Since the metabolism of oxycodone has not been fully established, the GLC characteristics of the metabolite(s) are yet unknown. However, from the expected metabolic pathways of iminoethanophenanthrofurans (8), it may be speculated that the metabolites have different retention in the GLC column than oxycodone under the same chromatographic conditions. Drugs usually coadministered with oxycodone such as acetaminophen, aspirin, caffeine, and phenacetin do not interfere in the assay. Without derivatization, acetaminophen and aspirin do not pass through the GLC column while phenacetin and caffeine appear to be eluted with the solvent.

The results of single-dose administrations of 4.5 mg of oxycodone hydrochloride and 0.38 mg of oxycodone terephthalate to human subjects are listed in Table II. The maximum plasma oxycodone levels ranged from 17.0 to 36.5 ng/ml in the six subjects. After 8 hr, the oxycodone levels ranged from 1.0 to 14.3 ng/ml.

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Interaction of the Antimalarial α-Dibutylaminomethyl-2,6-bis(trifluoromethylphenyl)-4-pyridinemethanol with Human Serum Albumin

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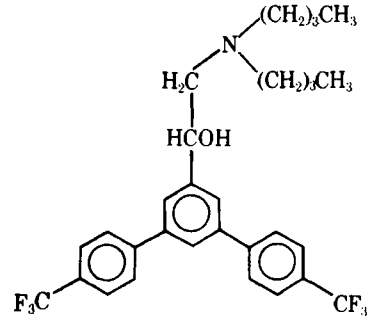
Abstract □ Binding of the antimalarial α-dibutylaminomethyl-2,6-bis(trifluoromethylphenyl)-4-pyridinemethanol with human serum albumin was studied using difference spectroscopy, fluorescence quenching, and equilibrium dialysis. Results indicated that the number of high affinity binding sites of the drug on protein is 0.45, with the total number of binding sites being 3.3–4.0. The binding constants were in the range of 0.57–4.00 × 10^6 M^{-1}. The drug was bound more strongly to a nonionic detergent than to either a cationic or anionic detergent. Interpretation of these data and fluorescence quenching results indicated that the drug is possibly bound to a hydrophobic site on human serum albumin.

Keyphrases □ Pyridinemethanol, substituted—binding to human serum albumin □ Binding, protein—substituted pyridinemethanol to human serum albumin □ Antimalarials—α-dibutylaminomethyl-2,6-bis(trifluoromethylphenyl)-4-pyridinemethanol, binding to human serum albumin □ α-Dibutylaminomethyl-2,6-bis(trifluoromethylphenyl)-4-pyridinemethanol—binding to human serum albumin

Interest in protein binding of drugs led to an investigation of the antimalarial α-dibutylaminomethyl-2,6-bis(trifluoromethylphenyl)-4-pyridinemethanol[1] (I). The drug is 22 times more effective than quinine and is active against highly chloroquine-resistant *Plasmodium berghei* in mice[2]. There is a lack of information about binding of such pyridinemethanols to serum proteins. Such data are important to an understanding of the distribution, metabolism, and excretion of these antimalarials since only the fraction of drug unbound exerts chemotherapeutic activity (1, 2).

The hydrophobic alkyl side chains, the aromatic π-electrons, the hydrogen-bonding site (OH group), and the possible electrostatic interactions suggest that the drug



I

may have a high affinity for serum albumin (3). The limited water solubility of the drug, even as the hydrochloride salt, makes the examination of binding affinity difficult. In this paper, the interaction of I with human serum albumin is examined by difference spectrophotometry, fluorescence quenching, and equilibrium dialysis.

EXPERIMENTAL

Apparatus and Chemicals—Compound I was supplied as the hydrochloride salt[3]. Its purity was verified by TLC in three different solvent systems (4) and by mass spectral analysis. Human serum albumin[4] was investigated for purity (5). The $E^{1\%}_{1cm}$ values for the albumin were 6.36 at 279 nm and 190 at 210 nm. Sorensen phosphate buffer, adjusted to pH 7.4, was used.

The dialysis membrane was prepared according to literature methods (6–8). Rigid, clear acrylic plastic dialysis cells (10 ml) were washed repeatedly with distilled water and sonicated overnight in distilled water. Electronic absorption spectra[5] were taken in 1-cm silica cells. Fluores-

[1] WR 148,946.

