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A strong ethidium binding site in the acceptor stem of most or all transfer RNAs.

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ABSTRACT

E. coli unfractionated tRNA and $tRNA^{phe}$ both contain a single strong ethidium binding site. Singlet-singlet energy transfer has been used to measure the distance between this site and dansyl hydrazine covalently attached to the 3' end of the tRNAs. The distance obtained is between 33 and 40 Å for both samples. This is completely consistent with results from earlier NMR studies which placed the single, strong ethidium binding site of yeast $tRNA^{phe}$ between base pairs 6 and 7 on the aminoacyl stem. From the known tertiary structure of $tRNA^{phe}$ it is possible to rationalize the unusual affinity of this site and its likely existence in all tRNAs.

INTRODUCTION

The trypanocidal drug, ethidium bromide (EB), has become a standard probe for analyzing nucleic acid structure (1). For DNA structural studies intercalative binding of ethidium bromide was developed by Vinograd and his collaborators into a diagnostic test for superhelical winding (2). Although it is well known that ethidium bromide also binds to double stranded RNAs (1), only a few studies of RNA conformation have utilized this probe. (3-8) Most of these studies have involved tRNA (3-6); other studies have been performed with the ribosomal RNAs, 5s and 16s (7 and 8).

Previous work has shown that there is a single, strong ethidium binding site on yeast $tRNA^{phe}$ (4,5) and unfractionated tRNA (6). The existence of this site on unfractionated tRNA ($tRNA_{unf}$) would seem to imply a single site on all tRNAs. The questions examined in this paper are whether this site is at the same location in all tRNAs and whether its existence can be rationalized in terms of the known tertiary structure of yeast $tRNA^{phe}$. Looking at cloverleaf models of tRNA secondary structure, the likely

regions for intercalation are the central double stranded regions. However, in the tertiary structure of yeast $tRNA^{phe}$ only the aminoacyl stem and the anti-codon stem are not involved tertiary H bonds (9,10). Thus these appear to be the most probable intercalation sites. NMR studies of yeast $tRNA^{phe}$ have located the ethidium bromide binding site between base pairs 6 and 7 on the aminoacyl stem (4). One would like to know if the location of this site is determined by sequence or structure. The technique which we have applied is singlet-singlet energy transfer between a fluorescent dye attached to the 3' end of tRNA and bound ethidium bromide. The distance between ethidium intercalated between base pairs 6 and 7 and a dye attached to the 3' end, as it exists in yeast $tRNA^{phe}$ is about 33 to 35 Å. This is a distance accessible to energy transfer measurements.

The studies described in this report will also give some insight about the structure of the 3' end of tRNA. X-ray diffraction studies cannot definitely place the 3' end (9). Recent oligonucleotide binding studies (11) have shown that the 3' end is much less available for binding oligonucleotides in aminoacyl $tRNA^{phe}$, implying some kind of structural constraints at this region. Therefore, any information about the structure or environment of the 3' end will be important in understanding tRNA function.

MATERIALS AND METHODS

1. Preparation of dansyl hydrazine tRNA.

E. coli B $tRNA_{unf}$ was purchased from Plenum. *E. coli* $tRNA^{phe}$ was a gift from Dr. A.D. Kelmers at Oak Ridge. This $tRNA^{phe}$ was found to be 50% chargeable with phenylalanine. The sample gave only one band on gel electrophoresis (10% acrylamide, pH8.3) before and after labeling with dansyl hydrazine.

Samples of tRNA were periodate oxidized under the following conditions (12). Lyophilized tRNA was dissolved in 1 to 10 ml. of 0.1 M sodium acetate, pH 5.0 to make solutions in a concentration range of 10^{-3} to 10^{-4} M. Sodium periodate was added to give a final concentration of 0.05 M and the mixture was incubated for 30 min. at 37° C. The reaction was terminated by the addition of 1 ml. of 2 M potassium chloride. The KIO_4 precipitate was removed by centrifugation at 3000 g at 4° C for 10 min. The

resulting supernatant was dialyzed against multiple changes of 0.1 M sodium acetate, pH 5.0 at 4° C overnight.

