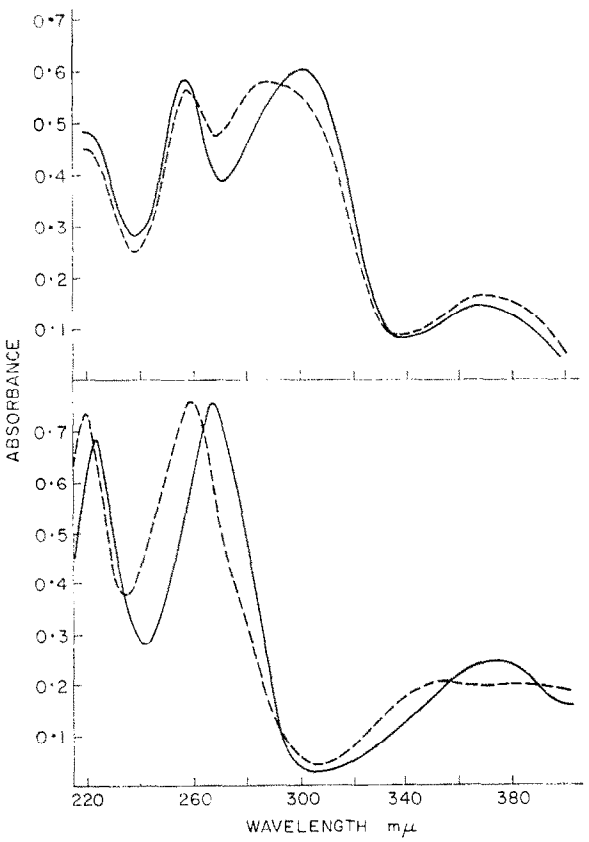

FIG. 5. Spectrophotometry of RF–MTX interaction. Upper graph: the u.v. absorption spectrum for MTX immediately upon addition to an RF solution (——) and after exposure to light for 1 hr (- - - -). Lower graph: the u.v. absorption spectrum for RF immediately upon addition to an MTX solution (——) and after exposure to light for 1 hr (- - - -).

The products of this apparent interaction between MTX and RF were found to be separable by column chromatography. RF and MTX at a 100:1 ratio were incubated in ungassed buffer (pH 8·4) for 1·5 hr at room temperature while exposed to room light. An excess of unlabeled MTX was added and the solution was fractionated on a DEAE cellulose column using a linear gradient (0·1 to 0·4 M) of NH_4HCO_3 (pH 8·3). The u.v. absorption was monitored at 254 mμ. As indicated in Fig. 6, ^3H-labeled material was eluted in four peaks: 55 per cent eluted with MTX (C), 12 per cent with

RF (B), and 23 per cent (A) and 9 per cent (D) were in unidentified peaks. These results were confirmed in two other similar experiments. Fractionation of ^{3}H-MTX alone, under these conditions, results in more than 95 per cent recovery in the MTX peak. Thus incubation of MTX with RF under these conditions produces new products which retain the ^{3}H label and whose elution pattern differs from that of MTX.

Time course of uptake of the RF–MTX reaction products. In these studies, labeled MTX derivatives were produced by incubating MTX with RF in ungassed buffer

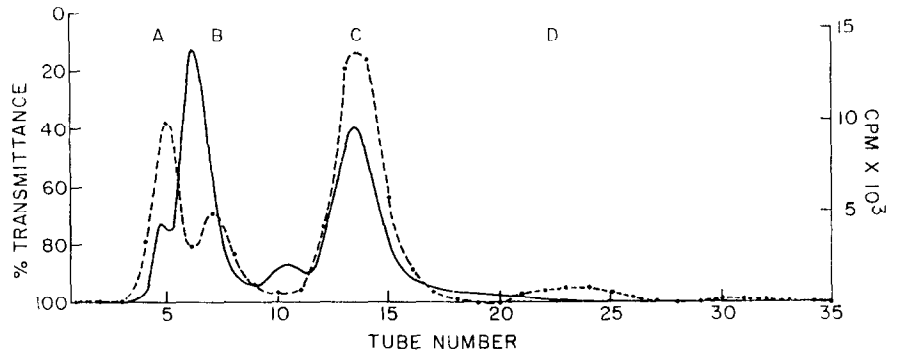

FIG. 6. Chromatography of the products of the RF–MTX chemical reaction. The u.v. absorption (——) was monitored at 254 mμ. Radioactivity is indicated by (- - - -).

(pH 8·4) at room temperature and exposed to room light for 1·5 hr prior to the experiment. Subsequent uptake of the ^{3}H label thus reflected both unchanged MTX and the labeled derivatives. In these experiments the binding capacity for MTX was not exceeded so that influx of unchanged MTX was unidirectional.