[2] P. S. Loizeaux, Walter Reed Army Institute for Research, personal communication, 1976.

[3] Walter Reed Army Institute of Research, Washington, D.C.

[4] Armour and Co.

[5] Cary spectrophotometer, model 118, Varian Instrument, Palo Alto, Calif.

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cence spectra were recorded on a spectrophotofluorometer[6] equipped with a corrected spectra attachment. Microliter syringes[7] were used to deliver quantities of solutions accurately. All other chemicals were the highest grade of commercially available materials.

Difference Spectrophotometry—The tandom technique (9) was used to study interactions between I (5×10^{-4} *M*) and albumin ($0.362–7.25 \times 10^{-5}$ *M*). Identical 50-μl increments of drug were added to the buffer cell in the reference beam and to the protein cell in the sample beam. To maintain a constant protein concentration in each beam after drug addition, a volume of protein equal to that of drug added but double the concentration of the protein already in the cell was added to the protein cell. Drug concentration was equalized by the addition of an identical volume of buffer to the buffer cell in the reference compartment. The resulting difference spectrum was then recorded for each added increment of drug over the albumin concentration range studied.

Drug–Surfactant Interactions—Difference spectroscopy studies were repeated in an identical manner to that already described except that a 0.025% cetrimonium bromide (cetrimide), 0.025% sodium lauryl sulfate, or 0.1% octoxynol[8] solution was used in place of the protein. These solutions represent cationic, anionic, or nonionic surfactants, respectively, at concentrations above the critical micelle concentration.

Fluorescence Quenching—Solutions of albumin were prepared over the concentration range of $1.45–14.5 \times 10^{-6}$ *M*. The fluorescence intensity of the native protein was recorded at excitation and emission wavelengths of 284 and 334 nm, respectively. The effects of 50-μl additions of drug (5×10^{-4} *M*) on the fluorescence of albumin were recorded.

The fluorometric titrations were performed as follows: 2.0 or 3.0 ml of a protein solution of an appropriate concentration, contained within a 1-cm quartz cell, was titrated by successive additions of drug solution. A concurrent titration of albumin and buffer was performed so that dilution effects could be considered and the magnitude of the fluorescence quenching could be adjusted appropriately. All fluorescence titrations were performed at an ambient temperature of 22 ± 1°.

Equilibrium Dialysis—Accurately pipetted samples (5–7.5 ml) of an albumin solution ($1.45–14.5 \times 10^{-6}$ *M*) were added to one-half of a dialysis cell, and an identical volume of I ($0.1–20 \times 10^{-6}$ *M*) in phosphate buffer was added to the other half. Equilibrium was obtained in 24 hr as determined by preliminary experiments. After dialysis was complete, an aliquot was removed from the drug side and concentrated sulfuric acid was added so that the final acid normality was 9.

The solution was then assayed fluorometrically using excitation and emission wavelengths of 322 and 381 nm, respectively (4). A blank buffer solution was concurrently assayed and subtracted from the sample reading. The total concentration of free drug was calculated from the resulting fluorescence intensity, taking into account drug bound to the dialysis membrane and cells.

RESULTS AND DISCUSSION

Compound I was shown to bind to human serum albumin by the techniques of difference spectroscopy, fluorescence quenching, and equilibrium dialysis. An example of difference absorption spectra obtained for the association of drug with protein is shown in Fig. 1. The spectra were characterized by positive peaks at 212, 243 ± 3, and 294 nm and negative peaks at 225 ± 4 and 324 ± 4 nm. The UV absorption spectrum of I at pH 7.4 was characterized by absorption bands at 296 (max) and 286 nm (10). Families of curves were generated by incremental additions of I to albumin solutions of fixed concentrations (0.025, 0.05, 0.1, 0.2, and 0.5%).

Absorbance titration curves (Fig. 2) were generated by plotting the change in absorbance (measured as the difference in intensity of the peak at 243 nm and the trough at 225 nm) *versus* concentrations of I added. Increasing concentrations of drug produced increasing absorbance changes that did not deviate from linearity until the drug to protein concentration was greater than 20. Limited drug solubility ($<10^{-4}$ *M*) prohibited a more accurate approximation of the saturation of protein binding sites. Thus, amounts of bound and free drug could not be determined since a complete saturation of protein is necessary for the calculations.

