Kinetic Analysis of Uptake and Phosphorylation of 5,6-Dichlororibofuranosylbenzimidazole (DRB) by Salivary Gland Cells of Chironomus tentans*

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The kinetics of transport and phosphorylation of the nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and the nature of monophosphate metabolites obtained were studied in salivary gland cells of Chironomus tentans. DRB, labeled by exchange with tritiated water, is transported across the plasma membrane, but only monophosphate derivatives emerge in the ethanol-soluble cell extract. Kinetic analysis of DRB uptake shows a rapid initial transport rate through the plasma membrane and an acquisition of equilibrium state between external and internal substrate concentrations within about 2 min. After that time, it is the phosphorylation of DRB within the cells that constitutes the rate-limiting step in the overall uptake process. The nonlinear dependence of transport step on extracellular DRB concentrations is consistent with the occurrence of saturable reaction capacities and thus a facilitated diffusion mechanism, in accordance with the Michaelis-Menten kinetics. The apparent $K_m$ value for the transport reaction falls in the region of 125 μM, while the apparent $V$ value is about 160 μmol/liter of cell volume x min.

The labeled DRB monophosphate fraction derived from the cells is not uniform, but rather it consists of monophosphate molecules in which the phosphate group is linked at different positions in the sugar moiety. Digestion of labeled DRB metabolites(s) with 3' and 5'-nucleotidases and separation by thin layer electrophoresis in borate buffer indicate the presence of 2',3'- and 5'-DRB monophosphate isomers. No measurable quantities of 3'-DRB monophosphate could be detected. The enzymes involved in phosphorylation of DRB display lower affinities, with $K_m$ values in the range of 250 to 400 μM, and considerably lower capacities, with $V$ values of 3 to 5 μmol/liter of cell volume x min, than the mediator proteins in the transport reaction. The results are discussed in relation to the question whether phosphorylated DRB metabolites may constitute the active form of DRB in the inhibition of the transcription of heterogeneous nuclear RNA producing genes in living cells.

A selective and reversible inhibition of the formation of heterogeneous nuclear RNA (hnRNA) by the purine nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) has been established in both insect (1–3) and mammalian cells (4) (for review, see Ref. 5). While the extent of inhibition in insect cells appeared to be almost complete (2), only about 70% of hnRNA was sensitive in mammalian cells (4, 6). Nevertheless, the labeling of cytoplasmic mRNA was suppressed by more than 95% in mammalian cells as well (6).

A prominent effect of DRB on some early event(s) in the course of transcription has been documented in several reports. Data from previous experiments with salivary gland cells of Chironomus tentans (2, 3, 7) and from HeLa cells (8) indicated a block of transcription at or near the site of initiation. However, a premature termination of nascent hnRNA chains was reported from later studies with adenovirus-infected (9) and uninfected (10) HeLa cells. Another interesting observation was the enhanced interferon production in DRB-treated human diploid fibroblasts (11).

While the biological effect of DRB on eukaryotic cells is now relatively well studied, there is essentially nothing known about its mode of action in living cells. The need for new information is obvious. An attempt in this direction is our effort to elucidate the intracellular fate of this analogue as well as the metabolic events that are interrupted or stimulated when DRB molecules are taken up by living cells. In a recent publication, we demonstrated that radio-labeled DRB does, in fact, enter salivary gland cells of C. tentans (12).

In addition, intracellular DRB is metabolically trapped by conversion to DRB monophosphate, but the phosphorylation reaction does not include the formation of DRB di- or triphosphates.

The present paper provides information on the kinetics of transport and phosphorylation of labeled DRB, and on the nature of DRB monophosphate derivatives formed intracellularly. It will be shown that the kinetics of DRB transport, as well as its metabolic trapping by phosphorylation, obeys the Michaelis-Menten equation. The initial velocity of transport is 50- to 100-fold higher than that of any of the studied phosphorylation reactions. By contrast, the apparent $K_m$ value for the transport step, 125 μM, is significantly lower than the $K_m$ value for phosphorylation of DRB to 5'-monophosphate (250 μM) or to 2'-monophosphate (400 μM).

