ASSOCIATIONS BETWEEN A FLUORESCENT DNA LIGAND, 4',6-DIAMIDINE-2-PHENYLINDOLE·2HCl (DAPI), AND RNA*

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The experiments performed in vitro have shown that DAPI and RNA form insoluble and indigestible complexes. This seems to explain the earlier observed retardation of drug accumulation in the nucleus of a living cell in the presence of RNA.

DAPI, 4′,6-diamidine-2-phenylindole·2HCl, binds to natural DNA as well as to synthetic polynucleotides: poly[d(A-T)·d(A-T)], poly[d(A-U)·d(A-U)], poly[d(I-C)·d(I-C)], to form specific fluorescent complexes. Either intercalation of DAPI between pairs of nucleotides [1 - 3] or its binding to the backbone of double stranded polynucleotides, similar to that displayed by distamycin and netropsin [4], have been proposed as a model of DAPI complexes formation. However, almost no information concerning DAPI/RNA binding is so far available.

DAPI, taken up by a living protozoan cell, paramecium, from a mineral medium, appeared in the nucleus after 5 to 15 min, as detected by fluorescence microscopy. RNA, when added to this medium, delayed by one hour DAPI accumulation in the nucleus [5]. In this case RNA, an indispensable component of paramecium culture medium [6], seemed to protect the cell against the drug. To elucidate the mechanism of the drug accumulation delay in the nucleus, the association between DAPI and RNA in vitro was examined.

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METHODS

Precipitation of RNA in the presence of DAPI. Two series of mixtures were made at different ranges of RNA concentration in 0.1 M acetic acid buffer, pH 5.5. The first series, which contained RNA at a final concentration of 6.45 mM in ribonucleotides, and different quantities of DAPI, was used for enzymatic studies and for some of precipitation studies. The second series, in which RNA and DAPI concentrations were about two to three orders of magnitude lower, was used solely for spectrophotometric studies. In both cases the initial RNA/DAPI molar ratio ($r^i$) ranged from 0.4 to 100. The samples were cooled at 4°C overnight and the precipitate was sedimented at 4000 g for 10 min. Both RNA and DAPI were assayed spectrophotometrically in the supernatants. The amount of RNA and DAPI in the precipitates were calculated by subtracting the quantities of RNA and DAPI in the supernatant from those in the initial mixtures.

Quantitative spectrophotometric measurements of DAPI and RNA. DAPI and RNA were assayed at $\lambda_{340}$ nm and $\lambda_{260}$ nm respectively. Absorbance measured at $\lambda_{260}$ nm corresponded to the sum of RNA and DAPI absorbances ($A_{260}^{R+D}$). The absorbances of RNA ($A_{260}^R$) and DAPI ($A_{260}^D$) were assumed to be additive over the range of RNA/DAPI molar ratio $4 \leq r^i \leq 100$, as supported by phosphorus determination in RNA (for details see Results). Molar absorption coefficient of DAPI at $\lambda_{260}$ nm was 1.52 times lower than that at $\lambda_{340}$ nm. Therefore to calculate RNA and DAPI concentrations, the following formulas were applied:

$$[R] = [A_{260}^{R+D}] - (A_{340}/1.52)]/5166,$$

$$[D] = A_{340}/27000,$$

where 5166 and 27000 are appropriate molar absorption coefficients of RNA and DAPI, respectively. The molarity of RNA solutions was calculated, assuming average nucleotide molecular weight to be 310. Spectrophotometric measurements were performed with use of a Zeiss VSU-2 spectrometer.

Differential spectrophotometry. A series of DAPI solutions, final concentration 25 μM, were titrated with the equimolar RNA solution so as to arrive at $r^i$ of 0.5 to 40. After removal of RNA + DAPI precipitate, absorbance of the supernatant solutions was measured in the range of $\lambda_{220}$ nm and $\lambda_{420}$ nm against the RNA solution in order to obtain the spectrum of DAPI. RNA concentration of the reference solution was set equal to that of the supernatant. The spectra of DAPI and RNA were recorded using a Beckman DU-6 scanning spectrometer.

