BIOLOGICAL ACTIVITY OF ETHIDIUM BROMIDE – TRANSFER RNA COMPLEXES.

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1. Introduction

It was now established that polycyclic drugs such as acridines or ethidium bromide (EB) are able to bind to polynucleotides other than DNA [1–4]. Their inhibitory action could therefore occur at levels other than the transcription of DNA.

The interaction of crude *E. coli* tRNA and EB has been studied by Bittman [5] who suggested that several kinds of tRNA–dye complexes could exist because of the multispecies nature of tRNA.

In a previous communication [6] we showed that proflavine considerably inhibits the loading of leucine on tRNA in a system containing *E. coli* tRNA and activation enzymes whereas there was no such effect on the loading of leucine in a system containing reticulocyte tRNA and activation enzymes. The inhibition by proflavine of the cross-reaction between *E. coli* tRNA and reticulocyte activation enzymes strongly suggested that the formation of a proflavine–tRNA complex could account for the observed effect.

On the other hand, it was also reported [7] that proflavine did not alter at the same levels the maximal loadings of leucine and valine on tRNA in *E. coli* systems, suggesting also some specificity of the dye.

In this paper, we describe the formation of complexes between EB and purified $tRNA_{Leu}$s and $tRNA_{Val}$s from *E. coli*. The latter were found to bind less drug by the presumed intercalation process [8] than unfractionated tRNA and $tRNA_{Leu}$s. Moreover, the extent of formation of valyl-tRNA in a reaction mixture was not affected by EB concentrations which yield respectively 50% and 30% inhibition when the loading on tRNA of leucine and of a mixture of 15 amino acids was performed.

On the other hand, the initial velocity of the loading reaction was very sensitive to the presence of the dye in the loading of both valine and leucine: in the case of valine, the inhibition was non-competitive.

2. Materials and methods.

Crude *E. coli* B tRNA was purchased from Schwarz BioResearch. 50% enriched $tRNA_{Leu}$s and 75% enriched $tRNA_{Val}$s were obtained according to Gillam et al. [9]. *E. coli* 112-12 activation enzymes were AS 3 fractions prepared according to Bergmann et al. [10].

Ethidium bromide was a gift from Dr Woolf of the Boots Pure Drug Co. and was used without further purification. Other chemicals, labelled compounds, reaction mixtures and spectrophotometric measurement of tRNA melting curves were previously described [11, 12]. EB concentrations and spectra were recorded with a Cary 15 spectrophotometer using 1 cm light path cuvettes at 20°. Values of r/c and r were derived according to Peacocke and Skerret [13] by measuring the absorbance at 480 nm of mixtures containing different amounts of tRNA and a constant concentration in EB.

3. Results and discussion

3.1. *Binding equilibria and the importance of tRNA secondary structure in the formation of EB–tRNA complexes.*

Fig. 1 represents Scatchard plots [14] corresponding to the formation of complexes between EB and *E. coli* crude tRNA, purified $tRNA_{Leu}$s and purified

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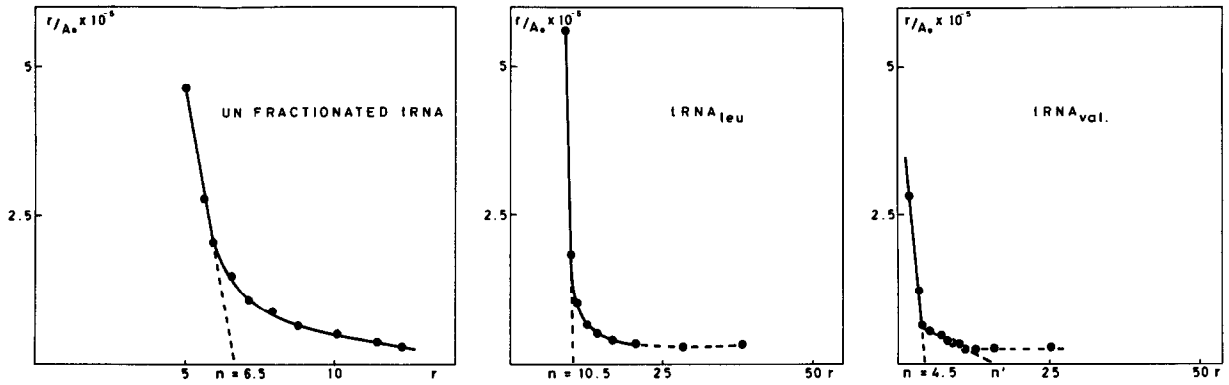