EXPERIMENTAL PROCEDURES

Materials

[1H]DRB, labeled by exchange with tritiated water without catalyst, was obtained from the Radiochemical Center (Amersham, England). The crude material was subjected to purification by preparative TLC as previously described (12). The specific activity of the purified DRB has been established in both insect (1–3) and mammalian cells (4) (for review, see Ref. 5). While the extent of inhibition in insect cells appeared to be almost complete (2), only about 70% of hnRNA was sensitive in mammalian cells (4, 6). Nevertheless, the labeling of cytoplasmic mRNA was suppressed by more than 95% in mammalian cells as well (6).

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The abbreviations used are: hnRNA, heterogeneous nuclear RNA; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; TLC, thin layer electrophoresis.

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product was about $6 	imes 10^3$ cpm/µg throughout the work. 5-DRB-monophosphate was a generous gift from Dr. D. Sluyter. 3'-Nucleotidase (type III, 3'-ribonucleotide phosphohydrolase, EC 3.1.3.6), 5'-nucleotidase (grade IV, 5'-ribonucleotide phosphohydrolase, EC 3.1.3.5), and various other normal nucleotides used as mobility markers were products of Sigma Chemical Co. (St. Louis, MO, USA). Polygram Cel 300 UV_a cellulose sheets were obtained from Machery-Nagel & Co. (Buren, GFR).

Biological Material, Labeling Conditions, and the Extraction of Nucleotides

Salivary glands were isolated from the fourth instar larvae of the midge C. tentans, and the explanted glands were transferred to 200 µl of modified Cannon's medium (13) supplemented with $^{3}H\text{-DRB}$ and incubated at 18°C for various time periods. After incubation, the glands were rinsed twice during a period of 3 to 5 s in a large excess of ice-cold fresh incubation medium containing unlabeled DRB. They were then extracted three times each in 100 µl of 70% ethanol as previously described (12). The intracellular water space per gland was estimated to be about 0.3 µl.

Enzyme Digestions

3'-Nucleotidase—An appropriate portion of ethanol-soluble cell extract was dissolved in a solution of 10 mM Tris-HCl buffer, pH 7.4, containing 2 mM MgCl$_2$ and 0.01 units of 3'-nucleotidase. The reaction mixture was then incubated for 30 min at 37°C and cooled before electrophoresis.

5'-Nucleotidase—The labeled extract was dissolved in a solution of 50 mM glycine/NaOH buffer, pH 9.1, together with 0.01 unit of 5'-nucleotidase, and the reaction was carried out at 37°C for 30 min.

Electrophoresis and the Measurement of Radioactivity

Thin layer electrophoresis (TLE; on precoated plastic sheets (Polygram Cel 300) was performed with 50 mM borate buffer, pH 9.2. The use of the borate buffer enabled 2'- and 3'-mononucleotides to be separated from their 5' counterpart (14). Electrophoresis in 1% agarose gel was performed with a 20 mM Tris-HCl buffer, pH 8.0 (2). At the end of the electrophoretic runs, the plastic sheets, or gel slabs, were cut and the slices (cutouts) were transferred to Packard scintillation vials, each containing toluene, soluene, and Permablend III. The radioactivity was measured in a Packard (3380) liquid scintillation spectrometer at an efficiency of about 35% and at a background of 10 to 12 cpm.