An example of data derived in the fluorescence quenching experiments is shown in Fig. 3. Linear portions of the titration curve extrapolated from the *x* and *y* axes gave the stoichiometric point from which the mole ratio of I bound to albumin was obtained. The association constants for the interaction of drug with protein were calculated from the curve using the methods of Attallah and Lata (11) (Table I).

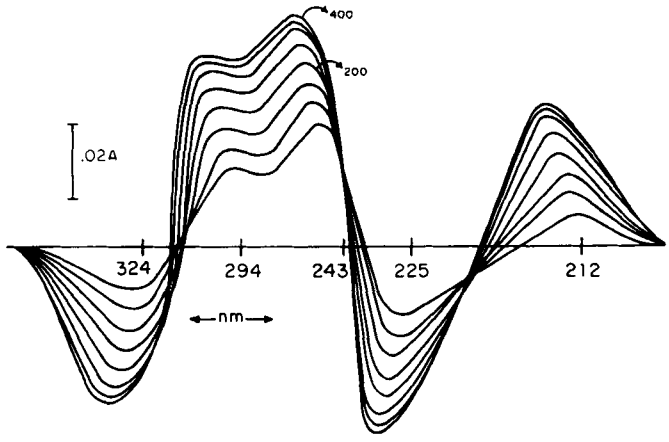


Figure 1—*Difference spectrophotometric titration curves of 3.62×10^{-6} M human serum albumin with I (5×10^{-4} M). The numbers 200 and 400 denote microliters of drug added.*

Data obtained from the equilibrium dialysis studies were graphed (Fig. 4) according to Scatchard (12).

Table II shows a comparison of binding sites and binding constants for the I–protein interaction obtained by the three experimental techniques. The data from the equilibrium dialysis experiments indicate that the number of high affinity binding sites was 0.45[9]. A number closer to 1.0, indicating a one-to-one interaction of drug to protein, would be more easily understood. One possible explanation for the low number is the dimerization of human serum albumin followed by its interaction with one drug molecule. However, this behavior is not likely at the low levels of protein utilized. Another explanation, the association of one drug molecule with two molecules of protein, is plausible because the drug has a high molecular weight and is relatively planar in conformation. Thus, it could conceivably bind between two protein molecules.

Data from the equilibrium dialysis experiments further indicate that the number of secondary binding sites was 2.85, yielding a total number of sites of 3.30. These values compare favorably with primary and sec-

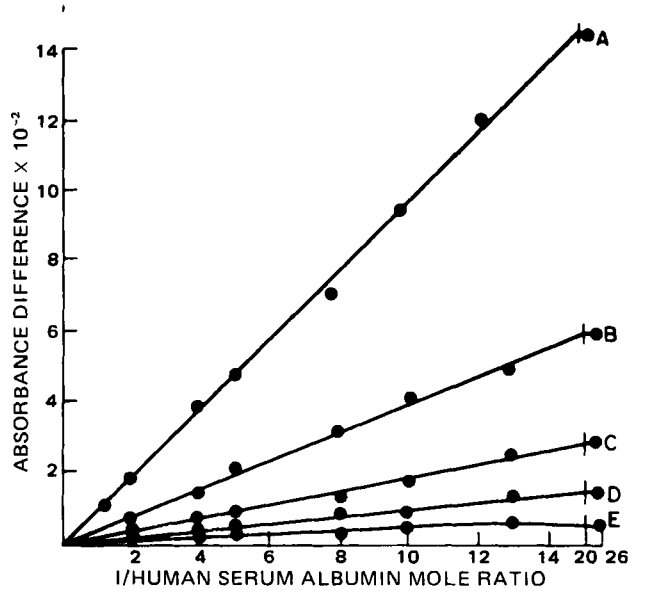


Figure 2—*Plots of absorbance difference [243 (peak)–225 (trough) nm] versus the mole ratio of I to human serum albumin. Slopes A–E represent the addition of increasing increments of I (5×10^{-4} M) to 3.62×10^{-6}, 7.25×10^{-6}, 1.45×10^{-5}, 2.90×10^{-5}, and 7.25×10^{-5} M human serum albumin, respectively.*

[6] Model MPF-4, Perkin-Elmer Corp., Norwalk, Conn.
[7] Hamilton Co., Reno, Nev.
[8] Triton X-100, Rohm and Haas, Philadelphia, Pa.

