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The intercalation of ethidium bromide in the chromonic lyotropic phases of drugs and nucleic acids

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Chromonic liquid crystalline phases are formed by a variety of drug and dye/water systems. In contrast to conventional lyotropic phases (where micelle formation underlies the mesogenic properties), in chromonic systems the molecules stack in columns. The different chromonic phases are different arrangements of these columns. We have examined the solution of ethidium bromide (EB) in the well-documented chromonic Intal/water system. EB is a widely used nucleic acid stain which changes colour when intercalated into DNA and which becomes fluorescent.

We have charted the changes in the temperature/composition phase diagram of the Intal/water system caused by adding EB. Although there are changes in the position of the phase boundaries, the overall pattern remains qualitatively the same—implying that the host phase is accepting EB as a similar chromonic molecule. The intercalation of EB molecules in the chromonic host phase results in optical effects—a metachromic colour change and fluorescence, similar to those occurring when the dye stains DNA.

These observations strengthen our belief that the central stack of bases in DNA can be regarded as being chromonic in nature.

1. Introduction

1.1. *Chromonic mesophases*

Recent studies in this department [1–5], and elsewhere [6, 7] have indicated that there is a well-defined family of lyotropic mesogens consisting of a number of drugs and dyes. In contrast to conventional amphiphile mesogens such as soaps, detergents and biological lipids, these newly defined *chromonic* [1–3] materials (see figure 1) have disc-like as opposed to rod-like molecules, are aromatic rather than aliphatic and the hydrophilic solubilizing ionic or hydrogen bonding groups are arranged around the periphery of the molecules and not at one end. They are the lyotropic analogues of the discotic mesophases. The chromonics have characteristic phase structures (see figure 2), phase diagrams, optical textures and X-ray diffraction patterns. The molecules can be considered as being insoluble in one dimension and the basic structural unit is a molecular stack rather than a micelle.

There are two principal chromonic phases, known as the N and M phases (although there are some additional rarer phases). The designations N and M date back to the early microscopy studies of Hartshorne on the mesophase of the anti-asthmatic drug disodium cromoglycate. The N phase was so-named because it forms a schlieren texture similar to that exhibited by the thermotropic nematics. It is a nematic array of columns and the only regular repeat distance shown by X-ray diffraction is the stacking repeat of 3·4 Å along each column. The M phase was so-named because it can form an optical texture similar to that of the hexagonal (middle) soap phase. X-ray investigations show that in this phase, along with the parallelism of the columns there is a transverse hexagonal ordering. Concentrated solutions of the M phase are much more viscous than the immediately preceding N phase. The phase change from $N \rightarrow M$ is first order and a sharp N/M phase boundary can usually be seen.

The chromonic system which has been explored most extensively is that of the drug, disodium cromoglycate (variously known as dscg, scg or INTAL™) shown in figure 1. The definitive study of the phase diagram, X-ray diffraction patterns and optical textures of this system was made by Hartshorne and Woodard in 1973 [8, 9]. However, there are many isolated references much earlier in the literature (in some instances dating back to the middle of the last century) describing systems that we would now term chromonic (Sandquist [10], Forneau [11], Balaban [12], Jelley [13] and others [14–16]). It now appears that these systems are widespread amongst drugs, dyes and similar compounds, but until the term 'chromonic' was coined in 1980 there was no general terminology for them, and they tended to be overlooked in general reviews of the liquid crystal literature.