Digestion of RNA. The supernatants and the precipitates obtained after centrifugation of the RNA/DAPI mixtures were exposed to RNase. The
samples were prepared by mixing of twofold concentrated standard solutions of RNA and DAPI at $r^1 = 40, 20, 13.3$ and $10$; after centrifugation RNA and DAPI concentrations were assayed in the supernatants. Then, the supernatants were diluted with buffer to final RNA concentration of 2 mg/ml i.e. 6.45 mM in nucleotides; the precipitates were washed three times with buffer and suspended in it. The RNA/DAPI molar ratio in examined supernatants ($r^2$) was 65; 29; 20; 16; and in precipitates ($r^3$) ~ 2.5. The reaction mixture, 0.3 ml in volume, contained: 300 µg of RNA, or 300 µg of RNA mixed with different quantities of DAPI and 0.00125 to 0.1 µg of RNase. All the components were dissolved in 0.1 M acetic acid buffer, pH 5.5. After 5 to 20 min of incubation at 30°C, an equal volume of ice cold 0.59 M perchloric acid containing 0.25% (w/v) uranil acetate was added. The samples were left in an ice bath for 30 min and then centrifuged at 2,000 g for 5 min. The clear supernatant was assayed at $\lambda_{340\text{nm}}$ and $\lambda_{260\text{nm}}$. To estimate the degree of RNA digestion by RNase, the quantity of the acid soluble nucleotides released by the enzyme from RNA in the presence of DAPI was compared to that released from DAPI-free RNA.

*Phosphorus assay.* The samples, containing 2 to 20 µg of P per 1 ml, were mineralized in concentrated H$_2$SO$_4$ with 1 - 2 drops of 5.27 M perchloric acid at 240°C. Phosphorus was assayed by means of a modified molibdenum-ejkonogen method [7].

*Materials.* High molecular, type XI, yeast RNA was purchased from Sigma Chemical Co. Pancreas RNase was from Serva Biochemicals. DAPI was synthesized and kindly provided by dr. Jan Kapuściński (Sloan-Kettering Cancer Center, New York).

**RESULTS**

On progressive addition of DAPI solution to a solution of RNA, a yellow sediment appeared. The extent of precipitation can be inferred from Fig. 1, where the spectra of supernatant RNA/DAPI mixtures are compared with the sum of spectra of the initial RNA and DAPI solutions, obtained by simultaneous recording of both solutions in two separate cells. Only 1% of the initial quantity of RNA and 25% of the initial quantity of DAPI could be recovered in the supernatants in which $r^1$ was below 0.1 while $r^2$ was 2.2. Thus, the precipitate contained RNA as well as DAPI.

The results of the quantitative spectrophotometric analysis were obtained on the assumption that the molar absorption coefficients of RNA and DAPI remained unchanged during the formation of a complex between the two compounds. Validity of this assumption was partially proved by the fact that the concentration of RNA calculated from the spectrophotometric data
was almost identical to that calculated from determination of the phosphorus content of RNA (Table 1). This appeared true for a wide range of $r_1$, from 4 to 40. Some differences, observed at $r_1 < 4$ never exceeded 5%. In that particular case the quantity of RNA in the supernatant approached zero so that the differences could originate from an experimental error as well as from slight changes in the absorption coefficient.

Spectral analysis of the RNA/DAPI mixture in the supernatant made at $r_1$ from 0.5 to 40 showed a red shift of both absorption peaks of DAPI (Fig. 2, Table 2). At $r_1 > 3.1$ the shift at $\lambda_{342}$ amounted to 7 - 8 nm. In all the samples in which the red shift was observed $r_1$ was greater than $r_1$ indicating that the changes were accompanied by an increase of [RNA] over [DAPI] in the supernatant (Table 2).
Table 1

RNA concentration in supernatants - comparison of results from spectrophotometric and phosphorus analysis

The results are mean values, ±S.D., from two experiments in duplicates

<table>
<thead>
<tr>
<th>$r^2$</th>
<th>RNA concentration established by:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spectrophotometry</td>
<td>phosphorus analysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(µg/10 ml)</td>
<td>(%)</td>
<td>(µg/10 ml)</td>
</tr>
<tr>
<td>Control (without DAPI)</td>
<td>326.7 ± 18</td>
<td>100</td>
<td>326.7 ± 13</td>
</tr>
<tr>
<td>40</td>
<td>323.0 ± 15</td>
<td>99</td>
<td>334.7 ± 16</td>
</tr>
<tr>
<td>10</td>
<td>306.0 ± 1</td>
<td>94</td>
<td>320.6 ± 24</td>
</tr>
<tr>
<td>4</td>
<td>293.0 ± 17</td>
<td>90</td>
<td>327.1 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>14.0 ± 19</td>
<td>4</td>
<td>33.3 ± 15</td>
</tr>
</tbody>
</table>

