5,6-DICHLORORIBOFURANOSYLBENZIMIDAZOLE (DRB) IS PHOSPHORYLATED IN SALIVARY GLAND CELLS OF *CHIRONOMUS TENTANS*

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1. Introduction

Since the original use of the nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) in studies on suppression of influenza virus multiplication [1], considerable information dealing with the effects of DRB has been accumulated (reviewed [2,3]). A selective influence of DRB on formation of heterogeneous nuclear RNA (hnRNA) in various cell systems has been documented [4,5], and more detailed studies furnished evidence in support of interference with some early events in transcription of hnRNA-producing genes [6]. Experiments with *Chironomus tentans* salivary gland cells [7,8] and HeLa cells [9] led to the conclusion that DRB inhibits chain initiation, whereas an examination of the effects of DRB on adenovirus transcription in HeLa cells pointed to an inhibition site 400–800 nucleotides downstream from the promotor site [10].

Clarification of the mechanism whereby DRB affects hnRNA synthesis obviously requires information about the intracellular fate of this analogue. Ideally, the effect of DRB should be examined in a cell-free RNA synthesizing system which includes RNA polymerase II. However, attempts to carry out the 'right experiment' have been hampered by lack of information as to the active inhibitory form of DRB.

DRB, as a purine nucleoside analogue, may be metabolized (e.g., phosphorylated), so that the inhibitory form consists of a DRB derivative, and not the nucleoside itself. A logical approach to this problem is to treat cells with radioactive DRB (labeled in the benzimidazole moiety) and to identify possible labeled DRB metabolites and the associated metabolic processes involved. In one such attempt to examine the intracellular fate of DRB [11], difficulties associated with exchange labeling of DRB were circumvented by incubating Ehrlich ascites cells in the presence of unlabeled DRB and [^{32}P]phosphate and subsequently analyzing the acid-soluble nucleotides by two-dimensional chromatography. With DRB monophosphate and triphosphate as reference substances, it was concluded that DRB is not phosphorylated in vivo.

We now describe the results of experiments which demonstrate that [^{3}H]DRB administered to salivary gland cells of *Chironomus tentans* is transported across the cell membrane and is, to an appreciable extent, metabolized to the phosphate. Furthermore, only the monophosphate of DRB, but no di- or tri-phosphates, are detectable in the ethanol-soluble cell extract, and no measurable fraction of [^{3}H]DRB is incorporated into any nucleic acid fraction.

2. Materials and methods

[^{3}H]Uridine (50 Ci/mmol) was a product of the Radiochemical Centre (Amersham). TLC aluminium sheets (precoated with aluminium oxide 150 F254 neutral, type T) were obtained from Merck (Darmstadt) and Polygram Cel 300 UV_{254} cellulose sheets from Machery-Nagel (Buren, FRG). Alkaline phosphatase (calf intestine, grade I, EC 3.1.3.1) and snake venom phosphodiesterase (PDE, EC 3.1.4.1) were products of Boehringer (Mannheim). The various normal nucleotides used as mobility markers were products of Sigma Chem. Co. (St. Louis, MO).

DRB-5′-monophosphate was prepared by phosphorylation of DRB by the procedure in [12]. Since this method occasionally gives a mixture including the 2′(3′)-phosphates [13], the product of phosphorylation was purified on a Dowex ($HCOO^-$) column, and the purified 5′-phosphate of DRB obtained as the free acid in crystalline form. It was hydrolyzed quantitatively to DRB by alkaline phosphatase or 5′-nucleotidase.

[^{3}H]DRB, labelled by exchange with tritiated water without catalyst, was obtained from the Radiochemical Center (Amersham). The crude preparation was subjected to purification by preparative TLC. Its chemical purity was high, as judged by its ultraviolet absorption spectrum, but its radiochemical purity was poor. The product was fractionated on aluminium oxide layers with *n*-butanol/H_2O (6:1), the [^{3}H]DRB band ($R_F \approx 0.6$) localized under a dark ultraviolet lamp and eluted overnight with 1 ml 70% ethanol at 4°C. The eluate was concentrated and rechromatographed, and the [^{3}H]DRB stored in 70% ethanol at 4°C. The purified radioactive product was tested for purity in 3 different separation systems (see section 3). Its specific activity was about 1 Ci/mmol.

