minimal. The data also demonstrates – not unexpectedly – that relatively high proportions of unbound molecules are available to the brain for an initial short period when high overall concentrations[7] of the drug prevail. This suggests that under these experimental conditions at least, the bulk of the Δ^1-THC enters the brain within 40 sec of i.v. administration. This short period encompasses the unstable situation prior to the equilibration of free drug with blood proteins. Once inside the brain, avid binding of the Δ^1-THC to protein and membranes and possible slow metabolism to 7-OH-Δ^1-THC[19] would be expected to contribute to a slow release and prolonged duration of action[20].

It is possible that an enhancement of the transformation of Δ^1-THC to CBN could be responsible for observed pharmacokinetic interactions between Δ^1-THC and CBN[6]. Table 2 illustrates the isotope ratios obtained from rats treated with pure labelled Δ^1-THC, and with labelled Δ^1-THC and cold CBN under identical experimental conditions to those used above and no enhancement of the dehydrogenation of Δ^1-THC is to be noted.

Table 3 illustrates the results of another experiment to simulate regular users; the rats were pretreated twice daily for 5 administrations of unlabelled Δ^1-THC before the pure labelled Δ^1-THC was finally administered. Again the difference does not appear to be sufficient to be the basis of differences in metabolism rates [21], but extended experiments should be undertaken to confirm this.

Thus the production of CBN from Δ^1-THC in rats may be viewed as competition for available free Δ^1-THC, the effect of which on the overall intoxication, is yet to be evaluated. Enhancement of this transformation does not appear to be the basis of either the THC/CBN pharmacokinetic interaction[6] or increased clearance rates of 'chronic users'[21].

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Spin label studies of ATP phosphoribosyltransferase of E. coli

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Summary. Covalently bound bromoacetamide nitroxides have been used to detect the conformational changes and enzyme association induced by its feedback inhibitor, histidine.

ATP phosphoribosyltransferase (EC2.4.2.17) is the first enzyme of the histidine biosynthetic pathway[2] and is allosterically inhibited by the end product, histidine[3]. It catalyzes the reversible reaction of ATP and 5-phosphoribosyl α-1-pyrophosphate to yield phosphoribosyl-ATP and pyrophosphate. Although the primary structure of the active site is unknown, Bell and Koshland showed that an SH group is necessary for catalytic activity from studies on iodoacetate inactivation in the Salmonella typhimurium enzyme[4].

Studies in our laboratory with the E. coli enzyme have dealt with conformational changes and association-dissociation effected by substrates and other ligands. The action of histidine on this enzyme has been studied by several techniques like fluorescence[5], gel filtration[6], equilibrium sedimentation[7] and steady state kinetics of the inhibition of the reaction catalyzed by the enzyme[8]. However, both processes induced by histidine, namely binding of the effector to the enzyme and enzyme association, could not be discriminated by any of the techniques mentioned above. In the work presented here, at very low histidine concentration the correlation time, τ, decreases, suggesting a conformational change in which the unpaired spin becomes a little freer. When the histidine concentration increases, τ also increases, apparently due to the enzyme aggregation induced by histidine.

Materials and methods. The spin labels 3-[(2-Bromoacetamido)methyl]-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxyl (No. 131), 3-[2-(2-Bromoacetamido)acetamido]-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxyl (No. 132), and 3-[3-(2-Bromoacetamido)propyl]carbamoyl) 2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxyl (No. 133), were purchased from Synvar, Palo Alto, California.

ESR parameters of the nitroxide spectra

	ΔH_1 (G)	ΔH_0 (G)	ΔH_{-1} (G)	$a_{1,0}$ (G)	$a_{-1,0}$ (G)	τ (ns)
Free label (No. 132)	1.2	1.2	1.3	16.2	16.2	0.07
Adsorbed label (No. 131)	2.4	2.3	2.8	16.2	16.3	1.14
Adsorbed label (No. 132)	2.0	2.0	2.3	16.1	16.3	1.06
Adsorbed label (No. 133)	1.6	1.5	1.9	16.3	16.4	0.69

–1, 0 and 1 denote the 3 lines of the nitroxide spectrum assigned to ^{14}N nuclear spin quantum states; M = –1 is the line corresponding to the higher field. a is the hyperfine splitting constant. ΔH is the peak-to-peak width.

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ATP phosphoribosyltransferase was purified basically according to the method of Parsons and Koshland[9], as previously described[6], including a Sephadex G-200 step. Protein was determined by the method of Lowry et al.[10], with insulin standards. The method of Campbell et al.[11] was used to label the SH function of the enzyme; after reaction with the label, the enzyme retained approximately 15% of its activity. ESR measurements were carried out on a JEOL JM-PE-3 spectrometer, working at 23 °C and at X band (9.53 GHz), using a modulation amplitude of 0.5 G and microwave power of 10 mW. The solutions were contained in a quartz cylindrical cell, standard for the JEOL equipment. The solvent was in all cases 50 mM Tris-HCl buffer, pH 8.0.

