Quantitation of the Specific Interaction of [14a-^{3}H]Cryptopleurine with 80S and 40S Ribosomal Species from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: The quantitative interaction of [14a-^{3}H]cryptopleurine with both sensitive and resistant ribosome preparations from yeast was studied by ultracentrifugation, and isotope dilution was used to determine the specificity of the interaction. A single binding site with high affinity for [14a-^{3}H]cryptopleurine was found for both 80S ribosomes and 40S ribosomal subunits. In addition multiple binding sites, each with a low affinity for the drug, were detected by using 40S, 60S, 80S, or 70S ribosomal species. These low-affinity sites seem to be nonspecific. The high-affinity binding site on the 40S ribosomal subunit is likely to account for the inhibition of translocation that is promoted by low concentrations of cryptopleurine. In contrast, the multiple binding sites located on the 60S ribosomal subunit, with a low affinity for the drug, could account for the inhibition of peptide bond formation that is observed at high cryptopleurine concentrations. The specific binding of [14a-^{3}H]cryptopleurine to both 80S ribosomes and 40S ribosomal subunits prepared from the resistant yeast strain is slightly reduced relative to values obtained by using corresponding preparations from the sensitive strain. This lower affinity cannot in itself explain the high resistance toward cryptopleurine that is observed both in vivo and in vitro. The resistance can, however, be explained if a conformational change occurs that both impairs the inhibitory action of the bound alkaloid at its specific ribosomal target site and reduces the binding affinity. The effects of a number of translocation or peptidyltransferase inhibitors on the binding of labeled cryptopleurine to different ribosome preparations were also studied, and the results are discussed in the text. Tylophorine, tylocrebrine, tubulosine, and emetine, as well as cryptopleurine, reverse the binding of [14a-^{3}H]cryptopleurine to 80S, 60S, and 40S ribosomal particles and also inhibit peptide bond formation at high drug concentrations. Tylophorine and tylocrebrine bind to both the nonspecific and high-affinity binding sites of cryptopleurine. In contrast, emetine and tubulosine might bind to a different but closely related target site.

Cryptopleurine, a phenanthroquinolizidine alkaloid isolated from *Cryptocaria pleurosperma* (de la Lande, 1948), is a potent inhibitor of eukaryotic protein synthesis in both intact cells and cell-free systems (Donaldson et al., 1968; Haslam et al., 1968). At low concentrations the alkaloid specifically blocks the translocation of peptidyl-tRNA from the ribosomal A site to the ribosomal P site that is dependent on elongation factor EF-2 and GTP (Barbacid et al., 1975; Carrasco et al., 1976; Bucher & Skogerson, 1976). A similar mechanism of action has also been described for the plant alkaloids tylophorine, tylocrebrine, emetine, and tubulosine [see review by Vázquez (1979)]. High concentrations of cryptopleurine are also known to inhibit peptide bond formation on eukaryotic ribosomes (Pestka et al., 1972; Barbacid et al., 1975). Some yeast and CHO cell mutants are cross-resistant to cryptopleurine, emetine, tylocrebrine, tylophorine, and tubulosine but not to other inhibitors of translocation like cycloheximide, pederine, and hygromycin B (Grant et al., 1974; Gupta & Siminovitch, 1977; Sánchez et al., 1977). Resistance is controlled by a single nuclear mutation and is associated with an altered 40S ribosomal subunit (Grant et al., 1974).

The dual action that cryptopleurine exhibits on ribosomal functions could indicate the presence of at least two different types of binding sites on the ribosome. However, over the range of cryptopleurine concentrations which inhibit polyphenylalanine synthesis, Bucher & Skogerson (1976) could not detect any specific binding site on yeast ribosomes as investigated by spectrofluorometric techniques. Instead they found multiple and apparently nonspecific drug binding sites on 40S, 60S, and 80S particles.

The availability of [14a-^{3}H]cryptopleurine with high specific activity has allowed us to carry out a study that was designed to improve our understanding of the interactions of cryptopleurine with different ribosome preparations. We have also studied the binding of cryptopleurine to ribosomes isolated from a cryptopleurine-resistant yeast strain in an attempt to understand the nature of the alteration(s) in the resistant particles. Competition experiments were performed by using tritiated cryptopleurine as a marker to determine if emetine, tubulosine, and the *Tylophora* alkaloids interact with similar ribosomal components. In addition we have studied the effect of these inhibitors on peptide bond formation by using ribosomes isolated from both cryptopleurine-sensitive and cryptopleurine-resistant yeast strains. The results obtained are presented and discussed in order to coordinate the apparently contradictory results obtained by different authors while studying the mode(s) of action of these plant alkaloids.

Materials and Methods

Yeast Strains. Isolation and culture of the haploid wild-type strain of *Saccharomyces cerevisiae* (strain Y166) and its spontaneous mutant CRY6 (resistant to cryptopleurine) have been described elsewhere (Sánchez et al., 1977).

Subcellular Fractions and Assays. Supernatant fractions (containing the soluble components for protein synthesis), wild-type and mutant ribosomes, and their subunits were prepared as described previously (Grant et al., 1974). Purities of 80S ribosomes and 60S and 40S ribosomal subunits were determined by sucrose gradient centrifugation, and their activities were tested in polyphenylalanine synthesis as described previously (Jiménez et al., 1975). One A_{260} unit corresponds to 17.86 pmol of 80S ribosomes, 54.92 pmol of 40S ribosomal subunits, and 26.44 pmol of 60S ribosomal subunits (Petermann, 1964). Peptidylpuromycin formation and the fragment

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reaction assays were carried out following methods previously described (Barbacid et al., 1975; Battaner & Vázquez, 1971).