2.1. *Biological material and labeling conditions*

Salivary glands were isolated from fourth instar larvae of *Chironomus tentans*, and the explanted glands incubated with [^{3}H]DRB in modified Cannon's medium at 18°C as in [6].

2.2. *Extraction of cells*

Following incubation the cells were rapidly rinsed in ~1 ml fresh medium and then extracted 3 times (for 20 min each) with 100 μl volumes of 70% ethanol. About 90% of the radioactivity was taken up in the first extract. Extraction with aqueous ethanol was employed in place of the usual cold $HClO_4$ to avoid the presence of salts in the extracts following neutralization of the acid. The extraction efficiency by both methods is similar.

2.3. *Separation techniques and measurement of radioactivity*

Three separation methods were employed to fractionate nucleotides:

(1) TLC on aluminium oxide coated aluminium sheets, with *n*-butanol/H_2O (6:1) for development;

(2) Thin-layer electrophoresis on Polygram Cel 300 plastic sheets, using 0.05 M citrate buffer (pH 5.2) at 20 V/cm for ~1.5 h in a Desaga (Heidelberg) thin-layer electrophoresis apparatus.

(3) Electrophoresis in 1% agarose gel performed as in [6].

At the end of the run, the sheets were cut and the strips transferred to Packard scintillation vials. To each vial 25 μl water was added, followed by 10 ml scintillator fluid (toluene and Permablend III, from Packard), and the vials cooled to 4°C before counting in a Packard (3380) liquid scintillation spectrometer. The counting efficiency was ~35% at a background level of ~10 cpm.

3. Results and discussion

3.1. *Radiochemical purity and biological activity of tritium labelled DRB*

Following purification (see above), the product was tested for purity by 3 different separation techniques. Figure 1 exhibits the distribution of purified labeled DRB after thin-layer chromatography on aluminium sheets (fig.1a) and after thin-layer electrophoresis on Polygram plastic sheets (fig.1b). Both the chromato-

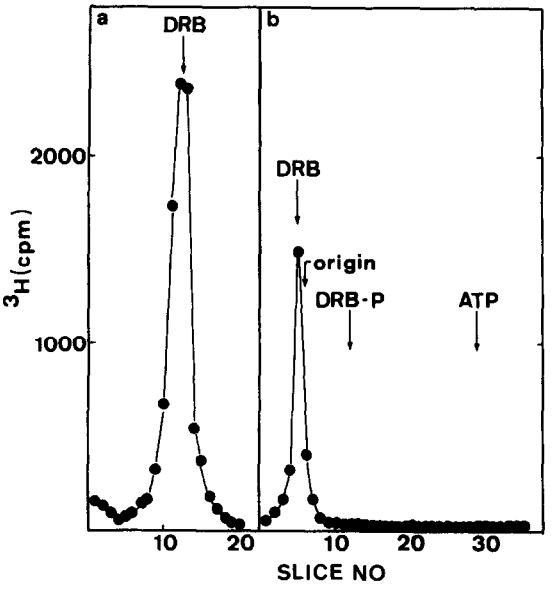

Fig.1. Thin-layer chromatography (a) and thin-layer electrophoresis (b) of [^{3}H]DRB after purification.

graphic and electrophoretic patterns contain one peak comigrating with added unlabeled DRB. Electrophoresis in 1% agarose gel at pH 8 led to similar results (not shown). The radiochemical purity of [^{3}H]DRB (the peak material compared with the total radioactivity) was estimated to be > 95%.

Possible structural changes arising during exchange labeling of DRB, and perhaps undetectable by the foregoing separation criteria, might affect the biological activity of the compound. To check this, salivary glands were incubated with [^{3}H]DRB for 60 min, and the distribution of labeled RNA, using [^{3}H]uridine as an RNA precursor, analyzed by gel electrophoresis. The results reflected the normal expected differential inhibition of hnRNA labelling [4], so that the exchange labeling of DRB did not affect its biological activity.