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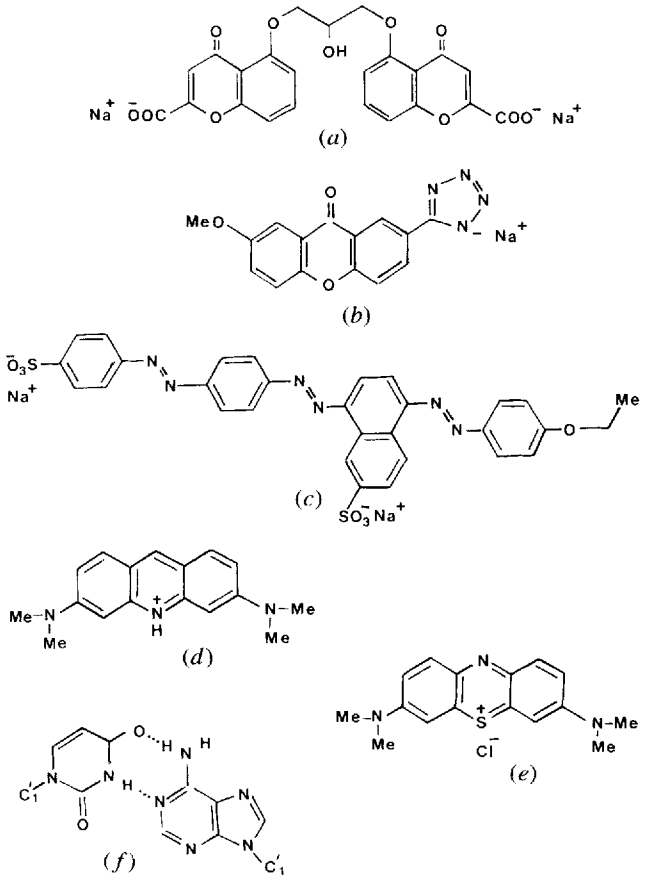

Figure 1. A selection of chromonic and potentially chromonic compounds. (*a*) The chromonic anti-asthmatic drug Intal (disodium cromoglycate). (*b*) The chromonic anti-allergic drug AH 7079 (Glaxo). (*c*) The chromonic dye Sirius Supra Brown RLL. (*d*) The potentially chromonic dye acridine orange (counter ion not shown). (*e*) The potentially chromonic biological strain methylene blue. (*f*) A pair of hydrogen bonded bases (adenine and uracil) in DNA.

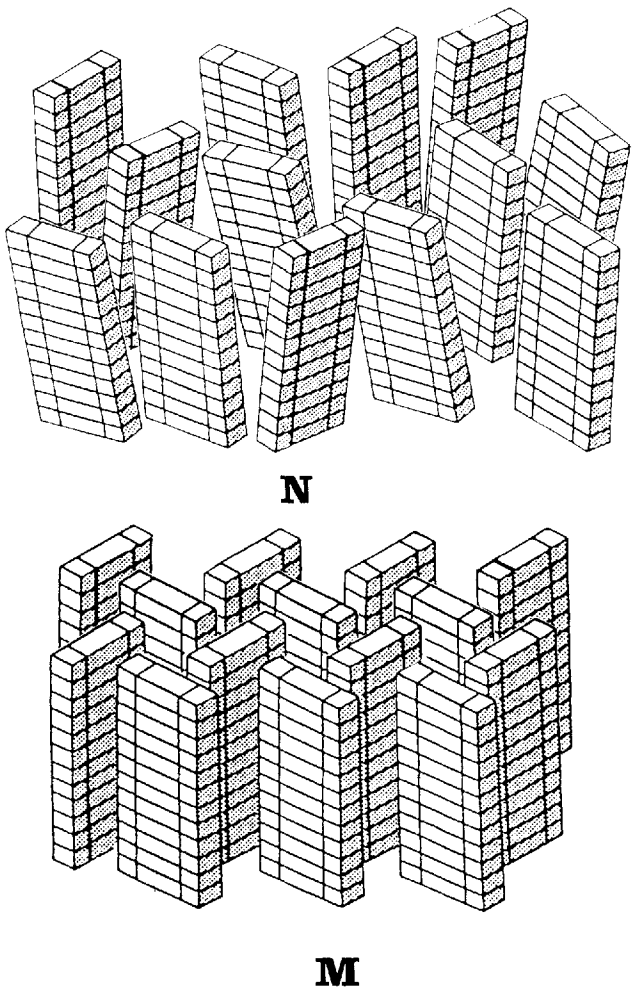

Figure 2. The structures of the chromonic N and M phases. In the N phase the molecular columns lie in a nematic array with orientational order only. In the more concentrated M phase, the columns lie in a hexagonal array.