[14a-^{3}H]Cryptopleurine Binding Assays. [14a-^{3}H]Cryptopleurine (1438 Ci/mol) was prepared and purified as described previously (Dölz et al., 1980). Binding to different ribosome preparations was studied at 0 °C by the ultracentrifugation method (Fernández-Muñoz et al., 1971) unless otherwise indicated. The standard reaction mixtures (2 mL volume) were made up in 5.5-mL polyallomer tubes (Beckman) and contained 50 mM Tris-HCl[1] buffer, pH 7.4, 12.5 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, [14a-^{3}H]cryptopleurine, and either polysomes or ribosomes or 60S or 40S ribosomal subunits as required. Concentrations of labeled ligand and ribosome preparations are given in the legends of the corresponding figures. The ribosome particles were sedimented by centrifugation in an SW 50.1 rotor (Beckman) at 30 000 rpm, for 1.5 h for polysomes and ribosomes and for 3 or 2.5 h for 60S or 40S ribosomal subunits, respectively. The extent of formation of the [14a-^{3}H]cryptopleurine–ribosome particle complex was calculated from the difference in radioactivity of 50-μL aliquots that were taken prior to and after centrifugation. In all cases the radioactivity of the samples was estimated as described previously (Fernández-Muñoz et al., 1971) with a counting efficiency for tritium of 28–35%. Data obtained from quantitative assays are presented as Scatchard plots (Scatchard, 1949).

For an accurate determination of the cryptopleurine binding capacity of 80S, 60S, and 40S particles, we have employed the isotope dilution method (Beato & Feigelson, 1972; Blondeau & Robel, 1975). The binding of [14a-^{3}H]cryptopleurine to the different ribosome preparations as estimated by the ultracentrifugation method was carried out in two different sets of experiments: (a) in the presence of a concentration T_1 of [14a-^{3}H]cryptopleurine and (b) in the presence of a concentration T_2 (ideally identical with T_1) of [14a-^{3}H]cryptopleurine isotopically diluted with a large excess (100–350-fold) of nonradioactive ligand. Under condition a the values of bound and unbound cryptopleurine are represented by B_1 and U_1, respectively. Under condition b the resulting data are represented by B_2 (bound nonspecifically) and U_2, respectively. Since we are mainly concerned with the determination of the number of specific binding site(s) per ribosome particle and the affinity constant(s) derived from the specific binding, we have carried out all the quantitative binding experiments by using a [14a-^{3}H]cryptopleurine concentration range of 5×10^{-9}–5×10^{-7} M. Within these concentrations, the labeled ligand is limiting with respect to the specific binding site.

It has been shown that the calculation to determine the amount of ligand bound specifically (B_s) which uses the equation $B_s = B_1 - B_2$ is incorrect because it leads to an underestimation of real values. Hence we have used the following equations for the calculation of B_s and U as proposed by Blondeau & Robel (1975):

$$B_s = \left(B_1 - \frac{T_1}{T_2}B_2\right)\left(\frac{T_2}{T_2 - B_2}\right)$$

$$U = \left(\frac{U_1}{T_2 - B_2}\right)\left(\frac{T_1}{1 + Kn}\right)$$

where Kn is a correcting factor that corresponds to the product of the affinity constant (K) times the number of binding sites (n) of the nonspecific components. The determination of Kn was based on the principle that, when the specific binding does not contribute to the binding measurement, it is verified that $Kn = B/U$. The technique used to measure Kn requires a concentration of [14a-^{3}H]cryptopleurine such that the specific receptor is completely saturated by nonradioactive ligand and therefore its contribution to the binding measurement is negligible (Richard-Foy et al., 1978). The experimental determination of Kn will be described under Results.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

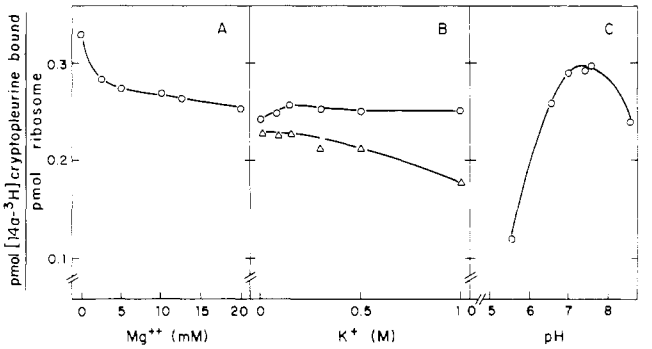

FIGURE 1: Characterization of [14a-^{3}H]cryptopleurine binding to ribosomes from the sensitive strain Y166. In addition to the specific components for each particular experiment the reaction mixtures (2 mL) contained 9×10^{-8} M ribosomes (A–C) or polyribosomes (B), 1 mM dithiothreitol, and 4×10^{-8} M [14a-^{3}H]cryptopleurine. Data were obtained by the ultracentrifugation method described under Materials and Methods. (A) Effect of Mg^{2+} ions: Reaction mixtures contained 50 mM Tris-HCl (pH 7.4), 80 mM KCl, and the indicated amounts of $MgCl_2$. (B) Effect of potassium ions: Reaction mixtures contained 50 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, and the indicated amounts of KCl. (○) Ribosomes; (△) polyribosomes. (C) Effect of pH: Buffers used were either 50 mM Tris-HCl (pH 7.4, 7.55, or 8.55) or Tris–maleate (pH 5.55, 6.55, or 7) containing 12.5 mM $MgCl_2$ and 80 mM KCl.