Figure 7 compares the uptake of ^{3}H label in cells exposed to MTX alone, MTX and RF pre-mixed, and MTX and RF mixed together only at the instant of exposure to the cells. Initially, uptake of label into cells exposed to the pre-mixed MTX–RF solution was faster than the control, but this rate rapidly fell to reach a constant value within 2 min. At this point, the rate approximated that of the cells in which MTX and RF were pre-mixed at the instant of exposure to the cells. The reduced rate as compared to the control represents the expected competitive inhibition of MTX influx by RF as described earlier.

The initial rapid uptake of label in the cells exposed to the pre-mixed MTX–RF is believed to reflect the rapid influx of the labeled MTX derivatives. The fall in rate with resumption of linear uptake is attributed to rapid equilibration of these derivatives in the intracellular water, with subsequent uptake now due to the unidirectional influx of unaltered MTX. The presence of exchangeable intracellular label under these conditions was demonstrated by a rapid efflux of label when cells exposed to the pre-mixed MTX–RF were resuspended into label-free medium at 37°. Thus the labeled MTX derivatives do not significantly bind to dihydrofolate reductase. Efflux of the labeled derivates is, however, temperature-dependent, with negligible loss at 0°.

Characteristics of the initial uptake process for the labeled MTX derivatives. As seen in Fig. 7, during the early seconds following addition of the pre-mixed RF–MTX,

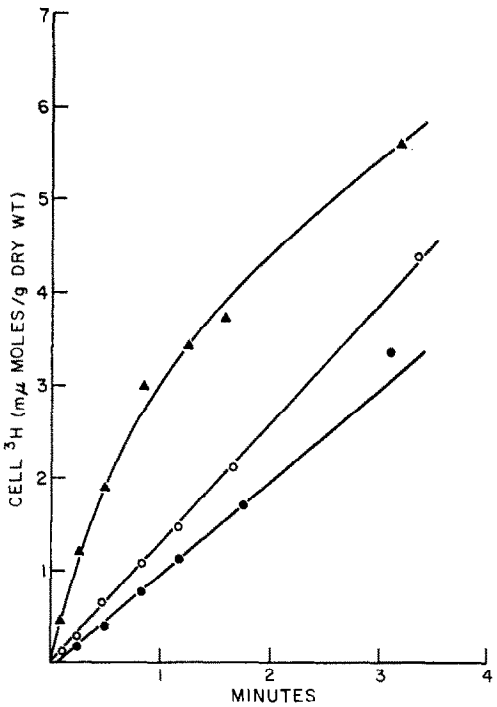

FIG. 7. Time course of ^{3}H uptake into cells exposed to: (1) MTX alone (○); (2) MTX and RF mixed together at the instant of exposure to the cells (●); (3) MTX and RF preincubated before exposure to the cells (▲). MTX and RF concentrations were 0·8 μM and 160 μM respectively.

total ^{3}H uptake approximates linearity and the labeled MTX derivatives account for the major part of uptake. In the studies to follow, the initial uptake rate for the labeled MTX derivatives was roughly approximated by measuring the difference between the total uptake of label and the uptake of MTX determined separately over a short flux interval. (a) The Q_{10} for uptake was estimated over a 5-sec flux period. MTX Q_{10} from 27 to 37° was 5 to 7. This was markedly different from the uptake of the MTX derivatives where in three experiments (in which RF and MTX were pre-mixed at 27°) uptake at 27° exceeded the value at 37° by an average factor of 2. (b) Although 2 mM unlabeled MTX, 5 mM folic acid, or 3·6 mM RF markedly reduced MTX influx, they had little effect on the uptake of the MTX derivatives. (c) The uptake of the MTX derivatives was unaltered in cells preloaded with 2 mM RF or 100 μM folinic acid.* Thus there was no evidence for exchange diffusion between folinic acid or RF and the MTX derivatives.

Using the augmentation of ^{3}H uptake as a quantitative estimate of the formation of the labeled MTX derivatives, it was found that the RF–MTX interaction occurred only slightly in the dark but increased linearly with time upon exposure to light. The rate of the MTX–RF interaction increased as the ratio of RF to MTX was raised in the preincubated solution, but this relationship was not quantitated.

* The cells were preloaded with folinic acid rather than with MTX because other studies[7] have shown that the unidirectional influx of MTX is enhanced in cells loaded with folinic acid, but is not significantly altered in cells loaded with MTX.

Interaction between RF and folic acid. To determine whether the RF interaction was unique for MTX or was shared by other folates, RF and ^{3}H-folic acid were incubated together in ungassed buffer (pH 8·4) for 1·5 hr at room temperature. An apparent interaction occurred, as the initial uptake of the ^{3}H label was six times greater for ^{3}H-folic acid pre-mixed with RF than for ^{3}H-folic acid alone.