Fig. 1. Scatchard plota for the binding of EB to tRNA. Solutions contained 1.3×10^{-5} M EB in 0.01 M tris-HCl buffer pH 7.4. Values of r and c were used to calculate the value of the association constant (k) using the Scatchard equation $r/c = kn - kr$ where r is the concentration of dye bound per molecule of tRNA, n is the number of binding sites per tRNA, c is the concentration of free dye and k is the association constant of the complex.

$tRNA_{Val}$s. The break in the plot indicates that at least two classes of complexes can be distinguished: complexes corresponding to a high energy of interaction (type I complexes) and complexes characterized by a much lower binding energy (type II complexes). The straight line through the points at low values of r gave an extrapolation to the intercept different in the three classes of tRNAs: the upper limits of type I complexes (n = number of EB molecules attached per tRNA molecule assuming an average molecular weight of 27,500 for the latter) were respectively found to be $n = 6.5$ (crude tRNA), $n = 4.5$ ($tRNA_{Val}$s) and $n = 10.5$ ($tRNA_{Leu}$s). In addition, $tRNA_{Val}$s present also an intermediate value $n' = 16$ between type I and type II complexes.

Table 1 lists these data together with the calculated values for the association constant (k) and for the energy of binding ($\Delta G_0 = -RT \ln k$). The binding energies are of the same order of magnitude as those found for the EB–DNA complexes although the values of n are lower in the case of tRNA [1].

It has already been shown [5] that EB increases the T_m of crude tRNA. Fig. 2a shows that this is also the case for purified tRNAs (here $tRNA_{Leu}$s – see also table 2). Furthermore, fig. 2b clearly shows that the formation of EB–tRNA complexes is strictly dependent upon the secondary structure of the polynucleotides: when temperature is increased, there is a marked release of bound EB as shown by the increase in absorbance at 480 nm. On the other hand, the release of the dye begins only with the melting of tRNA and the phenomenon is completely reversible if the mixture is allowed to cool.

Table 1
Parameters of binding of EB to tRNA in 0.01 M tris-HCl pH 7.4 at 20°.

	k (μM)	n	$-\Delta G_0$ (kcal/mole BE)
Crude *E. coli* tRNA	3	6.5(0.08)	7.6
Purified *E. coli* $tRNA_{Leu}$s	4	10.5(0.13)	7.3
Purified *E. coli* $tRNA_{Val}$s	9 k'=32	4.5(0.056) n'=16(0.19)	6.8 $-\Delta G'_0$=5.9
[a] *E. coli* DNA in 0.04 M tris-HCl pH 7.9	0.6	(0.18)	8.7 [b]
[a] T2 DNA in 0.04 M tris-HCl pH 7.9	0.5	(0.19)	8.9 [b]

[a] From [1]
[b] Calculated from [1]
The values in brackets are calculated per mole of nucleotide.