Results

Optimal Conditions for [14a-^{3}H]Cryptopleurine Binding. No significant changes in [14a-^{3}H]cryptopleurine binding to ribosomes were observed within the physiological range of pH values 7.0–7.6 (Figure 1). The strong inhibition of the binding reaction which was observed at low (5–6) pH values might result from changes in ribosome structure (Petermann, 1964). Neither Mg^{2+} nor K^+ ions have any significant effect on the binding of labeled cryptopleurine to 80S ribosomes. In the case of K^+ this study was extended to polysomes, as it has been shown previously that cryptopleurine does not inhibit the nonenzymic translocation that takes place at high concentrations of K^+ (Blobel & Sabatini, 1971; Jiménez et al., 1977). Since it is shown in Figure 1 that [14a-^{3}H]cryptopleurine binds to polysomes at high K^+ concentrations, the failure of the alkaloid to inhibit nonenzymic translocation might not result from a lack of drug interaction with the particles. Indeed binding of [^{3}H]cryptopleurine takes place at 37 °C at high K^+ concentration, although to a lower extent than at 0 °C (results not shown).

From these experiments we selected ionic conditions identical with those used for polyphenylalanine or polypeptide synthesis with the aim of correlating the binding properties of cryptopleurine with its inhibitory action on these reactions. In this respect, it was found that under these ionic conditions binding of [^{3}H]cryptopleurine to yeast ribosomes was identical at 0 and 30 °C (Dölz, 1981).

Binding of [14a-^{3}H]Cryptopleurine to Different Ribosome Preparations. Scatchard plots of data for equilibrium binding of cryptopleurine to different ribosome preparations from sensitive and resistant yeast strains and *Escherichia coli* are

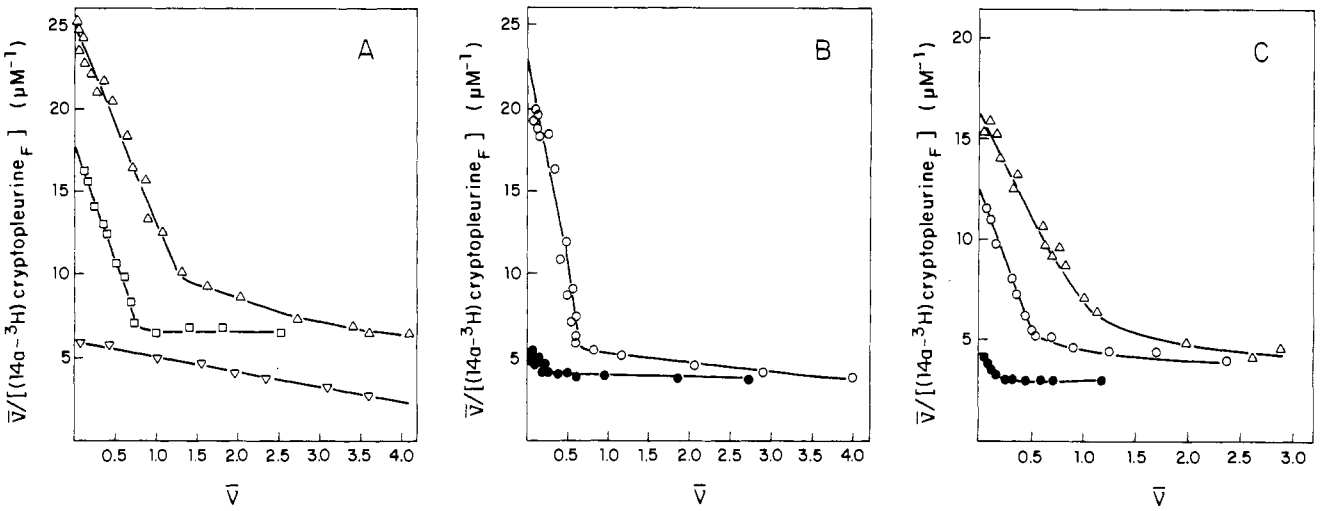

FIGURE 2: Scatchard plots for binding of [14a-^{3}H]cryptopleurine to different ribosome preparations. Data were obtained after sedimentation by ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained 50 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, 80 mM KCl, and 1 mM DTT using 9×10^{-8} M 80S ribosomes, 4×10^{-8} M polyribosomes, or 7×10^{-8} M 70 S ribosomes. [14a-^{3}H]Cryptopleurine concentrations ranged from 6 nM to 1 μM for 80S ribosomes, 10 nM to 1 μM for polyribosomes, and 30 nM to 7.5 μM for 70S ribosomes. (A) 80S ribosomes (△) and polyribosomes (□) from Y166 sensitive yeast strain and 70S ribosomes (▽) from *E. coli*. (B) 40S (○) and 60S (●) ribosomal subunits from Y166 strain. (C) 80S (△), 40S (○), and 60S (●) ribosome preparations from CRY6 yeast resistant strain.