3.2. *Phosphorylation of [^{3}H]DRB to monophosphate, but not to the diphosphate and/or triphosphate*

Explanted salivary glands were incubated with 65 μM of [^{3}H]DRB for 180 min and a portion of the nucleotide extract was subjected to thin-layer electrophoresis along with appropriate reference markers, viz. DRB-5′-monophosphate, 5′-AMP, ADP, ATP, UTP. The results, depicted in fig.2, demonstrate the presence of two main radiolabeled peaks. One migrates slowly, like nucleosides in general, towards the cathode, and overlapping the DRB marker. The faster-moving fraction migrates towards the anode, and closely mimics the electrophoretic mobility of the reference DRB-5′-monophosphate, although the two peaks do not perfectly coincide. It is also clear from fig.2 that there is no significant peak of labeled material which might be ascribed to higher phosphorylated derivatives, such as the diphosphate or triphosphate. The purine nucleoside analogue DRB consequently appears to be metabolized to monophosphate(s) in the salivary gland cells, but, in contrast to other known adenosine analogues [14], and normal nucleosides, the DRB-monophosphate is not further phosphorylated in our cellular system.

Identification of the labeled monophosphate-like fraction in DRB-treated cells was further supported by experiments in which, following treatment of the cells with [^{3}H]DRB, the ethanol extract was subjected to the action of alkaline phosphatase and snake venom phosphodiesterase, and the resulting product(s)

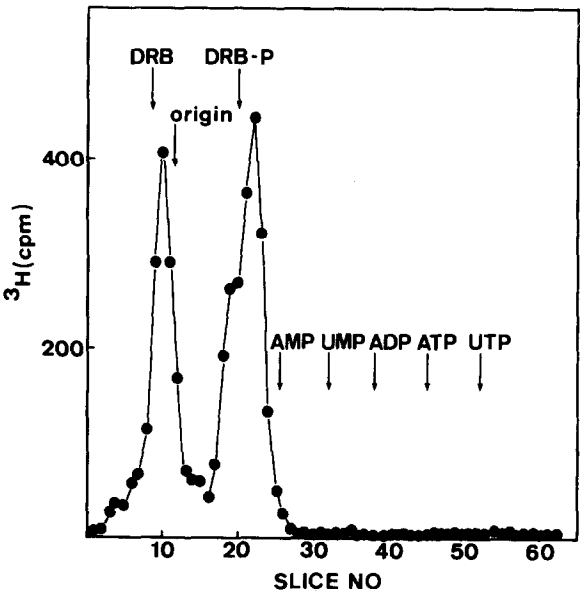

Fig.2. Electrophoretic analysis of labeled ethanol-soluble cell extract. Salivary glands from 10 larvae incubated with [^{3}H]-DRB for 180 min at 18°C. The cells were then extracted with 70% ethanol and the extract subjected to thin-layer electrophoresis on precoated plastic sheets. DRB, DRB monophosphate, AMP, ADP, ATP and UTP were used as reference substances. For other data see section 2.

analyzed in 1% agarose gel. The agarose gel system is preferable to thin-layer systems for analysis of the products of enzyme digestion, because the use of gel as supporting medium obviates the need for evaporation of samples to dryness before analysis. The gel electrophoretic pattern of labeled DRB extract in fig.3 displays the expected two main peaks, along with some additional labeled material distributed as a weaker broad peak between the two major peaks. Treatment of a portion of the same extract with alkaline phosphatase completely liquidates the DRB-monophosphate like peak. Simultaneously, the amount of label in the nucleoside (DRB) peak increases almost quantitatively by the amount present in the monophosphate peak. Hence dephosphorylation releases DRB from the monophosphate fraction. The labeled material located between the two main peaks is also partially susceptible to alkaline phosphatase, but its nature remains unknown and requires further study.