Fig. 2. Spectrophotometric differential analysis of DAPI mixed with different quantities of RNA. The uppermost curve represents absorption of pure dye in solution. The other curves from top to bottom (see the maxima on the right) represent the spectra of RNA/DAPI mixtures at $r^2 = 0.6; 3.1; 2.6; 10.3; 40.9$ respectively. The mixtures at $r^2 = 10.3$ and $r^2 = 40.9$ had been diluted 2.5 and 10 times respectively, for absorbance measurements. The measurements were made against an RNA reference solution (see Methods)
Table 2

The bathochromic effect in the spectrum of DAPI in function of RNA/DAPI molar ratio

<table>
<thead>
<tr>
<th>r^1</th>
<th>0.5</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.1</th>
<th>4.0</th>
<th>10.0</th>
<th>40.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>max I (nm)</td>
<td>342</td>
<td>342</td>
<td>342</td>
<td>342</td>
<td>350</td>
<td>350</td>
<td>353</td>
<td>353</td>
</tr>
<tr>
<td>max II (nm)</td>
<td>261</td>
<td>261</td>
<td>261</td>
<td>261</td>
<td>264</td>
<td>264</td>
<td>263</td>
<td>262</td>
</tr>
<tr>
<td>r^5</td>
<td>0.23</td>
<td>0.84</td>
<td>0.32</td>
<td>0.41</td>
<td>4.1</td>
<td>5.6</td>
<td>14.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>

Absorbance at the main absorption peak of DAPI (343 nm) plotted against r^1 was taken as a measure of the precipitation of RNA/DAPI complex. A series of DAPI solutions at a concentration of 250 μM were titrated with the equimolar RNA solution at r^1 from 1.0 to 4.0. The samples, diluted with 0.05 M acetic acid buffer, pH 5.5, to the final DAPI concentration of 25 μM were spun down and the supernatants were assayed spectrophotometrically. The lowest absorbance was observed at r = 2.5, indicating that one DAPI molecule bound to every two or three nucleotides (Fig. 3).

![Absorbance at λ_343](image)
The precipitation of both components, RNA and DAPI, at $r^1$ from 0.5 to 100 is shown in Fig. 4. It is quite clear that the maximal amount of RNA precipitated in the range of $r^1$ from 10 to 4 (see a large difference between white and black circles in Fig. 4). When DAPI was "in excess", at $r^1 < 2$, 97 to 100% of RNA precipitated. However, when RNA was "in excess", at $r^1 > 3$, not more than 30 to 50% of DAPI sedimented in the form of an insoluble complex with RNA.

![Graph showing the precipitation of RNA/DAPI complexes at constant initial RNA concentration and various $r^1$.](image)

Fig. 4. The precipitation of RNA/DAPI complexes at constant initial RNA concentration and various $r^1$. ●, initial RNA concentration; ■, initial DAPI concentration; ○, RNA and □, DAPI concentration in supernatant

When the carefully rinsed precipitate was resuspended in the initial volume of fresh acetic acid buffer, pH 5.5, about 5% of the complex was eluted during 24 h of extraction. The RNA/DAPI ratio in the redissolved complex was 3.0, i.e. very close to that found in the precipitate. Spectral analysis of the dissolved complex also showed a red shift of the main peak of DAPI to $\lambda_{347}$ (Fig. 5).

In order to determine whether the RNA bound in the insoluble complex can be digested by RNase, the suspension of the precipitate was exposed
Fig. 5. The spectrum of RNA/DAPI mixture in the supernatant (upper curve) and the spectrum of RNA/DAPI complex partially eluted from the precipitate, at pH 5.5 for 24 h (lower curve). The initial concentrations of RNA and DAPI: 0.052 and 0.026 mM, respectively, 

$$r^1 = 2.0; \text{ pH } 5.5$$

to a high concentration of the enzyme for 20 min. Contrary to the control, in which pure soluble RNA was used, the enzyme released no detectable quantity of nucleotides from the precipitate (Fig. 6).