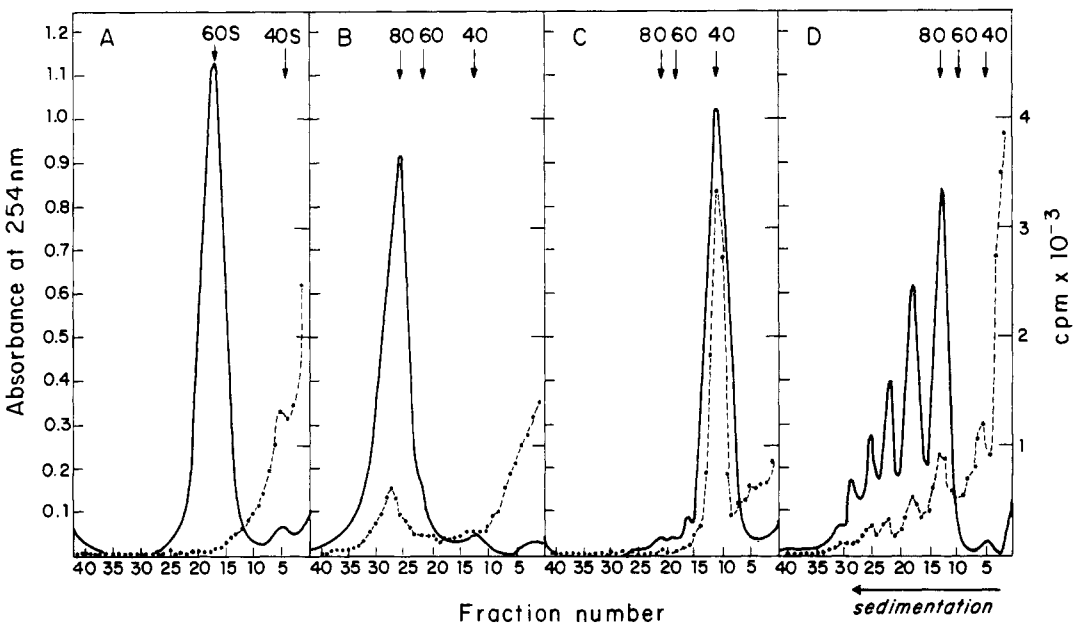

FIGURE 3: Interaction of [14a-^{3}H]cryptopleurine with different Y166 ribosomal preparations from the strain Y166 as studied by centrifugation through sucrose gradients. Reaction mixtures (0.2 mL) contained 50 mM Tris-HCl buffer (pH 7.4), 12.5 mM $MgCl_2$, 80 mM KCl, and 1 mM DTT with ribosome particles and [14a-^{3}H]cryptopleurine concentrations added as indicated. Samples were centrifuged at 0 °C through 5-mL 15–30% sucrose gradients prepared in the above buffer except for polyribosomes, in which case a 20 mM Tris-HCl buffer (pH 7.4) containing 30 mM $MgCl_2$ and 100 mM KCl was used. Centrifugation was in the SW 50.1 rotor at 32000 rpm (for 60S and 40S ribosomal preparations) or 40000 rpm (for 80S ribosomes and polyribosomes) for 4.25 h (60S), 2.5 h (80S), 4.5 h (40S), or 50 min (polyribosomes). Specific experimental conditions: (A) 80 pmol of 60S ribosomal subunits and 52 pmol of [14a-^{3}H]cryptopleurine; (B) 90 pmol of 80S ribosomes and 30 pmol of [14a-^{3}H]cryptopleurine; (C) 80 pmol of 40S ribosomal subunits and 52 pmol of [14a-^{3}H]cryptopleurine; (D) 100 pmol of polyribosomes and 100 pmol of [14a-^{3}H]cryptopleurine. The binding of [^{3}H]cryptopleurine to 80S ribosomes appears smaller than that to 40S particles, mostly due to the 3-fold higher absorbance of 80S ribosomes (see Materials and Methods).

shown in Figure 2. In these plots an upward curvature indicates the presence on yeast polysomes, 80S ribosomes, and 40S ribosomal subunits of two classes of binding sites. One class might be assumed to be specific (high affinity for cryptopleurine and a low binding capacity) and the other one nonspecific (multiple binding sites with low affinities). Hence, tracing the apparent asymptotes to the extremes of the curve will be misleading (Nørby et al., 1980). However, it is obvious that the radioactive alkaloid binds to sensitive 80S and 40S ribosomal subunit preparations (Figure 2A,B) with higher affinity than to the resistant ones (Figure 2C); thus there appears to be specific binding of the drug. However, this result is misleading since it is caused by a small amount of contaminating 40S ribosomal subunits which are present in the 60S ribosomal subunit preparations (see below and Figure 3A). Only the low-affinity, nonspecific, class of binding sites are apparent for 70S ribosomes from *E. coli* (Figure 2A) which is in agreement with the previous finding that it is only at high concentrations that cryptopleurine inhibits protein synthesis on *E. coli* ribosomes (Donaldson et al., 1968).

The results presented in Figure 3 show that the cryptopleurine–60S ribosomal subunit complex is completely disso-

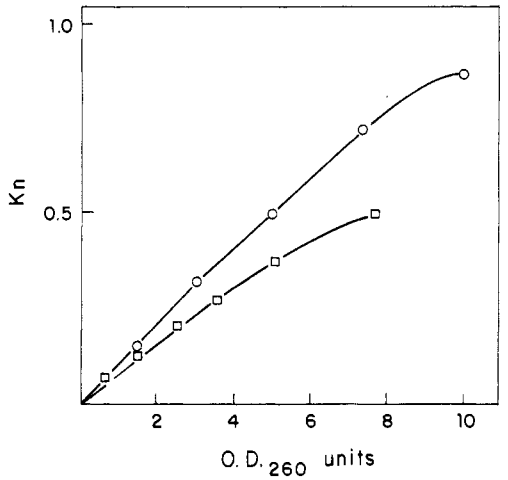

FIGURE 4: Variation of *Kn* values using different concentrations of 80S ribosomal particles from the strain Y166. Data were obtained after sedimentation by ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained 50 mM Tris-HCl buffer (pH 7.4), 12.5 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, 5×10^{-8} M [14a-^{3}H]cryptopleurine and 1.5×10^{-5} M unlabeled cryptopleurine. 80S ribosomes (○) with concentrations of 1.54, 3.08, 5.08, 7.39, and 10.01 OD_{260} units. 40S ribosomal subunits (□) with concentrations of 0.72, 1.55, 2.58, 3.61, 5.15, and 7.73 OD_{260} units. Levels of nonspecifically bound (RL_{ns}) and unbound (L) [14a-^{3}H]cryptopleurine were obtained for each particular experimental point, and from these data the different values of *Kn* were determined as deduced from the relationship $Kn = [RL_{ns}]/[L]$ (see Materials and Methods).