[9] Scatchard plots typically do not contain adequate numbers of *r* values less than 1.0, usually due to the method of analysis chosen for the investigation of the albumin–drug complex or the resultant free drug concentration. The fluorescence assay used in this study enabled the determination of many points below $r = 1.0$.

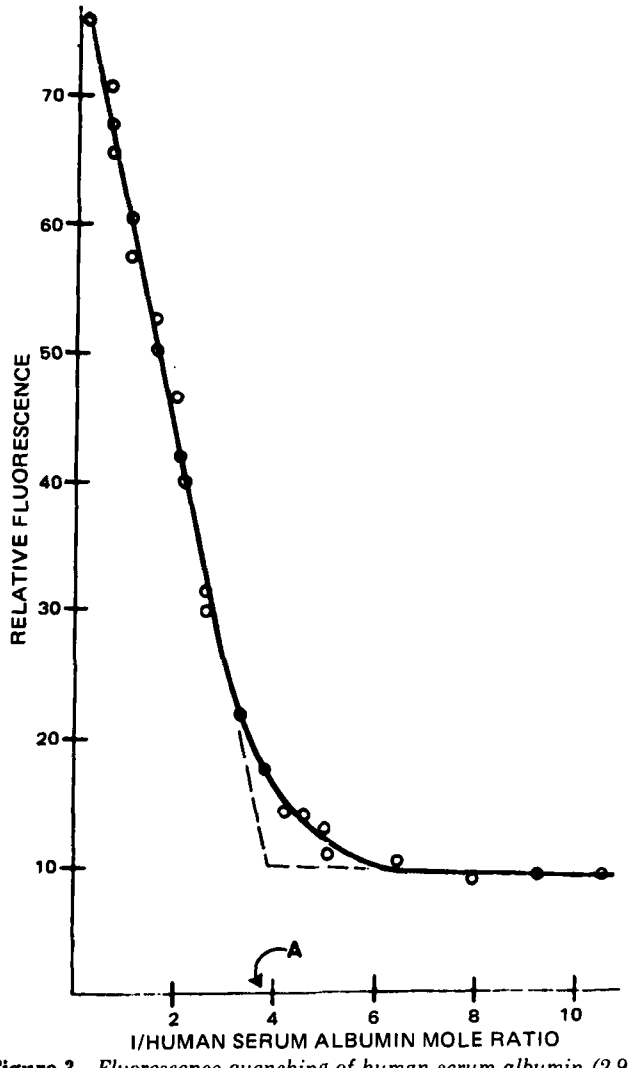


Figure 3—*Fluorescence quenching of human serum albumin (2.9 × 10^{-6} M) with increasing additions of I (5 × 10^{-4} M). Fluorescence intensity was monitored using excitation and emission wavelengths of 284 and 334 nm, respectively. The point at A represents 3.75 binding sites.*

ondary binding sites of 0.39 and 3.15, respectively, determined by Mais *et al.* (13) for quinidine, a drug conformationally similar to I. An earlier study (14) reported that quinidine binds to a single class of sites with $n = 1$ and $k = 7700$.

Fluorescence quenching experiments did not differentiate between primary and secondary binding sites but yielded an average total number of binding sites that compared favorably with the 3.30 from equilibrium dialysis experiments.

In contrast to equilibrium dialysis and fluorescence quenching, difference spectroscopy did not yield specific information concerning the total number of binding sites. At the lowest level of protein (0.025%) and the highest level of drug, a graph of spectral differences from 243 to 225 nm *versus* drug concentration revealed that the protein was becoming

Table I—Quenching of Native Human Serum Albumin Fluorescence by I [a]

Albumin Concentration, $M \times 10^{-6}$	Mole Ratio, n	Association Constant, $\times 10^{-6}\ M^{-1}$
1.45	3.50	1.64
2.90	3.75	1.22
7.30	4.00	1.58
10.2	3.60	1.35
14.5	4.00	0.57

[a] Excitation and emission wavelengths were 284 and 334 nm, respectively, in 1-cm quartz cells at 22 ± 1°.