RESULTS

Kinetics of DRB Transport—The uptake of natural nucleosides by different living cells usually takes place in two consecutive steps: a transport across the plasma membrane by a facilitated diffusion system including carrier proteins, and a metabolic trapping of nucleosides inside the cells by phosphorylation by means of nucleoside kinases. The rate of transfer through the plasma membrane is often estimated by measuring the long term rate of uptake assuming that it is the transport step rather than the phosphorylation which is rate-limiting (15, 16). Recent studies using improved experimental design, including rapid kinetic techniques, indicate, however, that the long term rate of nucleoside uptake reflects the velocity of phosphorylation and not the rate of facilitated transport rate across the plasma membrane (17, 18). Our experimental system and methodology enabled us to treat separately metabolic events like transport and intracellular phosphorylation of DRB. A typical experiment, in which the time course of DRB transport at an external concentration of 60 µM was investigated, is depicted in Fig. 1. Due to rapid initial transport rate and rapid acquisition of equilibrium state between external and internal substrate concentrations, the adoption of incubation periods as short as 20 to 120 s was necessary. As seen in Fig. 1, the linear portion of the transport curve with 60 µM DRB is about 30 s long. After about 2 min of incorporation, the level of intracellular DRB concentration approximately equals the one in the medium (steady state concentration). In this case, it was not feasible to estimate the extent of possible false enhancement of transport rates due to trapping of labeled DRB in the extracellular portion of plasma membrane (zero time values), and it could not be taken into account when the uptake rates were calculated. In view of the good conformity between external (real) and internal (calculated) DRB concentrations at steady state conditions, it appears unlikely, however, that the lack of correction for zero time values would have appreciably influenced the values for the transport rates. Data points used in the illustrations are average values of duplicate experiments. Labeled material which accumulates in the cells after different times, shorter than 5 min, consists essentially of unmetabolized inhibitor (data not shown). This implies that the transport step, rather than the phosphorylation reaction, is rate-limiting during the early phase of DRB uptake.

In order to study the dose-response relation of DRB transport and to establish kinetic parameters like the $K_{m}$ and $V$ values, salivary glands were labeled with $^{3}H\text{-DRB}$ at various concentrations for 30 s (Fig. 2A); total radioactivity in ethanol extracts was then measured. The initial rates of transport, expressed as micromoles of DRB/liter of cell volume x min, are plotted as a function of external DRB concentrations. The estimation of the initial velocities of the transport reaction by using identical periods of incubation (30 s), irrespective of substrate concentrations, relies on the assumption that the length of the linear portion of the transport curve is not appreciably shorter than 30 s. This might be shorter at DRB concentrations that are lower than 60 µM (e.g. at 15 and 30 µM) since, in general, the length of linear portion of the transport curve progressively decreases with decreasing substrate concentrations (18). Fig. 2 shows a nonlinear dependence of reaction velocities on extracellular DRB concentra-
Transport and Phosphorylation of 5,6-Dichlororibofuranosylbenzimidazole

FIG. 2. Uptake of DRB as a function of exogenous DRB concentration (A) and a Lineweaver-Burk plot of the data (B).

Initial transport velocities ($v_0$ in micromoles/liter of cell volume x min) were determined as described in the legend to Fig. 1. Only one incubation period (30 s) was used throughout the experiment. Concentrations of DRB were as follows: 15, 30, 60, 120, and 240 μM (A). The reciprocal of DRB concentrations (1/$v_0$) is plotted as a function of the reciprocal of initial velocities (1/$v_0$), and the Michaelis-Menten parameters for DRB transport were estimated graphically (B). For other data, see under “Experimental Procedures.”

Separation and Identification of Labeled DRB Monophosphate Isomers—The electrophoretic distribution of DRB monophosphate derived from Chironomus salivary gland cells after incubation with [3H]DRB was somewhat broad and indicated the presence of more than one component in the monophosphate peak (12). In an attempt to further analyze the composition and kinetics of appearance of DRB transport were estimated graphically (B). For other data, see under “Experimental Procedures.”