Lumichrome and ethanol lumiflavin interaction with MTX. The interaction between MTX and two congeners of RF (Fig. 8) was studied by comparing the initial uptake

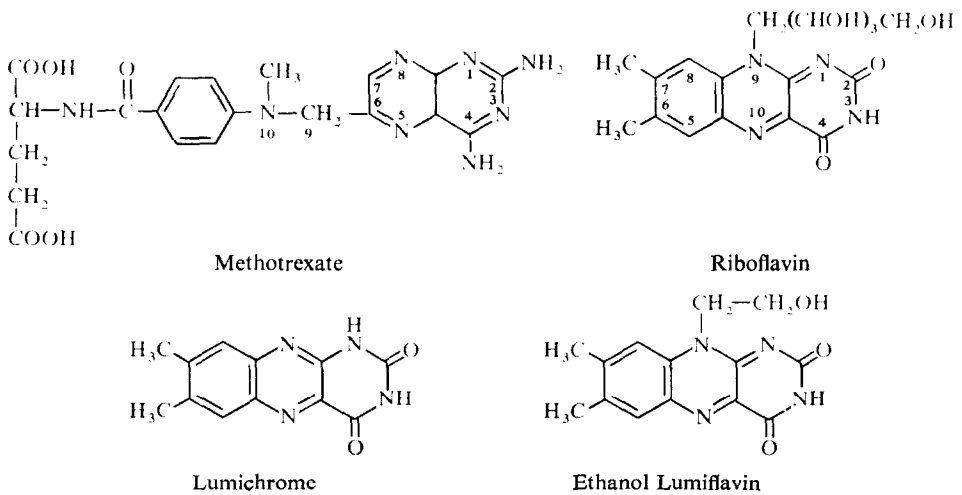

FIG. 8. Structures of MTX, RF, ethanol lumiflavin and lumichrome.

TABLE 1. RF, ETHANOL LUMIFLAVIN AND LUMICHROME INTERACTION WITH MTX*

Compound	I Compounds mixed at instant of exposure to cells: ^{3}H-MTX influx velocity (mμmoles/min/g dry wt.)	II Compounds premixed for 3 hr: Total ^{3}H uptake (mμmoles/min/g dry wt.)
MTX	0·214 ± 0·016	
MTX and RF	0·220 ± 0·010	0·709 ± 0·031
MTX and ethanol lumiflavin	0·220 ± 0·013	0·776 ± 0·034
MTX and lumichrome	0·218 ± 0·010	0·296 ± 0·012

* ^{3}H-MTX (0·1 μM) was incubated with either 1·5 μM RF, 1·5 μM ethanol lumiflavin or 1·5 μM lumichrome in ungassed buffer (pH 8·4) for 3 hr at room temperature while exposed to light. The uptake of ^{3}H label from these solutions (II) was then compared to the uptake of ^{3}H-MTX when these compounds were mixed with MTX at the instant of exposure to the cells (I). The flux interval was 60 sec. The data represent the mean ± S. D. of four measurements.

rate for MTX alone and for MTX pre-mixed with these compounds. Ethanol lumiflavin interacted with MTX to a somewhat greater extent than did RF (Table 1). Lumichrome, however, showed only a slight interaction. The simultaneous addition of any of these substances with MTX at the time of exposure to the cells did not alter MTX influx. This is expected because the extracellular MTX concentration employed

in these experiments was less than $\frac{1}{30}$ the influx K_t and the ratio of the flavin to MTX was too small to cause any influx inhibition (see Fig. 2). As with RF, it was necessary to preincubate the MTX with either ethanol lumiflavin or lumichrome in order to produce the labeled derivatives.

DISCUSSION

From these studies it is clear that there are two types of interaction between MTX and RF. When mixed together at the instant of exposure to cells, inhibition of MTX influx occurs at the cell membrane. When mixed together prior to exposure to cells, a chemical reaction occurs which results in the production of labeled MTX derivatives which rapidly enter the L1210 cell.

RF inhibition of MTX influx at the cell membrane is competitive, although its affinity for the carrier must be far less than that of MTX. The observation that addition of RF to cells containing free intracellular MTX led to efflux of MTX against an electrochemical potential gradient is compatible with countertransport and suggests further that RF may utilize this system, at least in part, to effect its own transport across the L1210 cell membrane. MTX unidirectional influx stimulation did not occur when cells were preincubated with RF, but the uptake and intracellular disposition of RF are not certain.

The chemical interaction between MTX and RF appears to result in the formation of several derivatives of MTX, which are separable by DEAE cellulose column chromatography. The reaction is time-dependent and strongly enhanced by exposure to light. Structurally, an isoalloxazine ring, as is present in riboflavin and ethanol lumiflavin, appears to facilitate this reaction; lumichrome, with an alloxazine ring, reacts only weakly (Fig. 8). Furthermore, a reaction can occur with folic acid as well as with MTX.