However, RNA remaining in the supernatant was fully accessible to the enzyme at low DAPI concentrations, $r^1 > 30$. Yet, at $r^1 = 15$, over 30% of RNA was not digested by RNase. For $r^1 < 10$ $r^1$ fell down dramatically (Table 2) which did not permit to attain a suitable substrate concentration in the supernatant, and made further analysis of RNA digestion impossible. The results expressed as functions of time and of RNase concentration are shown in Fig. 6.

**DISCUSSION**

Association between DAPI and RNA molecules has been considered so far as weak and nonspecific [1, 8, 9]. RNA/DAPI complexes did not exhibit fluorescence, unlike the complexes formed by DAPI with double stranded
nucleic acids [1 - 3]. The results presented in this work demonstrate that RNA and DAPI do form a complex in solution which precipitates in the pH range of 5.5 to 8.5. In the precipitated complex one DAPI molecule is bound per 2 - 3 nucleotide residues, irrespective of RNA/DAPI molar ratio in the initial solution (Figs. 3, 4). Similar precipitation of single stranded nucleic acids in the presence of other DNA ligands, acridines, was described by Kapuściński & Darżynkiewicz, [10, 11, 12].

The RNA and DAPI molecules remaining in the supernatant also form complexes as it can be inferred from the presence of the bathochromic effect in the spectrum of DAPI. Comparison of absorption spectra in Fig. 2 and Fig. 5 permits to conclude that the spectrum of redissolved RNA/DAPI complex still exhibits a bathochromic effect indicating that the same stable species is brought about to the solution. No changes in the DAPI spectrum could be observed when precipitation of the complex was the most intense. These changes, most probably, were an apparent effect of the increasing concentration of RNA over that of DAPI in the supernatant (Table 2).

Still, it is difficult to establish whether the red shift in the DAPI spectrum is accompanied by a hypochromic effect, as it was suggested by Chandra & Mildner for DNA/DAPI complexes [9, 13]. If it does, it must be weak and obscured by precipitation, which is mainly responsible for the lowering of absorbance at the two maxima in the RNA/DAPI spectrum. Preliminary experiments with dsDNA showed evidently precipitation of
DNA/DAPI complexes, at r < 4, especially when highly concentrated solutions were mixed. In spite of significant differences between DNA/DAPI and RNA/DAPI complexes we may still suppose that the hypochromic effect in the DNA spectrum, presented by Chandra & Mildner, was at least partially caused by precipitation.

A progressive decrease in RNA accessibility to RNase observed when the number of nucleotides per one DAPI molecule in the examined solution was less than 30 is probably correlated with the beginning of RNA condensation which precedes precipitation. At r^2 < 15, RNA concentration in supernatant quickly decreases and the precipitated RNA becomes completely inaccessible to the enzyme.

In the light of the presented results we may try to explain the delayed accumulation of DAPI in the cell nucleus in the presence of RNA in the extracellular medium by the following considerations. *Paramecium*, like other representatives of ciliate group of protozoa, takes up the food collected at the bottom of its oral cavity. It is just there that a specifically built and constantly modified membrane produces digestive vacuoles, or endosomes [14]. A newly formed digestive vacuole may intensely concentrate DAPI, a positively charged drug, within itself (in preparation). Similar concentration of other cationic dyes was described by Grębecki & Kuźnicki [15]. RNA, however, like other polyanions, is taken up continuously in the fluid phase endocytosis [16]. This would indicate that, as the drug concentration in the newly formed vacuole grows, the precipitation of the insoluble and indigestible RNA/DAPI complexes continues. In this way DAPI molecules are retained in the complex that fills the vacuole circulating in the cytoplasm till, after some time, it is egested out of the cell. Only a very small part of DAPI can dissociate from the complex, when the vacuole released into the cytoplasm is considerably acidified, down to pH 3, in the process of digestion [17], and only then it can be transported to the nucleus. Thus, the presence of RNA in the cell medium can effectively protect the cell against the penetration of that destructive cationic drug, which may interfere with the metabolism of nuclear chromatin.

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