The dansyl absorption is so masked by the massive nucleic acid absorption that there is only a small shoulder on the spectrum. That plus the relatively low intensity of the dye and absorption changes with solvent of the free dye, made the use of a radioactively labeled dye an advantage in characterizing the amount of dye bound. Tritium labeled dansyl hydrazine (specific activity 10 cpm/pmole) was prepared and used in these studies.

The oxidized tRNA was incubated with a 25 fold molar excess of ^{3}H dansyl hydrazine (DH) for 1 hour in the dark at 37° C. Parallel incubations of dye plus non-oxidized tRNA were utilized as controls. The free dye was removed by ethanol precipitations of the tRNA until no more free dye was found in the supernatant. For the sample as described above small quantities of dye continued to wash off. This continual loss of the dye was correlated with an instability of the bond connecting the dye to the tRNA (13). The labeling procedure was then modified to include a reduction of the bond in question. In order to keep the pH low during reduction, sodium cyanoborohydride was chosen as the reducing agent. Sodium cyanoborohydride (Alfa) was added to a final concentration of 1.3×10^{-4} M (a molar excess of at least 10 fold) and allowed to react for 30 min. to an hour at 37° C in the dark. The dye labeled tRNA was precipitated with ethanol at least 3 times and the final precipitate was dissolved in the binding buffer (see below), dialyzed overnight, and then stored frozen. This procedure has been done on at least 3 samples of tRNA; the degree of labeling has been comparable in each case with the total percent of labeling varying by about 10%. With $tRNA^{phe}$ various samples contained 0.4 to 0.6 dyes per tRNA. The samples used in the spectral studies were kept frozen in small aliquots in the binding buffer and defrosted immediately prior to use. The dye to tRNA ratio has routinely been checked and has remained constant.

2. Spectroscopic studies.

Fluorescence experiments were performed at 10° C in a Schoeffel RRS 1000 fluorimeter interfaced to a Tektronix E 31 computer and plotter. Lifetimes were measured on a single photon counting

apparatus as described previously (14). All tRNA spectra were measured on samples in binding buffer containing 20 mM Tris, 375 mM sodium chloride, 8 mM magnesium chloride and 400 mM ammonium sulfate, pH 7.5. The buffer and temperature are the same as were used in the NMR studies (4) to facilitate comparison of results. A major advantage of this high salt buffer is that it avoids nonspecific interactions between ethidium bromide and tRNA.

The concentrations of the solutions were estimated spectrophotometrically using the following extinction coefficients; $tRNA_{unf}$ $\varepsilon_{260} = 5.2 \times 10^5$, $tRNA^{phe}$ $\varepsilon_{260} = 5.4 \times 10^5$, and ethidium bromide $\varepsilon_{480} = 5.3 \times 10^3$ (15). All the solutions used for fluorescent studies were always checked at the exciting wavelength to be sure that concentrations were low enough to avoid any inner filter effects.

The EB binding was determined fluorimetrically, using the following relation;

$$F_{meas} = xF_b + (1-x)F_f \qquad (1)$$

where x refers to the mole fraction, F_b denotes the intrinsic fluorescence of bound EB, and F_f the fluorescence of free EB. F_f was evaluated by parallel titrations of EB in buffer. F_b was evaluated from the end points of the titrations. Two titration procedures were used: constant tRNA and varying EB or constant EB with the amount of tRNA varying. The constant EB titrations were done either by having a solution of EB with which the tRNA was diluted or by adding very small amounts of tRNA to a solution of EB. When necessary the concentrations were corrected for dilution effects. Once the amount of EB bound was determined, the data was calculated as the number of bound dye molecules per tRNA, $\bar{\nu}$, and that fraction divided by the concentration of free dye, c. Then the data was plotted according to the Scatchard relation:

$$\bar{\nu}/c = Kn - K\bar{\nu} \qquad (2)$$

A plot of $\bar{\nu}/c$ versus $\bar{\nu}$ gives a slope of -K (the association constant) and an intercept of n (the number of molecules of dye bound per tRNA).