Results. The spectra of nitroxides covalently bound to the enzyme are different from those displayed by the free spin label (not shown), with broader lines and higher values for the ratios of central line height to the others. In the case of bound nitroxide, the typical spectrum of a radical weakly attached to a large molecule is obtained, in which most of the unpaired spin density is localized on the nitrogen atom[12]. The hyperfine splitting constant (a) has, however, the same values in both cases (see the table), indicating that the dielectric constant of the solution surrounding the N–O group is the same. The parameters of the signal for the 3 nitroxides attached to the enzyme and for nitroxide No. 132 free in solution are presented in the table. When the nitroxide is bound to the enzyme, the line-width increases, probably due to dipolar interactions or to a worse average of nitroxide anisotropy. As the 3 ESR lines are well separated, indicating fairly rapid tumbling, the treatment of Stone et al.[13] as developed by Dwek[14] can be used for the calculation of the correlation time τ, from the square root of the ratios of the experimental derivative curves. The table clearly shows that the tumbling rate is slower when the spin is attached to the protein.

When spin labelled ATP phosphoribosyltransferase is titrated with histidine, the main spectral changes observed are in the ratios between the heights of the 3 lines. The variation of τ with histidine concentration is presented in figure 1 (histidine, at the concentrations used here, has no effect on the free nitroxide spectrum). The changes are small but reproducible and similar in the 3 nitroxides used. At very low histidine concentration τ decreases reaching a minimum at 10–25 μM, depending on the nitroxide used. At higher histidine concentration τ increases, being almost constant above 0.2–0.3 mM histidine. With the exception of signal height, the signal parameters do not change significantly for each nitroxide with histidine concentration. The change in signal height with histidine concentration for nitroxide No. 132 is shown in figure 2. A rapid decrease of the height is observed at low concentrations, with slight effect above 0.3 mM histidine. This pattern is similar to the one we reported previously in kinetic inhibition experiments[8], in which almost no inhibition was observed above 0.2 mM histidine and the maximum inhibition was not 100% but 70%.

Discussion. The enzyme group labelled by the nitroxides must be an SH group as far as haloacetamide nitroxides are specific for sulphydryl residues[15]. The attachment of the nitroxides to the E. coli enzyme must be covalent because of the structure of the label and the fact that they still remained bound after a long dialysis. The group(s) labelled in the enzyme must be in the active site or at least essential for enzymatic activity since the enzyme lost most of its activity after having been mixed with the label.

The variation of τ with histidine concentration (figure 1) is very similar in the 3 cases. At low histidine concentration, there is a decrease of the correlation time. Using the equation $\tau = 4\,\pi\,\eta\,r^3/3\,k\,T$, where η is the viscosity, r the

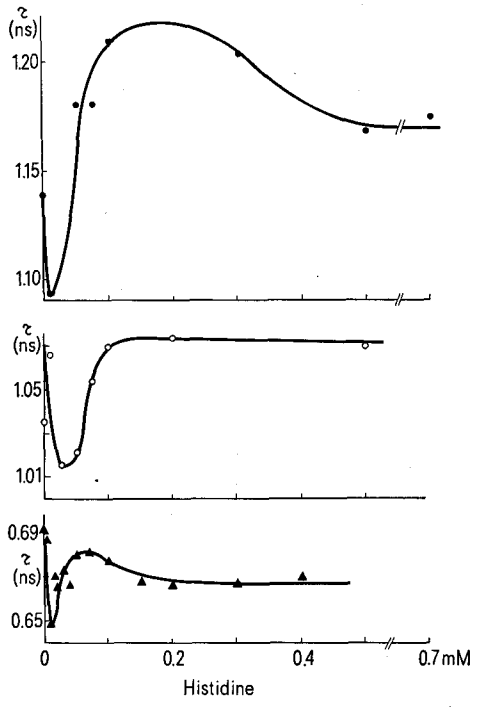

Fig. 1. Variation of the correlation time with histidine concentration, for nitroxides No. 131 (closed circles), No. 132 (open circles) and No. 133 (triangles).

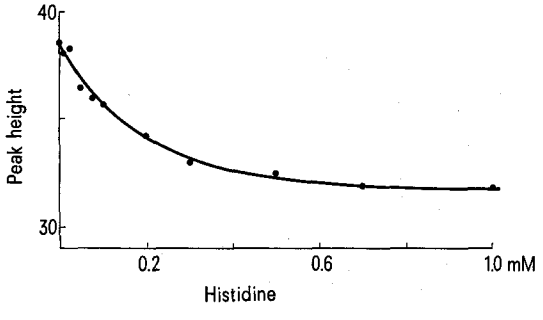

Fig. 2. Sum of the height of the 3 peaks of the spectrum of the nitroxide No. 132, in arbitrary units, vs histidine concentration.

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radius of the particle, k the Boltzman constant and T the absolute temperature, if τ decreases the radius r must decrease. This would mean that histidine is causing a conformational change in the enzyme that renders it tighter (smaller apparent size). This is in agreement with the conclusions of Bell and Koshland[4], who showed that histidine produced a higher exposure to the surrounding media of the essential SH group. The drop in the signal height (figure 2) can be explained as an increment of the dipolar interactions (there is a real possibility of several labelling sites per enzyme molecule): due to the conformational change the labels draw closer together.

