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EFFLUX IN ISOLATED HEPATOCYTES AS A POSSIBLE CORRELATE OF SECRETION IN VIVO: INDUCED EXIT OF THE FOLIC ACID ANALOG, METHOTREXATE, BY DIBUTYRYL CYCLIC AMP OR ISOBUTYL METHYL XANTHINE[1]

David A. Gewirtz[2], Joyce K. Randolph and I. David Goldman
Departments of Medicine and Pharmacology
Medical College of Virginia
Richmond, Virginia 23298

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Dibutyryl cyclic AMP and isobutyl methyl xanthine induce release of freely exchangeable methotrexate as well as a small component of apparently bound drug from freshly isolated rat hepatocytes; methotrexate polyglutamate derivatives are retained. These observations, as well as the energy dependence of methotrexate efflux induced by dibutyryl cyclic AMP suggests that this may represent the induction of a "secretory" phenomenon in which drug is released into the capillary sinusoid and/or the bile canaliculus when the hepatocyte is in its normal spatial orientation in the liver lobule in vivo. Because there is evidence that this folic acid analog and bile salts utilize the same transport mechanism in these cells, this phenomenon may have general physiological as well as pharmacologic relevance and the isolated hepatocyte may be a useful model system to study mechanisms of hepatic secretion at the cellular level.

The isolated rat hepatocyte has become an important system for the study, at the cellular level, of many biosynthetic and transport phenomena present in the intact liver (1-6). This system eliminates the contribution that Kupffer cells may make to the processes studied and, in the realm of membrane transport in particular, permits rapid quantitation of initial transport rates that are largely unaffected by large unstirred layers which surround hepatocytes in liver slices or hepatic perfusion models (7-10). Of particular interest is the unique potential of this system for study of control mechanism(s) of hepatic secretion - not yet explored nor understood at the cellular level. This report describes studies on the induction of net loss of the folic acid analog, methotrexate, from isolated hepatocytes that may simulate the "secretion" of methotrexate[3] into the bile

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3. Abbreviations: Methotrexate; 4-amino-10-methyl-pteroylglutamic acid; methotrexate + G1, 4-amino-10-methylpteroylglutamyl-γ-glutamic acid; methotrexate + G2, 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid; DEAE-diethyl aminoethyl; Bt_2cAMP or dibutyryl cyclic AMP-dibutyryl cyclic 3'5' adenosine monophosphate; IBMX-3-isobutyl-1-methylxanthine; DMSO-dimethylsulfoxide.

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canaliculus and/or the capillary sinusoid in vivo. Of particular interest is evidence that this agent and bile acids share common transport route(s) in the hepatocyte (8) so that the phenomenon herein reported may have broad physiological as well as pharmacologic relevance to the mechanisms by which a variety of compounds are transported out of liver cells and the control of these processes under physiological conditions.

Materials and Methods

Hepatocytes in suspension were prepared from male Sprague-Dawley rats by a modification of the collagenase perfusion method of Berry and Friend (11) as described previously (7). Cells were washed in the buffer utilized for the uptake studies (7) and stored on ice for no more than 1/2 hour before initiation of the experiments. Measurements of [^{3}H] -methotrexate uptake and efflux utilized techniques described in detail previouly (7,8,12) and in the legends to the charts. Determination of the ratio of intracellular water to dry weight 1.97 $\pm$ 0.41 l/mg (S.E.) and the concentration of ^{3}H in mol/l of intracellular water have been described (7).

[3',5'9-^{3}H] methotrexate with an initial specific activity of 30 Ci/mmole was synthesized by Amersham/Searle Corporation (Arlington Heights, IL). Both labeled and unlabeled methotrexate were purified by DEAE[3] cellulose column chromatography (12). Methotrexate and its polyglutamate derivatives were extracted by boiling with subsequent sonication of the washed cell pellet (13) and then separated by DEAE cellulose chromatography (12). Dimethyl sulfoxide, utilized as a solvent for the isobutyl methyl xanthine did not, alone, significantly perturb transport of methotrexate.

Results

Previous studies from this laboratory indicate that transport of the folic acid analog, methotrexate, into suspensions of freshly isolated hepatocytes is mediated by high and low affinity routes that are temperature sensitive, require extracellular sodium, and are inhibited by azide, ouabain and sulfhydryl group blocking agents (7). Intracellular methotrexate is found as both "freely exchangeable" and "less readily exchangeable" drug in the intact hepatocyte as distinguished by loss and retention, respectively, of the agent when hepatocytes loaded with methotrexate are separated from the medium by centrifugation and resuspended into a large volume of methotrexate-free medium (7). It is assumed that the less readily exchangeable methotrexate represents drug bound to intracellular constituents. Methotrexate is also rapidly converted to polyglutamate derivatives in isolated hepatocytes (13).