FIG. 3. Electrophoretic analyses of ethanol-soluble cell extract in borate buffer treated and untreated with 5'-nucleotidase. Salivary glands from 10 animals were incubated with [3H]DRB for 90 min at 18°C. The cells were extracted in 100 μl of 70% ethanol and two aliquots of 20 μl were evaporated to dryness. One sample was digested with 5'-nucleotidase, while the other was used as a control (Fig. 3). After addition of suitable reference substances (3'-AMP, 5'-AMP, and 5'-DRB-P), the samples were fractionated simultaneously by TLE in parallel lanes. The electrophoretic pattern of the undigested extract shows the presence of two relatively well resolved nucleotide peaks. The fast moving peak, comprising about 45% of the total nucleotide label, comigrates with unlabeled 5'-DRB-P. The other peak has a slower mobility rate and presumably represents either one, or both, 2'-DRB-P and 3'-DRB-P, as judged by comparison with the migration rate of the markers 5'-AMP, 3'-AMP (or 2'-AMP), and 5'-DRB-P. The identification of the fast moving peak as 5'-DRB-P was further strengthened by its sensitivity to the action of 5'-nucleotidase. As seen in Fig. 3, the enzyme treatment almost completely liquidated labeling of the fast moving component but left the slower migrating material essentially unaltered. Both peaks are eliminated and converted to DRB, however, when the cell extract is digested with alkaline phosphatase (12).

When in another experiment 5'-nucleotidase was replaced by 3'-nucleotidase, the slower moving radioactivity peak was reduced by about 20% (data not shown). But, as the 3'-nucleotidase preparation used was not entirely free from 2'-nucleotidase activity, the reaction sample was supplemented with unlabeled 2'-AMP in excess and the digestion with 3'-nucleotidase was repeated (Fig. 4). After this treatment, the electrophoretic analyses revealed no detectable effect on any of the labeled DRB monophosphate derivatives, indicating the absence of 3'-DRB-P in the cell extract. The activity of 3'-nucleotidase during incubation was checked by measuring its effect on unlabeled 3'-AMP included in the sample solution. The 5'-nucleotide portion of the label in Fig. 4 was estimated.
to be about 30%. Thus, on the basis of co-migration with the 5'-DRB-P reference and selective susceptibility to the action of 5'-nucleotidase, the fast moving peak in Figs. 3 and 4, was identified as 5'-DRB-P. Even though the identification of the slower migrating peak without availability to appropriate reference substance and purified 2'-nucleotidase is more indirect, the experimental data taken together strongly support its identity as 2'-DRB-P. First, the material in the slower moving peak is vulnerable to alkaline phosphatase and the products of the hydrolysis include normal DRB, making the presence of deoxyribonucleotide derivatives unlikely. Second, the order of migration in borate buffer for the adenosine nucleotide 5'-AMP and 2'-AMP and for the DRB nucleotide 5'-DRB-P and the putative 2'-DRB-P is the same. Finally, the slower moving peak is resistant to both 3'- and 5'-nucleotidases. The relative abundance of the DRB monophosphate isomers varies in different experiments. After 90 min of incubation with [3H]DRB, the following ranges could be established: 5'-DRB-P, 30 to 45%, and the putative 2'-DRB-P, 55 to 70%.

**Kinetics of Phosphorylation of DRB**—The time course of DRB phosphorylation in explanted salivary glands after incubation with 60 μM of nucleoside analogue and subsequent separation of DRB phosphates in 1% agarose gel is shown in Fig. 5. The accumulation velocity of the total phosphorylated metabolites reaches a measurable extent only after 5 to 10 min of incorporation. In contrast to the transport step in the overall DRB uptake, which attains a maximum rate within 1 to 2 min and then levels off, the phosphorylation reaction proceeds at a linear rate during a labeling period of 120 min. To study the dose-response relation of DRB trapping via phosphorylation and to establish K_m and V values, salivary glands were labeled with [3H]DRB at various concentrations for 45 min (Fig. 6). The cell extract was then fractionated by TLE in the presence of borate ions to permit quantitation of

![Graph showing time course of DRB phosphorylation](image)

**Fig. 5.** Time course of DRB phosphorylation. In each experiment, 20 salivary glands from 20 animals were explanted and incubated with 60 μM of [3H]DRB essentially as described in the legend to Fig. 1. The incubation periods adopted were between 7.5 and 120 min. After rinse with fresh medium and extraction with 70% ethanol, one-fifth volume of each sample solution was subjected to electrophoresis in 1% agarose gel to separate DRB from its phosphorylated metabolites. The radioactivity in the broad monophosphate peaks was then computed and plotted as a function of time. For other data, see under "Experimental Procedure."