Fluorescence polarization was measured on the Schoeffel

instrument with polarizers in the excitation and emission beams. The correction factor, G, was calculated from the intensities obtained with the excitation polarizer in the horizontal position and the emission polarizer in the horizontal (I_{HH}) and vertical (I_{HV}) positions. The data was expressed as either polarization, p, or anisotropy, r, according to the definitions:

$$p = \frac{I_{VV} - I_{VH}(G)}{I_{VV} + I_{VH}(G)} \quad \text{and} \quad r = \frac{I_{VV} - I_{VH}(G)}{I_{VV} + 2I_{VH}(G)} \qquad (3)$$

There is essentially no polarized scattered light in dilute solutions of tRNA so light scattering corrections were unnecessary for these samples.

Energy transfer efficiencies were determined directly from spectral data, as described in the Results. The value for R_0, the characteristic transfer distance, was calculated according to the relation:

$$R_0^6 = 8.81 \times 10^{-5} \quad \frac{\kappa^2 Q_D}{n^4} J \quad \text{Å}^6 \qquad (4)$$

where R_0 is the distance at which transfer is 50% efficient, Q_D is the quantum yield of the donor, n is the refractive index and J is the overlap integral between the donor and the acceptor. J is defined in terms of the emission spectrum of the donor (F') and the extinction coefficient of the acceptor (ε_A) as:

$$J = \int F'(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \; / \int F'(\lambda)\, d\lambda \quad \text{cm}^{-1}\text{M}^{-1}\text{nm}^4 \qquad (5)$$

κ^2 in equation 4 is a measure of the relative orientation of the two dyes.

RESULTS

Two tRNAs were chosen for this study, $tRNA_{unf}$ and $tRNA^{phe}$ from E. coli. Separate samples were periodate oxidized and reacted with dansyl hydrazine. After reduction with cyanoborohydride the samples were quite stable (no detectible changes within 5 months when stored frozen). The $tRNA_{unf}$ always showed a lower dye/tRNA ratio than the specific tRNA did. This is probably because it contains some aminoacyl tRNAs contaminating the sample and some of the tRNAs may lack an intact 3' end. We never could get higher than about 60% labeling with the $tRNA^{phe}$,

which could represent some damage to the original sample. It is interesting that this tRNA is only 50% chargeable, which could reflect 3' damage. Fluorescence parameters measured for the dansyl-tRNAs are summarized in Table I. For both samples the corrected excitation maximum was 308 nm, a considerable blue shift in exciting wavelength and the hypochromicity in the absorption may be the result of stacking of the planar dye with the bases at the 3' end.

Table I. Fluorescence parameters of dansyl labeled tRNAs.

	DH[a]	DH $tRNA^{phe}$	DH $tRNA_{unf}$
dye/tRNA	--	.55	.20
ε_{322} (x 10^{-3})	4.6	4.2	4.2
λ,ex, nm	328	308	308
λ,em, nm	500	505	505
Q[b]	--	.20	.21
τ, nsec	14	13	13

a. In 10 mM Tris, 20 mM NaCl, 10 mM $MgCl_2$, pH 7.2. The tRNA samples are in binding buffer.
b. The quantum yields were determined relative to quinine sulfate in 0.1 N H_2SO_4 (Q= 0.7) (16).

Ethidium bromide binding measurements were first performed on unlabeled tRNA so as not to waste the dye labeled samples. The fluorescence titrations were carried out on tRNA and oxidized tRNA to see if oxidation influenced the binding; it does not (data not shown). The ethidium fluorescence is enhanced about 25 times upon binding to the tRNA and the signal blue shifts from 612 to 600 nm. This is comparable to the fluorescence changes with DNA upon binding EB so intercalative binding seems likely. After the magnitude of the binding constant was found for the unreacted tRNA, the titrations were performed with the labeled samples. Figure 1 shows the Scatchard plots obtained with the labeled and unlabeled samples. For all three samples there is only one binding site per tRNA. The association constants were 2.5 x 10^6 M^{-1}, 3.0 x 10^6 M^{-1}, and 3.3 x 10^6 M^{-1} for DH $tRNA^{phe}$, DH $tRNA_{unf}$, and $tRNA_{unf}$ respectively. These binding constants are comparable to DNA in low salt but about 10 times higher than those for DNA