Figure 1 illustrates that when cells are first treated with 10 mM sodium azide following which methotrexate is added, net accumulation of this folic acid analog is markedly depressed.[4] However, when azide is added at a time when

4. This is, in part, related to inhibition of methotrexate polyglutamate synthesis by azide (unpublished observation).

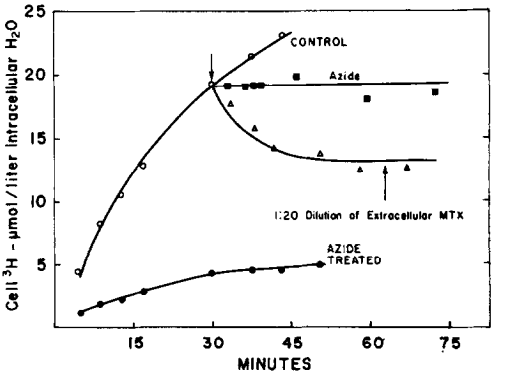

Figure 1. The effect of azide on the time course of uptake of [^{3}H] after incubation of cells with ^{3}H-methotrexate. Uptake of [^{3}H] by hepatocytes incubated with 1 μM [^{3}H]-methotrexate alone (O) is compared with uptake by cells pretreated for 5 min. with 10 mM sodium azide (at 37° C) (●). After 30 min. (indicated by the upper arrow) portions of control cells were diluted 1:20 with methotrexate-free buffer (Δ) to determine exchangeable intracellular [^{3}H]. At 30 min., another portion of control cells was exposed to 10 mM Na azide (■).

exchangeable intracellular methotrexate has already reached a steady state (e.g. 30 minutes, see reference 7), there is cessation of net drug accumulation but no net loss of methotrexate from the cell despite the persistence of a large apparent transmembrane electrochemical potential difference for methotrexate under these conditions (Figure 1 and reference 7). Similarly, antimycin A (10 μM), azide (10 mM) + iodacetate (1 mM), or ouabain (1 mM) terminate net methotrexate accumulation but do not induce net loss of exchangeable drug from the cell.

In contrast, addition of 2.5 mM dibutyryl cyclic 3'5' adenosine monophosphate (Bt_2cAMP) or 2.5 mM 3-isobutyl-1-methyl xanthine (IBMX) to cells at the steady state with exchangeable methotrexate (15 to 60 minutes after addition of methotrexate) results in a rapid and marked net loss of methotrexate from the cells (Figure 2).[5] In fact, the loss of net cell methotrexate induced by Bt_2cAMP (48.19 ± 2.3% of total intracellular methotrexate - from 19 experiments) is greater than the freely exchangeable intracellular methotrexate level (38.84 + 2.77 of total intracellular drug - from 25 experiments) suggesting that efflux of bound as well as free drug from the cell must have been induced. This is confirmed in Figure 3A where cells incubated with 1 μM ^{3}H-methotrexate for 15 minutes were separated by centrifugation, washed and resuspended into a like volume of

5. When these agents are added before, or simultaneously with methotrexate, uptake of methotrexate is reduced (inset to Figure 2) as observed with metabolic poisons.

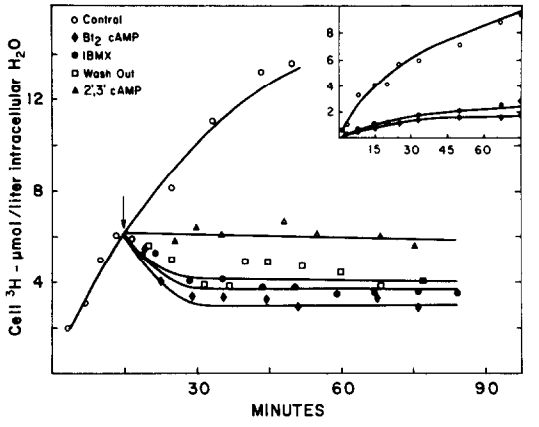

Figure 2. Induction of net methotrexate efflux from hepatocytes incubated with [^{3}H]-methotrexate. After 15 min. of incubation with 1 µM [^{3}H]-methotrexate portions of the cell suspension (indicated by arrows) were exposed to 2.5 mM dibutyryl cyclic 3'5' adenosine monophosphate (Bt_2cAMP), 3-isobutyl-1 methyl-xanthine (IBMX). cyclic 2'3' adenosine monophosphate (2'3' cAMP), or were centrifuged and resuspended into a like volume of methotrexate-free buffer to determine exchangeable [^{3}H].
Inset. Uptake of 1 µM [^{3}H]-methotrexate by control hepatocytes (O), hepatocytes exposed to 2.5 mM Bt_2cAMP (♦) or IBMX (●).

methotrexate-free buffer to permit loss of freely exchangeable intracellular methotrexate. Addition of Bt_2cAMP at this point resulted in a further net loss of intracellular methotrexate.[6] Likewise, IBMX induces the release of additional intracellular drug subsequent to a "wash out" procedure. When efflux of methotrexate is first induced by exposure of hepatocytes to Bt_2cAMP or IBMX, subsequent washing and resuspension of the cells into methotrexate-free buffer results in loss of additional intracellular drug, indicating that Bt_2cAMP or IBMX, alone, do not induce the release of all exchangeable methotrexate (Figure 3B).