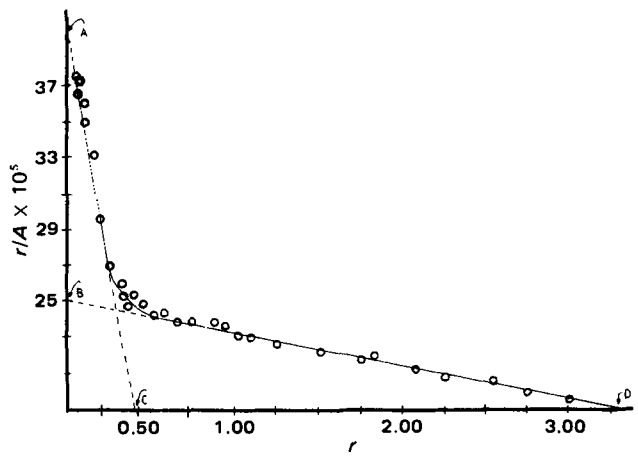


Figure 4—*Scatchard plot of* r/A versus r *for the human serum albumin–I titration for an albumin concentration of 1.45 × 10^{-6} M. A and B represent the binding constants for primary and secondary binding sites, respectively; C and D represent the number of primary and secondary binding sites, respectively.*

saturated with drug at a 20:1 drug to protein ratio. Binding constants for I calculated from both equilibrium dialysis and fluorescence quenching experiments were comparable and were in the range of 0.57–4.00 × 10^6 M^{-1}.

To elucidate the types of binding sites of I on albumin, the characteristics of curves generated by adding I to solutions of surface-active agents were studied. Difference spectra were generated as described previously, except that surface-active agents were substituted for protein. Addition of drug to an anionic surfactant produced negligible changes in absorbance. Addition of drug to a cationic surfactant produced small absorbance changes at 267 (peak) and 258 (trough) nm. Difference spectra with a nonionic surfactant (Fig. 5) produced a bathochromic shift similar to that observed between drug and albumin, indicating that binding was possibly occurring at a nonionic or hydrophobic site on the protein. Troughs appeared at 320 and 275 nm, and a peak appeared at 248 nm. The intensity of the peak was proportional to the quantity of drug added.

The hydrophobic or hydrophilic nature of binding sites on human serum albumin also was studied by other investigators (15, 16). Sudlow *et al.* (15) used dansyl sulfonamide and dansyl sarcosine as specific fluorescence markers to study two distinct binding sites on the albumin for anionic drugs such as warfarin, iophenoxic acid, and flufenamic acid. Gabay and Huang (16) used difference spectroscopy and fluorescence quenching experiments to demonstrate binding of phenothiazines to hydrophobic sites on human serum albumin. Sjoholm and Ljungstedt (17) found that the portion of albumin containing tryptophan is hydrophobic in nature.

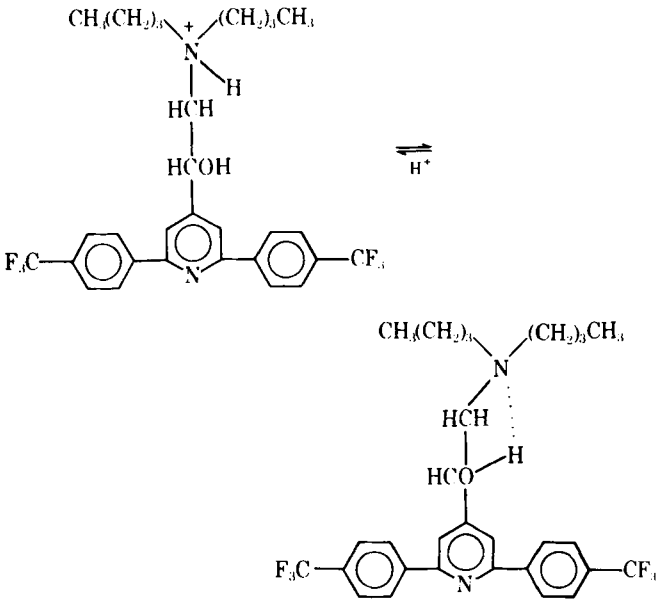
The observed fluorescence quenching of albumin by I in the present studies could indicate that the drug is being bound near tryptophan at a hydrophobic site. Since the fluorescence of albumin arises mainly (>90%) from the aromatic tryptophan residue (one tryptophan per albumin molecule) and since that region of the albumin is hydrophobic (16, 17), a decreased fluorescence on titration of the albumin with I indicates that the tryptophan region is involved. Also binding studies of several tranquilizers and depressants with pKa values similar to I (pKa 8.3) revealed that those drugs with high binding constants ($\geq 10^5\ M^{-1}$) are bound hydrophobically (18).