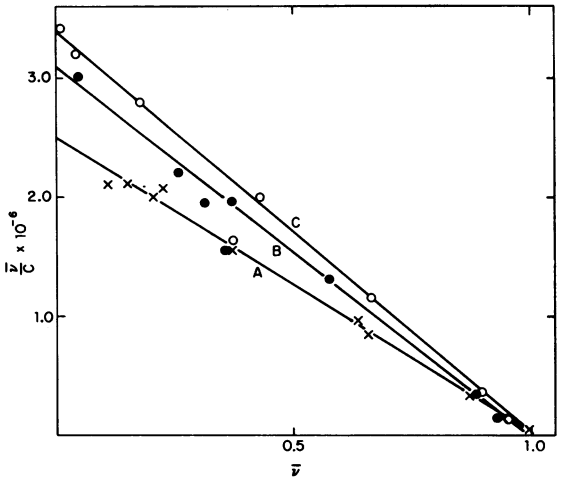

Figure 1. Scatchard plot of the binding of ethidium bromide to DH $tRNA^{phe}$ (A), DH $tRNA_{unf}$ (B), and $tRNA_{unf}$ (C).

in similar salt conditions to those used here.

To measure energy transfer and compute distances the following sequence of steps is usually required.

1. Choose the dyes such that there is a good overlap between the donor emission and the acceptor excitation spectra.
2. Calculate R_0 using κ^2 of 2/3 to see if the spectral overlap of the two dyes gives a reasonable distance range.
3. Insure that a reasonable stoichiometry exists between the two dyes; such that energy transfer occurs and is sensitive to the stoichiometry.
4. Estimate relative orientation of the transition dipoles of the two dyes from polarized fluorescence measurements.
5. Calculate the distance range based upon the range of κ^2 obtained from the polarization data.

The following section illustrates these steps.

The two dyes used here were chosen to satisfy the overlap requirement. The dansyl hydrazine emission is a broad peak centered at 505 nm and the ethidium bromide absorption or excitation is in the same region. Figure 2 shows how good the overlap actually is for these two dyes. The overlap integral in equation 5 can now be calculated.

$R_0(2/3)$ is calculated, the R_0 obtained using κ^2 equal to 2/3

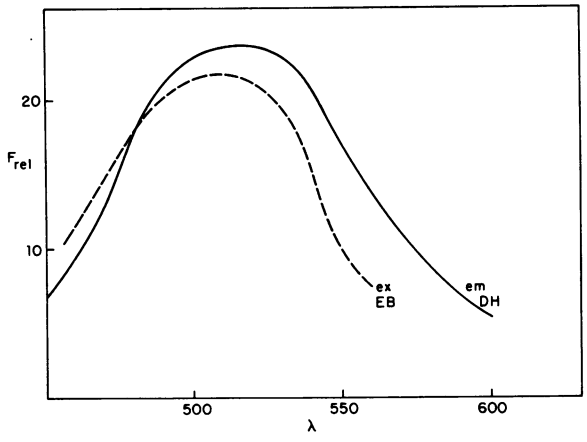

Figure 2. The overlap of the emission spectrum of the donor (em DH) and the excitation spectrum of the acceptor (ex EB).

(see equation 4). This value was calculated for dansyl hydrazine and ethidium bromide to be around 28 Å. Thus these two dyes are a good pair for energy transfer. It is important to realize that 2/3 is only an average value for κ^2 and this step is only an approximation to see if the desired distance measurements are feasible. The sensitivity of energy transfer measurements is optimized when the distance is equal to R_0. Thus the DH-EB dye pair is an excellent choice for the distance of 30 to 40 Å expected from the yeast $tRNA^{phe}$ tertiary structure.

The stoichiometry of binding is known and controllable by varying the total EB concentration and using the EB binding constants determined earlier. Energy transfer can be detected if the donor emission is quenched upon binding the acceptor or the acceptor fluorescence is enhanced by the presence of donor. Since not all of the tRNAs present in the solution possess donor, we have chosen to measure donor quenching. This means that only that fraction of the tRNA containing donor is actually observed. Donor quenching must be proportional to the fraction of tRNAs containing ethidium bromide.

Some raw energy transfer results are shown in Table II. As expected the presence of EB leads to a decrease in the dansyl fluorescence. Quenching of dansyl increases progressively with the amount of bound ethidium. The efficiency of transfer can be calculated as follows:

$$E = (1 - F_{DA}/F_D)\ 1/\bar{\nu} \qquad (6)$$

Table II. Dansyl hydrazine fluorescence at 505 nm as a function of the amount of ethidium bromide bound.