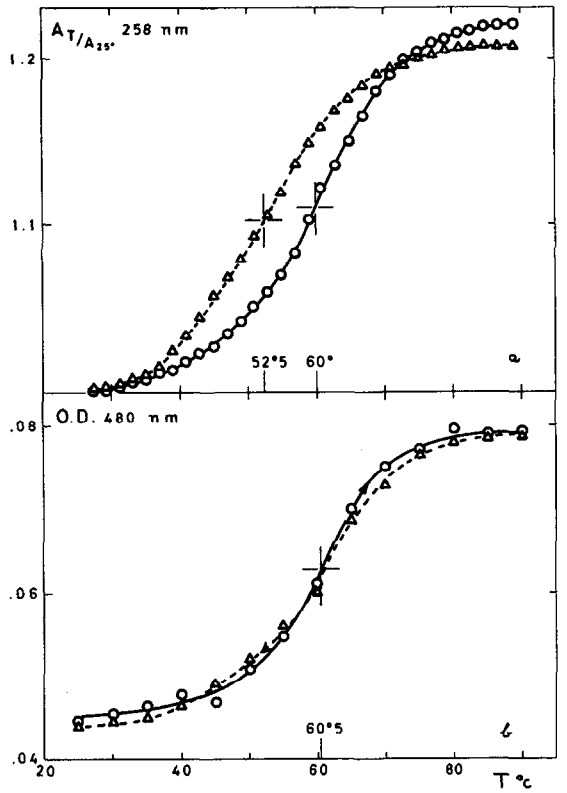

Fig. 2. a) Effect of EB on the melting of purified *E. coli* $tRNA_{Leu}^{s}$ (41 μg/ml) △- - -△ in 0.01 M tris-HCl buffer pH 7.4; ○—○ in the same buffer plus 4 μg/ml EB.

b) Effect of heat on the A_{480} of a mixture of 5 μg/ml EB and 75 μg/ml purified *E. coli* $tRNA_{Leu}^{s}$ in 0.01 M tris-HCl buffer pH 7.4.

3.2. *Effects of EB on the aminoacylation reactions of tRNAs.*

It has been reported that EB inhibits the aminoacylation of rat liver tRNA by phenylalanine, valine and methionine to different extents [15]. In *E. coli* systems, we also found that EB affects the loading of amino acids on tRNA while it is without effect on the leucine-dependent ATP—PP_i exchange. However, the dye has a dual effect on the loading reaction in that it has a more marked action on the rate than on the extent of the reactions. This difference is very striking in the case of the esterification of valine where up to 50 μg/ml of EB has no effect on the extent of the reaction whereas it considerably diminishes its initial velocity. The same phenomenon although less clear-cut is observed for the esterification of leucine. Our observations also indicate that the inhibitory effect of the dye increases the most rapidly at low concentrations in conditions such that EB is bound to tRNA essentially as type I complexes [16]. The observations summarized here above will be detailed elsewhere [17]. It is thus clear that EB affects differently the loading of different tRNAs.

Table 2
Effect of EB on melting temperatures of various tRNAs.

	T_m in 0.01 M tris-HCl buffer pH7.4 (°C)	T_m in the same buffer + 4 μg/ml EB (°C)
Crude *E. coli* tRNA	56	62
$tRNA_{Leu}^{s}$	52	60
$tRNA_{Val}^{s}$	50	60

tRNA concentrations were 40 μg/ml

Table 3 is an attempt to correlate the effects of the dye with the number of EB molecules that are bound to tRNAs as type I complexes: there is apparently a relationship between the effect on the extent of the reaction and the number of EB molecules that given tRNAs can bind according to the type I mechanism; the larger n, the larger the amount of tRNA that has become unable to bind amino acids at the equilibrium of the reaction.

The correlation is however less clear-cut when the effects of the dye on the initial velocity of the aminoacylation reaction are considered; in the three cases studied, the inhibition is very important and less related to n.

As the maximal amount of valine that can be loaded on a limiting quantity of tRNA does not vary with EB concentrations up to 50 μg/ml, this system is particularly convenient for the study of the effect of the dye on the initial velocity of the reaction. Fig. 3 represents the Lineweaver-Burk plot for the inhibition by EB of the loading of valine on tRNA: this inhibition is of the non-competitive type.