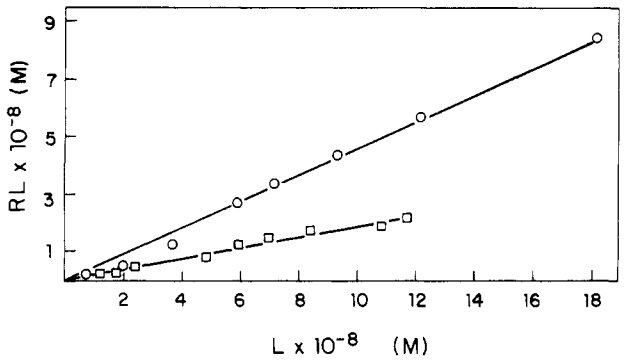

FIGURE 5: Variation of *Kn* values using different concentrations of [^{3}H]cryptopleurine. Data were obtained after ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained the same ionic components described in Figure 4 and either 5 or 1.7 OD_{260} units, respectively, of 80S or 40S ribosomal particles from yeast strain Y166. In the presence of 80S ribosomes (○) the concentrations of labeled and unlabeled cryptopleurine ranged respectively from 1.6×10^{-8} to 4.3×10^{-7} M and from 4.8×10^{-6} to 1.3×10^{-4} M. In the presence of 40S ribosomal subunits (□) these concentrations varied from 1.0×10^{-8} to 1.4×10^{-7} M and from 1.4×10^{-6} to 2×10^{-5} M, respectively. Levels of nonspecifically bound (RL_{ns}) and unbound (L) [14a-^{3}H]cryptopleurine were determined experimentally as described under Materials and Methods. The values of *Kn* are given by the slopes of the resulting graphs.

ciated (Figure 3A) by centrifugation of the reaction mixtures through sucrose gradients, indicating that the binding of the alkaloid to the 60S ribosomal subunit is of very low affinity. In contrast the binding of [14a-^{3}H]cryptopleurine to polysomes, 80S ribosomes, and 40S ribosomal subunits remains stable (Figure 3B–D), indicating the presence in these particles of a high-affinity binding site. Strong binding of cryptopleurine to the small amounts of 40S ribosomal subunits that were present in preparations of 80S and 60S particles (see Figure 3A,B,D) was also detected.

Specific Binding of [14a-^{3}H]Cryptopleurine to Sensitive and Resistant Ribosomes. In order to calculate the specific amount of cryptopleurine bound to yeast ribosomes, we have

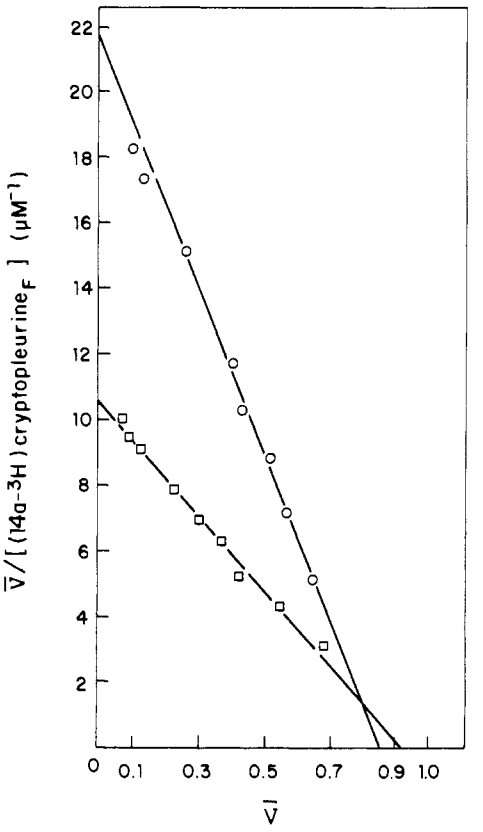

FIGURE 6: Scatchard plots for binding of [14a-^{3}H]cryptopleurine to 80S ribosomes from sensitive (Y166) and resistant (CRY6) yeast strains as determined by isotope dilution. Data were obtained after ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained 8.9×10^{-8} M 80S ribosomes and [14a-^{3}H]cryptopleurine with concentrations ranging from 1.6×10^{-8} to 3.2×10^{-7} M. When required, a concentration range between 4.8×10^{-6} and 9.6×10^{-5} M for unlabeled cryptopleurine was used. The ionic conditions were those described under Materials and Methods. The amounts of specifically bound and free cryptopleurine were calculated as described under Materials and Methods. The *Kn* value used was 0.48 which was calculated as described in the text. (○) Sensitive and (□) resistant 80S ribosomes.

chosen the isotope dilution method in its differential approach. For this purpose it is necessary to know the value of *Kn* which varies according to the experimental conditions used (Blondeau & Robel, 1975; Truong & Baulieu, 1971). The results obtained indicate that up to high ribosome concentrations *Kn* varies linearly (Figure 4). This behavior seems to be general and has been described for some ligand–protein interactions (Truong & Bailieu, 1971; Richard-Foy et al., 1978). At the concentrations of 80S ribosomes and 40S ribosomal subunits (5 and 1.7 OD_{260} units, respectively) used in the experiments for the determination of the values of both *Kd* and *n* of the specific component, the values of *Kn* were 0.48 and 0.17, respectively (Figure 4). Furthermore *Kn* (slope of the straight line) (Figure 5) may be considered also to be constant at a fixed ribosome concentration at least in the range of [14a-^{3}H]cryptopleurine concentrations which were diluted isotopically in the experiments for the determination of the *Kd* and *n* values of the specific component of the ribosome particles.