DH $tRNA^{phe}$: $\bar{\nu}$	$\frac{F_{DA}}{F_D}$	DH $tRNA_{unf}$: $\bar{\nu}$	$\frac{F_{DA}}{F_D}$
.94	.79	.95	.90
.87	.93	.84	.91
.80	.94	.80	.95
.50	.96	.45	.96

$\bar{\nu}$ is the fraction of tRNA containing bound EB; F_{DA} and F_D are the fluorescence intensities of DH tRNA in the presence and absence of EB.

where F_{DA} and F_D are the intensities of the donor fluorescence in the presence and absence of acceptor, respectively.

To compute distances it is necessary to have an estimate of the relative orientation of the two dyes (κ^2). Dale and Eisinger (17) have suggested a method for setting limits on the value of κ^2 using fluorescence polarization data. We have applied this method to our data. In order to use the method we need to know the limiting anisotropy of our samples. Solutions of DH $tRNA^{phe}$, DH $tRNA_{unf}$ and $tRNA_{unf}$ plus EB were prepared with either glycerol or sucrose dissolved in the binding buffer. The polarization was then measured as a function of viscosity of the solution and expressed as a Perrin plot. A value for the rotational relaxation time of the tRNA can be calculated from the slope of the plot and the limiting anisotropy can be calculated from the intercept. (see Figure 3) Table III reports the numerical values for these parameters.

The rotational relaxation times of both dansyl and ethidium on the tRNA are close to previous estimates for EB bound to yeast $tRNA^{phe}$ (5). These results are consistent with the times expected for rotation of a rigid molecule with the known size and shape of tRNA. This indicates that complete randomization of the orientation of both DH and EB requires tRNA motion. The limiting anisotropy of rigid ethidium and dansyl can be expected to be around 0.4. The values obtained of 0.07-0.09 for dansyl indicate considerable local motion is possible on a time scale rapid compared with tRNA motion. However, this motion does not

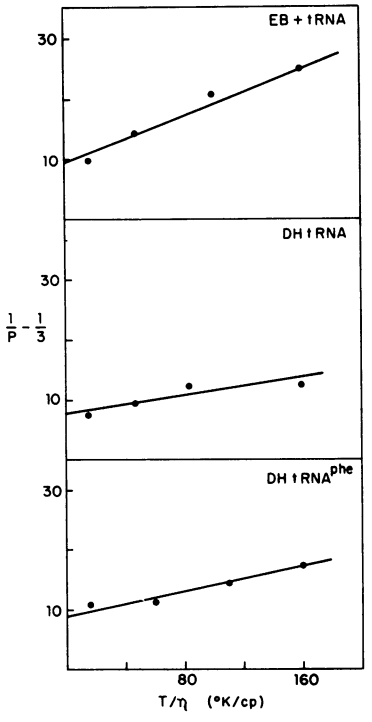

Figure 3. Perrin plots of fluorescence polarization. DH $tRNA^{phe}$ and DH $tRNA_{unf}$ were excited at 308 nm and emission observed at 505 nm. The EB + tRNA was excited at 505 nm and the emission observed at 600 nm.

Table III. Fluorescence polarization data.

	DH $tRNA^{phe}$	DH $tRNA_{unf}$	EB[c] + tRNA
Limiting anisotropy[a]	0.076	0.089	0.072
Rotational relaxation time, nsec[b]	20	21	23
Cone half angle of surface distribution.	39°	38°	40°
Cone half angle of volume distribution.	57°	55°	58°

a. Obtained from the intercept of the Perrin plot, Figure 3.
b. Calculated from the slope of the Perrin plot.
c. Fluorescence of the free EB was negligible so no correction was necessary.

occur over a broad enough range of orientations to obliterate all the original photoselection of a population of oriented molecules. The value of 0.07 obtained for the limiting aniso-

tropy of EB bound to tRNA is comparable to that found for EB intercalated in DNA, i.e. 0.16 (18) or 0.097 (19). These low values must reflect some flexibility in the site of the bound dye.