To characterize the effect of Bt_2cAMP and IBMX on intracellular methotrexate polyglutamate derivatives, the levels of intracellular methotrexate and methotrexate polyglutamates was assessed both before and after the exposure of hepatocytes to Bt_2cAMP and IBMX. Table I demonstrates that the level of

6. In order to further confirm that Bt_2cAMP does indeed induce additional methotrexate release in excess of the total exchangeable intracellular drug fraction, hepatocytes washed free of [^{3}H] methotrexate and resuspended into a 10-fold larger volume of methotrexate-free buffer were subsequently exposed to 2.5 mM Bt_2cAMP. Under these conditions, the addition of Bt_2cAMP results in the release of 17.02 $\pm$ 1.03% of the remaining intracellular radiolabel ($p < 0.01$ in 4 experiments).

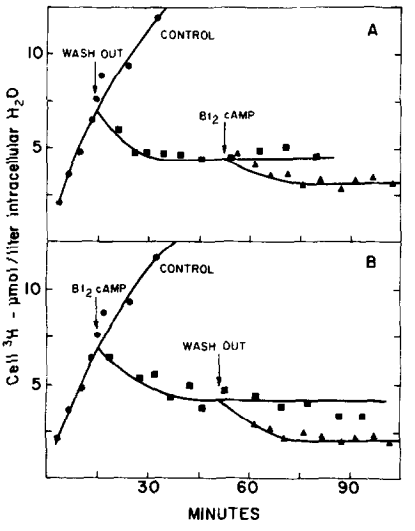

Figure 3. Efflux of freely exchangeable and less readily exchangeable [^{3}H] induced by dibutyryl cyclic AMP. A. Cells incubated with 1 µM [^{3}H]-methotrexate for 15 min. were washed and resuspended into a like volume of methotrexate-free buffer to induce efflux of exchangeable [^{3}H]. After an additional 35 min. of incubation a portion of the cells was exposed to 2.5 mM Bt_2cAMP. B. Cells were incubated with 1 µM [^{3}H]-methotrexate for 15 min. and exposed to 2.5 mM Bt_2cAMP. After an additional 35 min. (arrow) the cells were washed and resuspended in a like volume of methotrexate-free buffer.

methotrexate within the cell decreases by approximately 50% after exposure to either Bt_2cAMP or IBMX. In contrast, the total level of methotrexate polyglutamate derivatives is not reduced; in fact, the level of methotrexate + $G2^3$ is increased significantly ($p < 0.05$).

Induction of methotrexate efflux from hepatocytes by Bt_2cAMP appears to be an energy-dependent phenomenon. As indicated in Figure 4, the addition of 10 µM antimycin A to cells which have accumulated methotrexate terminates further drug uptake but does not result in net loss of drug from the cells, similar to what is observed with azide (Figure 1). However, antimycin A (or azide) reduce the rate at which methotrexate leaves the hepatocyte upon subsequent exposure to 2.5 mM Bt_2cAMP.

Hepatocytes with exchangeable methotrexate were also exposed to various compounds structurally similar to Bt_2cAMP to determine if other adenine nucleotides were effective in inducing methotrexate efflux. Figure 3 shows that addition of 2.5 mM 2'3' cyclic adenosine monophosphoric acid (2'3' cAMP) terminated net uptake of methotrexate but did not stimulate efflux of metho-

The Effects of Bt_2cAMP[3] or IBMX[3] on Net Levels of Methotrexate and Methotrexate Polyglutamate Derivatives

	Methotrexate	Methotrexate + G1[3] (pmoles/mg cell protein)	Methotrexate + G2[3]
Control (7)*	22.15 ± 2.66	7.21 ± 1.09	1.73 ± 0.32
Bt_2cAMP (4)	11.49 ± 1.66	9.19 ± 1.43	3.42 ± 0.49
IBMX (5)	11.17 ± 1.93	8.72 ± 1.65	2.37 ± 0.32

Table 1. Intracellular levels of methotrexate and methotrexate polyglutamates were measured 30 minutes after exposure to 1 μM ^3H methotrexate (Control) followed by an additional 30 minutes in the presence of 2.5 mM Bt_2cAMP or 2.5 mM IBMX. *Values in parentheses indicate number of separate experiments performed on different days. Levels of intracellular ^3H are expressed as pmoles/mg cell protein ± standard error of the mean.

trexate from the cell. Similarly, 3'5' cAMP and the adenosine nucleotide 5' AMP terminated methotrexate uptake but did not induce drug efflux. Furthermore, the addition of various other compounds which are known to raise intracellular levels of cyclic AMP in the hepatocyte did not induce methotrexate efflux. These compounds included glucagon (10^{-10} to 10^{-7} M), and isoproteronol (10 and 100 μM).