It is not clear which portions of the structure of I are involved in hydrophobic binding to the albumin. Previous data obtained at pH 7.4

Table II—Binding Sites and Binding Constants for the Interaction of I and Human Serum Albumin

Method	Binding Site, n	Binding Constant, $\times 10^{-6}\ M^{-1}$
Equilibrium dialysis	Primary (0.45)	4.00
	Secondary (2.85)	0.76
Fluorescence quenching	Total (3.5–4.0)	0.57–1.64
Difference spectroscopy	>20[a]	—[b]

[a] Does not represent n but represents total sites occupied before the protein is saturated with drug. [b] Preliminary estimates of k, using methodology similar to that in enzyme kinetics yielding K_m, suggest a value of $7 \times 10^5\ M^{-1}$.



Scheme I

showed that the monocation is the predominant species (10) and that the neutral form of the drug (occurring to the extent of 10–12%) exists as an intramolecular-bonded species (Scheme I). Additional studies also demonstrated that the pyridinic nitrogen is not protonated at pH 7.4 (10). Further investigation indicated that the pKa of the pyridinic nitrogen is 0.0, a low pKa for such a nitrogen (the pKa of pyridine is 5.2). The low value was attributed to both steric and inductive effects of the two trifluoromethylphenyl substituents. These effects should diminish possible binding of albumin at the pyridinic nitrogen of I and further support the possibility for hydrophobic forces being responsible for the drug–albumin interaction.

There is possibly additional attachment of I to human serum albumin at pH 7.4 *via* the quaternary nitrogen functionality. Most antimalarials exist as mono- or dications at physiological pH (19) and can potentially

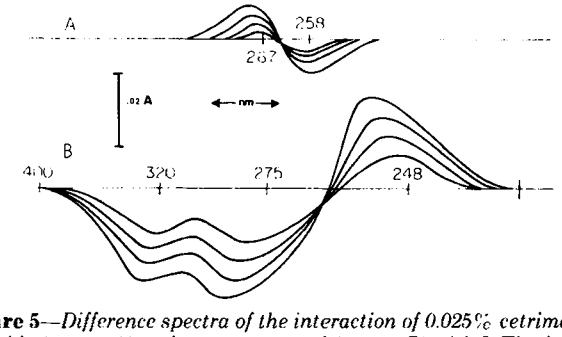


Figure 5—*Difference spectra of the interaction of 0.025% cetrimonium bromide (curves A) and 0.1% octoxynol (curves B) with I. The families of curves were generated by titrating each substance with 50-μl increments of I (5 × 10⁻⁴ M).*

interact with protein *via* the charged site. In the case of chloroquine (pKa 7.78), both species are available at pH 7.4; however, Parker and Irvin (20) showed that only the monocation interacts reversibly with bovine serum albumin. Evidence in this study for the possible attachment of I to albumin *via* the quaternary nitrogen functionality is the occurrence of red shifts of up to 10 nm obtained from difference spectroscopy experiments on addition of drug to albumin (or of albumin to drug). These shifts may represent a small change in the tertiary or quaternary structure of the protein. These types of shifts were also reported in the difference spectral study of chloroquine (19).

In summary, binding sites and binding constant data for I by equilibrium dialysis and fluorescence quenching methods are comparable. The data indicate that human serum albumin has between three and four binding sites per molecule for I, with a binding constant of approximately $1 \times 10^6\ M^{-1}$. Fluorescence quenching experiments and drug–surfactant interaction studies revealed that binding of the drug is possibly occurring at a hydrophobic site on the albumin. Difference spectroscopy data provided some evidence for additional attachment of drug to albumin *via* the charged exocyclic nitrogen.

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