Using the method of Dale and Eisinger (17) the fast flexible motions of dyes reflected by limiting anisotropies of less than 0.4 are modeled as distributions of transition dipole moments on the surface or volumes of cones. The angles expected for such cones based upon the measured anisotropy are summarized in Table III. From these angles, using the graphical results in reference 17, limiting values for κ^2 can now be obtained. These values in turn allow limits to be calculated for R_0. Once a value for R_0 has been found, the actual distance, R, can be calculated from the efficiency of transfer, E.

$$E = (R_0)^6 / [(R_0)^6 + R^6] \quad (7)$$

These results are shown in Table IV.

The range of values reported for R represents a maximum range. The largest values represent the unlikely case of transition moments oriented nearly along a line between the two dyes. Statistically this is an extremely unlikely orientation. It is definitely ruled out in the case of intercalated EB and stacked DH. From the structure of yeast $tRNA^{phe}$ the transition moments are likely to be arranged as parallel stacks rather than linear. This consideration indicates that the distance is likely to be closer to the lower limits of R given in Table IV. A more realistic estimate of the measured distance is around 35 ± 3 Å. We have assumed that the dansyl residue is stacked on the 3' end.

Table IV. Computation of the distance between ethidium bromide and dansyl hydrazine.

	DH $tRNA^{phe}$		DH $tRNA_{unf}$	
Transfer efficiency[a]	0.18		0.16	
κ^2 Models	Surface	Volume	Surface	Volume
Range of κ^2	.30-1.5	.45-1.5	.40-1.7	.40-1.5
Range of R_0	24-30 Å	25-30 Å	25-33 Å	25-30 Å
Range of R	29-39 Å	32-39 Å	33-44 Å	33-40 Å

a. The transfer efficiency has been corrected for the amount of acceptor bound.

This is consistent with the dansyl spectral and polarization data cited earlier. Using the published models of the structure of yeast $tRNA^{phe}$ as a guide (9,10), the distance between EB intercalated between base paired residues 6 and 7 and DH stacked over the adenosine residue at the 3' end is between 33 and 34 Å. The flexibility of dye attachment at the 3' end would offer a variation of about 2 Å in the distance. Thus our results show clearly that the ethidium binding site is likely to be between residues 6 and 7 in most or all tRNAs.

DISCUSSION

The binding of ethidium bromide to tRNA has been previously described by many authors with varying results. One binding site per tRNA has been found using various salt conditions but usually including magnesium (3,4,5). However, without magnesium multiple sites for binding EB exist (3). We chose the salt and temperature conditions reported by Jones and Kearns (4). These conditions should insure that tRNA will be in the native conformation (20). Native tRNA binds ethidium bromide very strongly as judged by the binding constant of 10^6 M^{-1}. At ionic strengths comparable to the ones used here for tRNA, the DNA-ethidium bromide association constant is in the range of 10^5, an order of magnitude lower than that found for tRNA (1). This 10 fold difference in the binding affinity could reflect intrinsic differences in the binding sites. Sobell and coworkers (21) have determined the structure of several model intercalation complexes (ethidium-dinucleoside monophosphate crystals). They propose that intercalative drug binding is accompanied by a change in the ribose ring puckering from the normal C3' endo to a structure which is C3' endo (3'-5') C2' endo on both strands of the binding site. Certain residues of tRNA (as exemplified by yeast $tRNA^{phe}$) are already in the 2' endo configuration, e.g. 7,9,17,19,21,46,48, and 60 (9). If 2' endo sugar puckers are a necessary requirement for intercalation, tRNA would then naturally possess sites predisposed to intercalative binding. The only region in tRNA which satisfies both requirements of C2' endo ribose puckering and double strandedness is between bases 6 and 7.

The universality of EB binding is suggested by its existence and identical location on both yeast and E. coli $tRNA^{phe}$ and

unfractionated E. coli tRNA. Preliminary results on $tRNA_f^{met}$ indicates that the site is also present on this tRNA. The EB site is another case of a structural feature common to all tRNAs. Most of the common structural features of tRNAs have been shown to be involved in function. The site between bases 6 and 7 could offer a perfect intercalative site for aromatic amino acids. Any of the protein-tRNA interactions common to all tRNAs could use this site to hold the tRNA. It remains to be seen whether EF-Tu, ribosomal A or P sites or aminoacyl tRNA synthetases have discovered this site and exploited it in recognizing tRNA preferentially over other cellular RNAs.

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