Our results present additional evidence that type I complexes between EB and tRNA might be similar

Table 3
Correlation between the effects of EB on the aminoacylation reactions of tRNAs *and the amounts of dye that these tRNAs bind as type I complexes.*

tRNA	*n*	"Plateau" of the reaction in % of the control without EB	Reaction rate in % of the control without EB
$tRNA_{Val}^{s}$	4.5	95	50
Crude tRNA	6.5	70 [a]	33 [a]
$tRNA_{Leu}^{s}$	10.5	48	30

The EB/tRNA ratio in the reaction mixtures is 1.1×10^{-4} / 7.2×10^{-6}

* These values represent the average of 15 amino acids assayed : Ala, Val, Ser, Glu, Arg, Ile, Leu, Thr, Lys, Asp, Gly, His, Phe, Tyr, and Try.

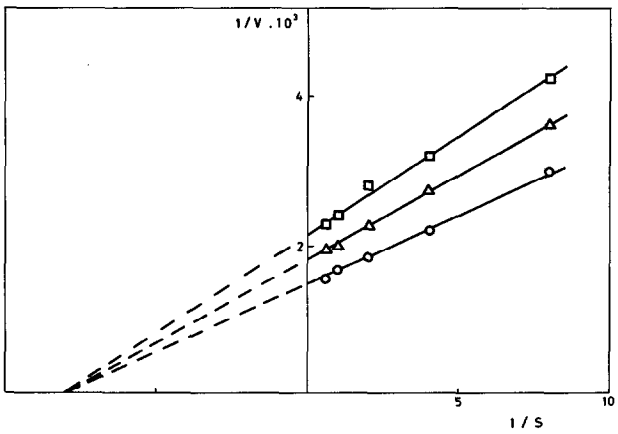

Fig. 3. Effect of EB on the initial velocity of the loading of valine on tRNA. Lineweaver-Burk plot. o—o no EB; Δ—Δ EB 10 μg/ml; □—□ EB 20 μg/ml; S is in mg/ml of tRNA.

to the intercalation process postulated for DNA [1, 8] since EB–tRNA complexes depend upon the integrity of tRNA secondary structure.

Our data agree qualitatively with those of Bittmann [5] although our values of *n* were lower than his. The origin of this discrepancy might be the difference in tRNAs used: all of our tRNA samples presented hyperchromicities of about 25% when heated at 90° whereas those used by Bittmann [5] showed only 15% hyperchromicity at the same temperature.

The effects of EB on biological activity and structure of tRNA are very similar to those observed with proflavine which were first reported by Wérenne et al. [3] and Grosjean et al. [4]. In addition, we found that different tRNA species can bind EB following a different stoichiometry when type I complexes are considered: a positive correlation can be established between *n* and the maximal amount of aminoacyl-tRNA formed. It should also be pointed out that not only *n*, but also the localization of the bound molecules could be important in the biological effect of the dye.

The differential effect of EB on the extent and the initial velocity of the aminoacylation of tRNAs is not yet fully understood; at least, it is clear that no kinetic studies can be undertaken with tRNA aminoacylation systems where the extent of the reaction is reduced by the presence of EB. We mentioned that this disadvantage is avoided in the loading of valine where it was found that the inhibition was non-competitive instead of a competitive as found by Landez et al. [15] in the case of the aminoacylation of rat liver tRNA by phenylalanine. These authors however showed that the drug also reduced the extent of the reaction, making thus impossible an exact evaluation of the substrate (tRNA) concentration in the inhibited system.

A mechanism of non-competitive inhibition might mean that it is the release of the final product of the reaction – the aminoacyl-tRNA – which is hindered by the binding of the dye to tRNA. This postulated mechanism would agree with the results of Yarus and Berg [18] who found that the limiting step of the aminoacylation of tRNA is the release of the aminoacyl-tRNA from the enzyme–substrate complex. Finally, the correlation between the biological effects of EB and the number of EB molecules bound to tRNA as type I complexes is supported by the recent results of Tao et al. [19] who showed that yeast $tRNA_{Phe}$ binds only one EB molecule per tRNA by the intercalation process, the ability of this tRNA to load phenylalanine being unaffected by the presence of the dye.

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