RF has been observed to interact with a number of other compounds including phenols,[8, 9] indoles, [9-12] amino acids,[13] purines,[10, 14-16] pyrimidines,[16] and other molecules containing complex ring structures.[17, 18] There are several mechanisms by which RF may enter into these reactions: (1) RF, in a light-induced excited state, participates in charge-transfer reactions with a number of compounds, leading to formation of RF–substrate complexes which may be detected by shifts in absorption spectra. In the reaction between RF and indoles (tryptophan),[9] or phenols,[8, 9] 1:1 RF substrate complexes are formed which have been isolated in crystalline form. (2) Also described is a light-dependent oxidation–reduction reaction between RF and a number of substrates: for example, RF may oxidize amino acids to keto acids,[13] adenine to hypoxanthine[15] and indoleacetic acid to indolealdehyde.[11] These reactions are time-dependent and may increase over several hours. The nature of the RF reaction with MTX is not known, but alterations in the MTX molecule during conditions of severe oxidation have been described.[19]

Although the labeled MTX derivatives were not identified, some characteristics of their transport were evaluated. These substances appear to be taken up by the L1210 cell by a mechanism which differs from that of MTX. Thus uptake was much faster than that of MTX. Uptake was not inhibited by the presence of an excess of either RF or MTX, and the temperature sensitivity of the uptake process was vastly different from that of MTX. That the uptake of the labeled derivatives was enhanced at low temperatures is unexplained, but this observation, together with the finding that

efflux of the derivatives is negligible at 0°, suggests that transport is by a mediated process rather than by simple diffusion.

In view of the findings reported in this paper, it is of interest to compare the experimental conditions employed by Hakala,[5] in which riboflavin inhibited the apparent uptake velocity of MTX, and initial studies from this laboratory,[1] in which the apparent influx of MTX was stimulated by RF. Hakala measured the uptake of MTX by determining the dihydrofolate reductase-bound MTX (after setting it free) by titration in a folate reductase assay system. In those experiments Sarcoma 180 cells were kept in an incubator in tissue culture medium containing MTX and varied concentrations of riboflavin for 24 hr without exercising protection against light. From the low ratios of riboflavin to MTX employed (maximum 27:1), it is unlikely that there was inhibition of MTX uptake by competition at the cell membrane. Instead, a photochemical breakdown of MTX probably occurred with the production of derivatives that did not bind to dihydrofolate reductase. Hence the measured uptake of unaltered MTX would have been reduced. By contrast, in earlier studies from this laboratory, MTX transport was obtained by measuring uptake of ^3H label; apparent stimulation of MTX influx was due to the chemical interaction between these compounds producing labeled derivatives which entered the cell more rapidly than did MTX itself.

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REFERENCES

1. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, *J. biol. Chem.* **243**, 5007 (1968).
2. I. D. Goldman, *J. biol. Chem.*, **244**, 3779 (1969).
3. M. T. Hakala, *Biochim. biophys. Acta* **102**, 210 (1965).
4. W. C. Werkheiser, *J. biol. Chem.* **236**, 888 (1961).
5. M. T. Hakala, *Biochim. biophys. Acta* **102**, 198 (1966).
6. Y. Inui and H. N. Christensen, *J. gen. Physiol.* **50**, 203 (1966).
7. I. D. Goldman, *Biophys. J.* **9**, A229 (1969).
8. D. E. Fleischman and G. Tollin, *Biochim. biophys. Acta* **94**, 248 (1965).
9. J. F. Pereira and G. Tollin, *Biochim. biophys. Acta* **143**, 79 (1967).
10. J. E. Wilson, *Biochemistry, N.Y.* **5**, 1351 (1966).
11. B. Nathanson, M. Brody, S. Brody and S. B. Broyde, *Photochem. Photobiol.* **6**, 177 (1967).
12. I. Isenberg and A. Szent-Gyorgyi, *Proc. natn. Acad. Sci. U.S.A.* **44**, 857 (1967).
13. P. Byrom and J. H. Turnbull, *Photochem. Photobiol.* **6**, 125 (1967).
14. J. A. Roth and D. B. McCormick, *Photochem. Photobiol.* **6**, 657 (1967).
15. K. Uehara, T. Mizoguchi and S. Hosomi, *J. Biochem., Tokyo* **59**, 550 (1966).
16. M. A. Slifkin, *Biochim. biophys. Acta* **103**, 365 (1965).
17. J. Posthuma and W. Berends, *Biochim. biophys. Acta* **112**, 422 (1966).
18. M. T. Hakala, *Fedn Proc.* **28**, 856 (1969).
19. D. R. Seeger, *J. Am. chem. Soc.* **71**, 1753 (1949).