Discussion

These observations suggest that Bt_2cAMP or IBMX activate a specific cellular mechanism which results in efflux of the folic acid analog, methotrexate, from the isolated rat hepatocyte. The data indicate that this is not simply a countertransport phenomenon in which Bt_2cAMP or IBMX inhibit influx, permitting net loss of methotrexate from the cell to occur, since a variety of other agents that markedly inhibit net methotrexate uptake, including other cyclic nucleotides, nucleotides and metabolic poisons terminate net drug accumulation but do not result in the net loss of methotrexate from hepatocytes. Rather, the data suggests that this Bt_2cAMP- or IBMX-induced net loss of methotrexate from the hepatocyte may represent a mechanism by which methotrexate is "secreted" into the capillary sinusoid and/or the bile canaliculus when the cells are in their normal spatial orientation within the intact liver lobule. Evidence suggesting a "secretory" phenomenon include: (i) Bt_2cAMP and IBMX induce additional MTX efflux subsequent to the release of exchangeable methotrexate from the cell,

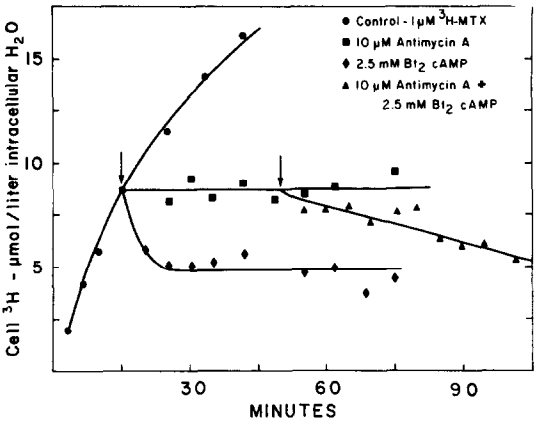

Figure 4. Inhibition of Bt_2cAMP induced efflux of [^3H] by antimycin A. Cells incubated with 1 μM [^3H]-methotrexate for 15 min. were exposed (at the arrows) to either 2.5 mM Bt_2cAMP alone, or 10 μM antimycin A followed 30 min. later by 2.5 mM Bt_2cAMP.

suggesting the ejection of bound intracellular methotrexate. This raises the possibility that bound and free drug may be present, in part, in intracellular vesicles which are released from the cell by an exocytotic mechanism which may be an integral component of the "secretory" process. (ii) The level of intracellular methotrexate polyglutamate derivatives is not reduced. This indicates that only specific components of the intracellular drug pool may be released from the cell. This observation further reinforces the conclusions of a previous report (13) that methotrexate polyglutamate derivatives do not readily exit from hepatocytes. (iii) Efflux of methotrexate induced by dbcAMP is energy-dependent in that it is slowed by metabolic poisons. Since it has been shown that methotrexate is secreted into the bile (14,15) it is not unexpected to observe some correlary of this process in the isolated hepatocyte.

Since there is evidence that methotrexate and bile salts may share similar transport mechanisms in isolated rat hepatocytes (8), the demonstration of apparent secretion of methotrexate in this system may have broad physiological as well as pharmacologic relevance. Further, since naturally occurring tetrahydrofolate cofactors are stored within the hepatocyte for ultimate release for utilization by rapidly proliferating peripheral tissues, it is possible that these observations may have some relevance to the mechanism by which these naturally occurring substances as well as other substrates stored within the liver are released into the circulation. There is, however, no evidence that these compounds share the same influx mechanism (7,31,32).

Dibutyryl cyclic AMP and isobutyl methyl xanthine have been shown to act as secretagogues or to augment basal release rates in various endocrine and exocrine tissues (16-21). Although Bt_2cAMP and IBMX may induce methotrexate efflux by increasing intracellular levels of cyclic AMP (22-25), the inability of other compounds which also raise intracellular cyclic AMP levels in the hepatocyte, such as glucagon and isoproteronol, to similarly induce net methotrexate efflux suggests the involvement of some other mechanism (26-30). The specific mechanism by which Bt_2cAMP and IBMX induce net efflux of methotrexate from the hepatocyte remains undefined and is presently under study. Nevertheless, the present data indicate the existence of a release or "secretory" mechanism for specific pools of intracellular methotrexate in the isolated hepatocyte and suggest yet another important application for the isolated rat hepatocyte as a model for studies, at the cellular level, of secretory phenomena in the intact liver.

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