![Graph showing formation of DRB monophosphate isomers](image)

**Fig. 6.** Formation of DRB monophosphate isomers expressed as a function of exogeneous substrate concentration (A) and a Lineweaver-Burk plot of the data (B). Initial phosphorylation velocities (μM, micromoles/liter of cell volume × min) were determined as described in the legends to Figs. 1 and 5. An incubation period of 45 min was used throughout the experiment. The concentrations of DRB were 15, 30, 60, 120, and 240 μM. The monophosphate isomers were separated by TLE in borate buffer and the label in the monophosphate peaks was calculated (A). The reciprocal of DRB concentrations (1/S) is plotted as a function of the reciprocal of initial velocities (1/V). The Michaelis-Menten parameters for phosphorylation were then determined graphically (B). ○—○, 2'-phosphate; ●—●, 5'-phosphate.
2'-DRB-P (slower migrating peak) and 5'-DRB-P constituents. The rates of phosphorylation, expressed as micromoles of DRB phosphorylated/liter of cell volume \( \times \) min, are plotted as a function of external DRB concentrations (Fig. 6A). It is clear from Figs. 1 and 6A that the rate of transport of DRB across the plasma membrane is considerably more rapid than that of the formation of the putative 2'-DRB-P and 5'-DRB-P separately or, taken together, at all investigated exogenous substrate concentrations. However, the ratio of the rate of transport to that of conversion to DRB monophosphate isomers decreases with increasing DRB concentration. The velocity of the formation of 2'-DRB-P derivative remains higher than that of its 5'-monophosphate counterpart even at increased DRB concentrations. The nonlinear dependence of the reaction velocities on extracellular DRB concentrations during phosphorylation is in line with the kinetic features of the transport step and indicates the existence of saturable enzymatic reactions (nucleoside kinase activity) in agreement with the Michaelis-Menten theory.

The experimental data in Fig. 6B, expressed in Lineweaver-Burk plots, show a linear correlation between the reciprocal of DRB concentration in the medium (1/\( S \)) and that of the initial uptake velocity (1/\( u_0 \)). The kinetic parameters (apparent \( K_m \) and \( V \)) for the transport and phosphorylation events are recorded in Table I. The \( K_m \) value for the transport step, about 125 \( \mu M \), is clearly lower than corresponding values for any of the two measured phosphorylation reactions in the course of the formation of the putative 2'-DRB-P (400 \( \mu M \)) or 5'-DRB-P (250 \( \mu M \)). Also, the capacity that transport-promoting proteins possess toward DRB distinctly differs, although in the opposite direction, from that of the phosphorylating nucleoside kinases. The \( V \) value for the transport step (160 \( \mu M \)/liter of cell volume \( \times \) min) is more than 30-fold higher than corresponding \( V \) value for any of the DRB phosphorylating reactions (Table I).

**DISCUSSION**

The experiments described in this communication contribute information on questions concerning the uptake and intracellular fate of the nucleoside analogue DRB. The initial transport rate of DRB molecules across the plasma membrane is rapid. The transport reaction attains, however, the steady state level between the external and internal DRB concentrations within 1 to 2 min. The nonlinear dependence of transport step on extracellular DRB concentrations is in consonant with the Michaelis-Menten kinetics and the existence of a facilitated diffusion mechanism in DRB uptake. The great majority, if not all, of DRB molecules transported into the cells becomes phosphorylated to monophosphate isomers. The rate of phosphorylation reaction is considerably slower than that of the transport step. The accumulation of monophosphate metabolite occurs at a linear rate during at least 120 min of incubation. An interesting aspect of the DRB uptake is its intracellular trapping by phosphorylation to monophosphates only.