The results of experiments to determine the parameters of the specific binding of cryptopleurine to sensitive and resistant ribosome preparations are presented in Figures 6 and 7. Sensitive and resistant 80S ribosomes present one specific binding site for cryptopleurine ($n^s = 0.84$ and $n^r = 0.92$) (Figure 6) with $Kd^S_{80} = 3.9 \times 10^{-8}$ M and $Kd^R_{80} = 8.7 \times 10^{-8}$ M, respectively. Sensitive and resistant 40S subunits also show

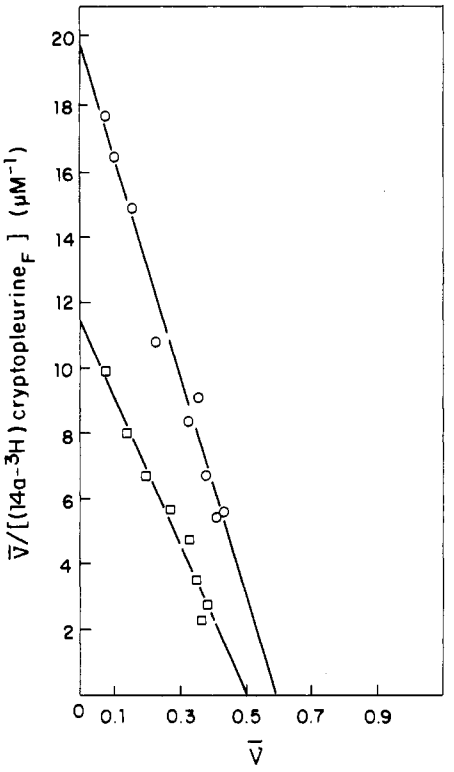

FIGURE 7: Scatchard plots for binding of [14a-^{3}H]cryptopleurine to 40S ribosomal subunits from sensitive (Y166) and resistant (CRY6) yeast strains as determined by isotope dilution. Data were obtained after ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained 9.3×10^{-8} M of 40S ribosomal subunits and [14a-^{3}H]cryptopleurine with concentrations ranging from 1.4×10^{-8} to 2.2×10^{-7} M. In the samples where isotope dilution was required, a range from 3.5 to 55 μM was used. The ionic conditions were those described in the legend to Figure 4. The amounts of specifically bound and free cryptopleurine were calculated following the equations described in the legend to Figure 6. The *Kn* value used was 0.17 and was calculated as described in the text. (○) Sensitive and (□) resistant 40S ribosomal subunits.

one specific binding site for cryptopleurine ($n^s = 0.58$ and $n^r = 0.50$) (Figure 7) with $Kd^S_{40} = 2.93 \times 10^{-8}$ M and $Kd^R_{40} = 4.44 \times 10^{-8}$ M, respectively. The fractional number of specific binding sites bound per receptor might be explained if a certain percentage of the specific binding sites was inactivated on the 40S ribosomal subunits used in our experiments. A further possibility is that the isotope dilution chosen (250-fold) led to an underestimation of B_s. However, this does not seem to be the case since, in the absence of any isotope dilution, less than one specific binding site per 40S ribosomal subunit was detected (Figure 2). No specific binding site could be detected on 60S ribosomal subunits using the isotope dilution method (results not shown), which is in agreement with the finding that cryptopleurine binds to the 60S ribosomal subunit with very low affinity (Figure 3).

Effect of Protein Synthesis Inhibitors on the Binding of [14a-^{3}H]Cryptopleurine. Cryptopleurine, tylocrebrine, tylophorine, tubulosine, and emetine are all known to have a similar mechanism of action since they inhibit the EF2- and GTP-dependent translocation on 80S ribosomes [Vázquez, 1979 (review)]. Furthermore ribosomes from yeast and CHO cell mutants resistant to cryptopleurine show cross-resistance to the other alkaloids. Since this resistance is expressed on the 40S ribosomal subunit, it was concluded that all these inhibitors might act at identical or overlapping site(s) on this subparticle [see review by Vázquez (1979)].

We have observed that tylocrebrine, tylophorine, tubulosine, and emetine prevent the binding of [14a-^{3}H]cryptopleurine to 80S, 60S, and 40S ribosomal particles (Figure 8). However, none of the graphs show the typical sigmoidal profile of a competition reaction for an identical binding site even if the competing drug is unlabeled cryptopleurine (Figure 8B,C). These results strongly indicate the presence of multiple nonspecific ribosomal binding sites for all the inhibitors. This might be expected since the binding of cryptopleurine on the 60S ribosomal subunit is also blocked by the other alkaloids, although higher concentrations of unlabeled cryptopleurine, tylophorine, and tylocrebrine are required. Much higher concentrations of tubulosine and emetine are required to promote inhibition of cryptopleurine binding to each ribosome particle (Figure 8), and this inhibition is less pronounced in the case of the 60S ribosomal subunit. Practically identical results were obtained when the competition reactions were performed on 80S, 60S, and 40S species from either the sensitive or the resistant strains (Dölz, 1981).