At higher histidine concentrations (over 15–25 μM), τ increases. However, the signal height goes on decreasing throughout the range of histidine concentrations used, which means that the increase of dipolar interactions continues as an effect of histidine action. The only possible explanation, for these 2 simultaneous facts, is to assume that the enzyme size increases as a consequence of histidine action and that this increase (association) causes more dipolar interaction between the nitroxides bound to the enzyme molecules. It is known that histidine associates the E. coli enzyme from dimer to hexamer[6,7]; in those studies, histidine, in the range 0.05 to 1 mM, was able to aggregate the enzyme as shown in ultracentrifugation experiments[7] and 0.4 mM histidine readily associated it as followed by gel filtration[6]. In the present report, using a finer technique, we have been able to detect association of the enzyme with as little as 10 μM histidine. By calorimetric measurements of the binding of histidine to the enzyme, we have detected association at even lower concentrations (manuscript in preparation).

The nitroxide chain length does not affect the hyperfine splitting constant but produces different line-widths and correlation times, yielding broader lines and slower correlation rates as the nitroxide chain length is reduced. Labelling of the protein with one nitroxide or another affects the position of the minimum. This position depends on the relative contribution of the 2 superimposed processes, conformational change of the protomer and its association, to the overall effect produced by the feedback inhibitor histidine.

Interaction of extrinsic fluorescent probes with E. coli glutamine synthetase[1]

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Summary. Binding of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) to adenylylated ($E_{\overline{11}}$) glutamine synthetase is cooperative and time-dependent, with 3 dye sites per subunit. In fluorescence polarization experiments TNS and pyrene butyrate give normalized Perrin plots that indicate a symmetrical arrangement of dye excited state dipoles, relative to the rotational axis of the oblate ellipsoid of the dodecameric native enzyme.

Glutamine synthetase plays a key role in nitrogen metabolism for a variety of organisms[2–4]. The enzyme from E. coli (12 subunits of 50,000 daltons each) has been studied extensively with regard to its regulation and physical characteristics[5]. Covalent attachment of an AMP group to any subunit markedly alters its pH optimum, metal ion specificity, substrate binding constants, and sensitivity to bound feedback modifiers[6]. The present study is a continuation of attempts to probe critical features of this complex system by optical spectroscopy, the first of which involved introduction of chromophoric rare earth ions in the active site region[7].

Experimental. Adenylylated glutamine synthetase ($E_{\overline{11}}$) was prepared according to Shapiro and Stadtman[8]. 2-p-Toluidinyl-naphthalene-6-sulfonate (TNS) was prepared and recrystallized by the procedures of McClure and Edelman[9]. Sucrose (ultrapure) was a product of Schwarz/Mann. 1-Pyrene butyrate was a product of Eastman, and was recrystallized three times from ethanol-water. Fluorescence data were obtained with a Turner 430 spectrofluorometer (bandwidth 7.5 nm), equipped with thermostatted ($\pm$ 0.1 °C) cell holder and polarization filters, connected to a Sargent SLRG recorder.

Enzyme (0.5 mg/ml) was buffered at pH 6.5 with 10 mM 3,3-dimethylglutarate and 1 mM $MnCl_2$. % sucrose was varied by mixing 2 buffer solutions (A = 0%, B = 50% sucrose) in different ratios to the same volume (2.0 ml). Concentrations of TNS were varied similarly. Viscosities of sucrose solutions were determined at 37 °C with an Ostwald viscometer and compared to published[10] values. % sucrose (η, cp) were- 5% (0.815), 10% (0.955), 20% (1.305), 35% (2.65), 50% (9.55), respectively.

Binding data were analyzed by Scatchard[11,12] plots, by the equation

$$Y/[D] = K(n - Y)$$

where Y is the average moles of dye (D) bound, and n is the number of binding sites for D, per mole protein.

Polarization of fluorescence of enzyme-bound fluorescent dyes was calculated by the equations[13,14]:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where I_{VV}, I_{VH}, etc, refer to the intensity of the emission with polarizing filters on the excitation and emission sides oriented both vertically or vertically-horizontally, respectively. G is the grating correction factor, equal to (I_{HV}/I_{HH}). The normalized form of this equation:

$$\frac{U}{Uo} = \frac{1/P - 1/3}{1/Po - 1/3} = 1 + \frac{3\tau}{\varrho h}$$

where Po is the polarization observed where T/η is zero, or at infinite viscosity, was used for plots of the data as U/Uo vs T/η.

Results and discussion. Binding of TNS. As reported earlier by Miller et al.[15], binding of TNS to glutamine synthetase occurs with such enhancement of fluorescence that unbound dye is essentially non-fluorescent. The binding process is a biphasic process and time-dependent: rapid initial complexation of TNS with enzyme gives a maximal fluorescence emission within the mixing time, then this signal decays to about 70% of the original value in a first-order process having $t_{1/2} \simeq 1$ min. The initial kinetically-favored product apparently provides