The absence of measurable quantities of DRB di- or triphosphates in vitro makes previous suggestions on the active inhibitory form of DRB in intact cells unlikely. It was proposed that DRB, as a purine nucleoside analogue, may exert its inhibitory action at some early stage of the formation of hnRNA by competition with normal nucleoside triphosphates like ATP or GTP (2). In view of the present results, the effect of DRB triphosphates in a cell-free system would have little significance for an in vivo situation.

The conversion of DRB to more than one type of monophosphate raises the question whether any, or both, of the observed monophosphate metabolites are involved in the inhibitory reaction. At present, no definite answer can be given to this question. There are, however, circumstances which render DRB monophosphates unlikely as candidates for the active components in the inhibition of hnRNA synthesis.

The first one is the relatively slow rate of accumulation of DRB-monophosphate metabolites (measurable amounts only after 5 to 10 min of incorporation) in view of the immediacy of the inhibitory effect on synthesis of Balbiani ring RNA. Uniformly labeled growing nascent chains (19) up to 10\(^7\) nucleotide length were abolished within 5 min after addition of 65 \( \mu M \) of DRB which is consistent with an almost immediate effect on hnRNA synthesis. The second circumstance is the rapidity of the reversal of transcription block when DRB is withdrawn from the medium (3), while the phosphorylated derivatives are intracellularly trapped and show a linear rate of accumulation for at least 120 min (Fig. 5). It is, of course, not possible to preclude the possibility that the inhibitory form of DRB is a phosphorylated metabolite which constitutes only a minute fraction of the total DRB phosphates and possesses a high turnover rate and thereby escapes detection. This would also be true of any other nonphosphorylated metabolites, if they exist. It should be mentioned here that no measurable quantities of DRB or its metabolites could be observed associated with any ethanol or perchloric acid-p precipitable cell component (12).

A common feature of nucleoside analogues is their involvement in suppression of various metabolic events in living cells, after phosphorylation inside the cells to nucleoside triphosphate metabolites (20). It is thus often the triphosphate metabolite that interferes with one or several transcription step(s) by competition with one or several endogenous nucleoside triphosphates. The purine nucleoside analogue DRB appears to be different from most of the other established analogues by 1) its inability to undergo phosphorylation to di- and/or triphosphates (12); 2) its inhibitory effect being probably mediated not through the phosphorylated metabolites; and 3) the emergence of two DRB monophosphate isomers, in particular, the formation of the unusual 2'-nucleotide metabolite. Even if there is no phosphorylated DRB derivative which directly affects the transcription process, it is conceivable that the phosphorylation step in itself acts as a trigger for the inhibitory action. Such a condition might be induced by depletion of some phosphorylated factor(s) coupled to the regulation of hnRNA-producing genes. It is, finally, an open possibility that DRB is an allosteric inhibitor implying that unmodified DRB molecules constitute the active inhibitory form.

It is of interest to note that, contrary to our data in Chironomus, no measurable intracellular phosphorylation of DRB was observed in Ehrlich ascites cells (21). Hence, an extension of the present type of study to other cell systems will obviously be necessary before a more general conclusion as to the intracellular fate of DRB can be drawn.

**TABLE I**

<table>
<thead>
<tr>
<th>Type of nucleotide</th>
<th>( K_m ) ( \mu M )</th>
<th>( \mu M/1 ) cell vol ( \times ) min</th>
<th>( V )</th>
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</thead>
<tbody>
<tr>
<td>Free substrate</td>
<td>125</td>
<td>160</td>
<td>1/50</td>
</tr>
<tr>
<td>5'-Monophosphate</td>
<td>250</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2'-Monophosphate</td>
<td>400</td>
<td>5</td>
<td></td>
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\^1 E. Egyházi, A. Ossoinak, M. Holst, and K. Rosendahl, unpublished experiments.
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**REFERENCES**