We did not observe inhibition of [14a-^{3}H]cryptopleurine binding to 80S ribosomes prepared from the sensitive strain by any of the other inhibitors of protein synthesis that we have tested including cycloheximide (up to 10^{-3} M concentration), pederine (up to 1.5×10^{-4} M), hygromycin B (up to 2×10^{-3} M), and the peptide bond formation inhibitors anisomycin, trichodermin, anthelmycin, and sparsomycin (up to 10^{-3} M) (results not shown).

Effects of Cryptopleurine, Tylocrebrine, Tylophorine, Emetine, and Tubulosine on Peptide Bond Formation. The finding that cryptopleurine interacts with the 60S ribosomal subunit could explain its inhibitory effect on peptide bond formation (Pestka et al., 1972; Barbacid et al., 1975). As tylocrebrine, tylophorine, tubulosine, and emetine can displace nonspecifically bound cryptopleurine from its site(s) on the 60S ribosomal subunit, we expected that all these compounds might also inhibit peptide bond formation at high drug con-

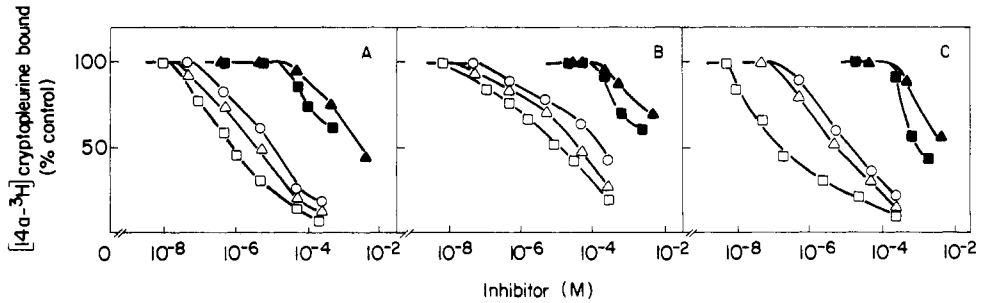

FIGURE 8: Effects of a number of alkaloid inhibitors of enzymic translocation on the binding of [14a-^{3}H]cryptopleurine to 80S ribosomes and 60S and 40S ribosomal subunits from the sensitive (Y166) yeast strain. The experiments were carried out by ultracentrifugation as described under Materials and Methods. Reaction mixtures (2 mL) contained 3.5×10^{-8} M [14a-^{3}H]cryptopleurine and the ionic components described in the legend to Figure 4. 80S ribosomes and 60S and 40S ribosomal subunits were present in the relevant tubes at 8.9×10^{-8}, 9×10^{-8}, and 9.3×10^{-8} M final concentrations, respectively. (□) Cryptopleurine; (△) tylocrebrine; (○) tylophorine; (■) tubulosine; (▲) emetine.

Table I: Effects of Cryptopleurine, Tylocrebrine, Tylophorine, Tubulosine, and Emetine on Peptide Bond Formation[a]

		peptide bond formation (% control)			
		fragment reaction		peptidyl-[^{3}H]puromycin formation	
inhibitor	concn (M)	Y166	CRY6	Y166	CRY6
crypto-	2.0×10^{-4}	82	84		
pleurine	5.0×10^{-4}			47	50
tylocrebrine	5.0×10^{-4}			61	59
	1.0×10^{-3}	76	81		
tylophorine	5.0×10^{-4}			66	64
	1.0×10^{-3}	80	83		
tubulosine	5.0×10^{-4}			68	71
	2.0×10^{-3}	72	78	50	54
emetine	5.0×10^{-3}			54	57
	8.7×10^{-3}			44	39
	1.0×10^{-2}	67	69		

[a] The reaction of UACCA-[^{3}H]Leu-Ac (52 Ci/mmol) with 1 mM puromycin on 80S ribosomes (fragment reaction) was carried out as described by Battaner & Vázquez (1971). 0.08 pmol of Ac-[^{3}H]Leu-puromycin was synthesized in the controls in the absence of added drug. Formation of peptidyl[^{3}H]puromycin using yeast polysomes and [^{3}H]puromycin was assayed as described by Jiménez et al. (1977). 3.6 pmol of peptidyl[^{3}H]puromycin was synthesized in controls in the absence of added drug.

centrations. This interpretation was indeed correct, although the inhibitory effects were more apparent on the puromycin reaction than on the fragment reaction (Table I). Furthermore, the five alkaloids inhibited peptide bond formation on both sensitive and resistant ribosomes. The 60S ribosomal subunit of mutant ribosomes is thus sensitive to the alkaloid, supporting the notion that resistance to cryptopleurine in the yeast strain CRY6 is induced by a single nuclear mutaiton which is expressed in the 40S ribosomal subunit (Grant et al., 1974). Moreover, these results may, at least partially, explain the finding that resistance of CRY6 ribosomes to cryptopleurine is never complete, since the alkaloid, added at concentrations that inhibit peptide bond formation, also inhibits the growth of resistant yeast cells (Grant et al., 1974).