When a portion of the cell extract was treated with

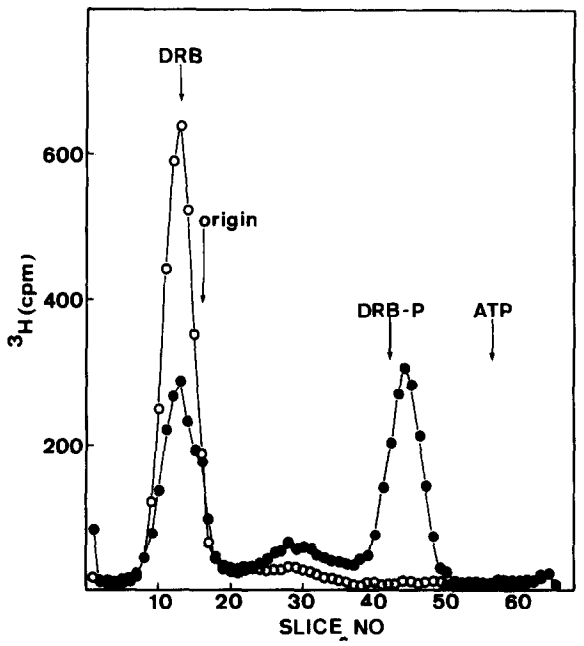

Fig.3. Electrophoretic analyses of labeled ethanol-soluble cell extract with and without treatment with alkaline phosphatase. Salivary glands from 10 animals were incubated with [^{3}H]DRB for 180 min at 18°C. After ethanol extraction, ~10% of the extract was evaporated to dryness. Next, the material was dissolved in 20 μl of a solution containing 0.1 M glycine buffer (pH 10.5), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 2 μg each of DRB, DRB monophosphate and ATP and 0.02 U alkaline phosphatase, and the sample solution was kept at 37°C for 15 min. After cooling, the sample was subjected to electrophoresis in 1% agarose. Another 10% of the ethanol extract was treated without enzyme in an otherwise similar procedure: (-○-) alkaline phosphatase; (-●-) control.

phosphodiesterase in 0.2 M Tris–HCl buffer (pH 9.2) for 15 min at 37°C with ATP as an internal reference, no significant hydrolysis of the labeled DRB phosphate fraction could be detected (result not shown). Hence, on the basis of comigration with the DRB-monophosphate marker, sensitivity to alkaline phosphatase, and resistance to snake venom phosphodiesterase, the product of intracellular metabolism of DRB is identified as a monophosphate.

Our results differ from those in [11], where no measurable intracellular phosphorylation of DRB was found. However, their procedure differed from ours in two important respects:

(i) The use of a different cellular system, Ehrlich ascites cells;

(ii) Incubation in this system of cold DRB with [^{32}P]phosphate, and looking for ^{32}P-labeled DRB.

Nonetheless, it is difficult to reconcile these conflicting results, the more so in view of the relatively good conformity in the mode of inhibitory action of DRB in insect cells and in mammalian cell systems like HeLa and L cells. It will obviously be necessary to establish whether DRB exerts a selective and reversible inhibitory effect on hnRNA formation in intact Ehrlich ascites cells, in a manner resembling that established in other cell types, with a site of action at or close to the initiation site, before one can meaningfully evaluate the significance of the lack of phosphorylation of DRB in such cells [11] as compared to the phosphorylation observed in the present study.

3.3. *DRB is not incorporated into nucleic acids*

Since various nucleoside analogues may be incorporated into nucleic acids following their intracellular phosphorylation [14], and thereby interrupt RNA synthesis, it becomes of interest to establish whether this occurs with DRB. Salivary glands were labeled for 180 min in the presence of 65 μM [^{3}H]DRB, followed by analysis of the ethanol-insoluble radioactivity. Less than 5% of the total label was insoluble and remained bound to the cell structure. Solubilization of the cells in sodium dodecyl sulphate solution, followed by electrophoresis in agarose gel [6] did not reveal any appreciable incorporation of [^{3}H]DRB in any particular nucleic acid fraction (result not shown). This failure of salivary gland cells to incorporate DRB into nucleic acids is consistent with the absence of formation of di- and triphosphates of DRB. Hence the transcription block by DRB does not appear to depend on competitive processes between DRB triphosphates and endogenous nucleoside triphosphates, in agreement with in vitro results [11].

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