Discussion

Our results show that cryptopleurine interacts with 80S ribosomes and 40S ribosomal subunits isolated from sensitive yeast cells and has two different classes of binding sites. One of these is specific for the alkaloid and is a single binding site with a high affinity for cryptopleurine ($Kd^S_{80} = 3.9 \times 10^{-8}$ M and $Kd^S_{40} = 2.9 \times 10^{-8}$ M). The other class contains multiple, nonspecific binding sites with very low affinities for the alkaloid. Only nonspecific binding sites are located on 60S ribosomal subunits. The weak affinity of the nonspecific sites for cryptopleurine is indicated by unstable binding of the alkaloid to 60S ribosomal subunits during centrifugation in sucrose gradients. From these results we conclude that the inhibition of translocation that takes place at low concentrations of cryptopleurine is the result of a specific high-affinity interaction of this compound with the 40S ribosomal subunit. In contrast, the inhibition of peptide bond formation that occurs at high cryptopleurine concentrations is likely to result from the nonspecific interactions of the alkaloid with the low-affinity sites present on 60S ribosomal subunits.

The conclusion that there is a single high-affinity binding site for cryptopleurine on 40S ribosomal subunits that controls the specific inhibition of protein synthesis by the alkaloid is consistent with the finding that a single nuclear mutation induces cryptopleurine resistance in yeast and that this mutation is expressed in the small ribosomal subunit (Grant et al., 1974). Since earlier methods, using spectrofluorometric techniques, detected only multiple binding sites for cryptopleurine (Bucher & Skogerson, 1976), it must be assumed that this technique was inadequate to allow detection of the single high-affinity binding site for cryptopleurine.

The binding of cryptopleurine to both 80S and 40S ribosomal particles prepared from yeast strains that were either sensitive or resistant to this alkaloid was qualitatively similar. Nevertheless the high-affinity binding sites located on 80S ribosomes and 40S ribosomal subunits have respectively 2.2 and 1.5 times less affinity for cryptopleurine than have the corresponding sites on sensitive particles. These differences in affinities are not, however, high enough to explain the levels of resistance to cryptopleurine observed both in vivo and in vitro (Grant et al., 1974; Sánchez et al., 1977). Therefore, it might be possible that conformational change(s) in mutant ribosomes could lower the inhibitory effectiveness of the bound alkaloid. We have indeed observed that in mutants cross-resistant to trichodermin and anisomycin, the binding affinities of the latter antibiotic are identical for sensitive or resistant ribosomes (Jiménez & Vázquez, 1975). Furthermore, studies using affinity labeling techniques suggest that there are conformational differences in the peptidyltransferase center of these mutant and wild-type ribosomes (León-Rivera et al., 1980).

Our results have shown clearly that both tylophorine and tylocrebrine block the binding of [14a-^{3}H]cryptopleurine to 80S, 60S, and 40S ribosomal particles noncompetitively. Profiles for the inhibition curves are not sigmoidal but are biphasic and less steep than might have been expected at the higher concentrations of the unlabeled inhibitors. This shift of the graphs, which is also observed in the presence of unlabeled cryptopleurine, can only be attributed to the presence of nonspecific binding sites for radioactive cryptopleurine located on 80S ribosomes. We calculate that, under our experimental conditions, about 30% of the labeled alkaloid is indeed bound nonspecifically to ribosomes (results not shown). However, biphasic curves are also caused by the presence of nonspecific binding sites for tylocrebrine and tylophorine as evidenced by comparing the inhibition curves obtained when 80S or 40S ribosomal particles are used on the one hand and 60S ribosomal subunits on the other. This comparison illustrates that, in addition to these nonspecific binding sites, tylophorine and tylocrebrine have a common high-affinity binding site shared with cryptopleurine on the 40S ribosomal subunit. This type of phenomenon is well documented since binding of a number of radioactively labeled ligands (i.e., catecholamines, muscarinic acid, steroid hormones, etc.) to both their specific and nonspecific receptor sites is inhibited by chemically related antagonistic drugs (Cuatrecasas et al., 1974; Levitzki et al., 1975; Beld et al., 1980; Richard-Foy et al., 1978). Obviously under these experimental conditions any attempt to calculate the apparent dissociation constant of the interacting ligand (on the basis of the amount of cold antagonist that displaces 50% of the labeled ligand) could result in serious errors (Jacobs et al., 1975; Levitzki et al., 1975).

It is not clear if emetine and tubulosine share an identical specific binding site with cryptopleurine on the 40S ribosomal subunit. Only very high concentrations of tubulosine or emetine reverse binding of [14a-^{3}H]cryptopleurine to ribosome particles. The high concentrations of either tubulosine or emetine which are required to displace [14a-^{3}H]cryptopleurine from the ribosomes would indicate a very low affinity for these

two drugs under our experimental conditions. Furthermore no specific binding of ^{3}H-labeled emetine to reticulocyte ribosomes could be detected previously (Grollman, 1968). Although cryptopleurine-resistant ribosomes are cross-resistant to emetine and tubulosine, the drugs might bind at different sites of a specific center of the 40S ribosome subunit which is involved in translocation (Söllhuber et al., 1980). This center might be distorted in the presence of a mutant ribosomal protein, an effect that causes resistance to cryptopleurine and cryptopleurine-related drugs.

The inhibition of peptide bond formation by high concentrations of cryptopleurine, tylophorine, tylocrebrine, emetine, and tubulosine is most probably mediated by nonspecific binding of several molecules at low-affinity sites on the peptidyltransferase center of 60S ribosomal subunits. These sites need not be related to the binding sites for a number of other specific inhibitors of the peptidyltransferase center such as trichodermin and related compounds [Vázquez, 1979 (review)]. In support of this conclusion is the finding that mutants resistant to trichodermin are sensitive to cryptopleurine and vice versa (results not shown). Furthermore, none of the peptidyltransferase inhibitors tested (trichodermin, anisomycin, anthelmycin, and sparsomycin) are able to inhibit the binding of [14a-^{3}H]cryptopleurine to yeast ribosomes.

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