1.2. *Nucleic acid mesophases*

We are currently engaged in presenting the case that the chromonic family can also be extended to include nucleic acids. The liquid crystalline behaviour of concentrated nucleic acid solutions was first observed 30 years ago [17, 18] and has been subsequently extensively studied. The interest and importance of this subject for chemical and biological sciences is apparent in the increasing number of papers in the last 10 years. Two mesophases were described by Livolant [19, 20] as occurring in buffered solutions with calf thymus fragments: a cholesteric phase formed by dilute solutions, and a columnar hexagonal phase found at higher concentrations. Recently similar behaviour has been found in oligodeoxyguanylates [21] and other dinucleotides [6]. The optical textures of the cyclic dinucleotide (d(cGpGp) are closely similar to those exhibited by the chromonics and DNA. The hexagonal packing seen by Livolant using electron microscopy is consistent with the chromonic mesophase structure suggested by Attwood and Lydon [3], as is the 3·4 Å repeat of B-DNA.

It has also recently been reported [22] that 'peptide nucleic acid' (a DNA analogue with a backbone consisting of *N*-(2-aminoethyl)glycine units) can mimic DNA to form a duplex with Watson–Crick base pairs. This would reinforce the assumption that the most important feature in the formation of DNA is the self assembly of the base pairs to form a chromonic stack.

1.3. *Intercalation*

One of the remarkable properties of liquid crystals is *miscibility*, whereby apparently chemically dissimilar compounds that have similar mesogenic properties are able to form mixed mesophases in all proportions. For

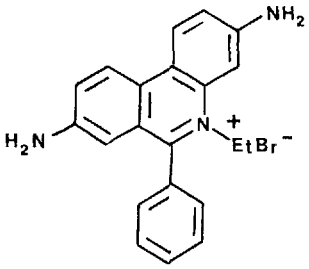

Figure 3. The molecular structure of the DNA marker ethidium bromide (EB).

some classes of mesophase, this phenomenon is very well defined. It is used, for example, to characterize smectic phases according to 'Sackmann's Miscibility Criterion'. In chromonic systems, miscibility is synonymous with intercalation whereby the guest molecule is incorporated into the chromonic column. Many biologically active compounds such as antibiotics, carcinogens and anti-carcinogenic agents owe their activity to the incorporation of their molecules into chromonic stacks of nucleic acid bases. We would expect there to be correlation between the ease of intercalation of a drug and its medical effectiveness. At present however, it is not clear which factors qualitatively determine this—it could be some combination of size, charge and polarizability. We expect therefore, that investigations into intercalation in chromonic systems will have profound implications concerning the design of future generations of drugs.

This paper concerns the intercalation of one such compound, the widely used biochemical reagent ethidium bromide (EB) shown in figure 3. In the past it had some medical applications as an anti-trypanocidal agent, but its major present use is as a fluorescent chromatographic marker for nucleic acids. EB itself does not fluoresce, but in the presence of nucleic acids it emits bright red light when illuminated with UV light. It is thought that the EB molecules intercalate between the base pairs and that it is the interaction between the π systems which gives rise to the fluorescent properties. Complexes of DNA and EB have increased viscosity, a lower sedimentation coefficient and a higher melting point than the uncomplexed DNA. The drug absorbs in the visible region at 480 nm (red), but after forming a complex with DNA, the wavelength is increased to 518 nm and the specific absorption is decreased. This is an example of metachromasy—a general phenomenon seen with most intercalated chromophores.

The drug is interesting for two reasons. Firstly, because it inhibits DNA-dependent nucleic acid synthesis, and it is hoped that details of the interaction may add to our understanding of this process. Secondly, the triple ring system is flat and aromatic, very similar to that of the other chromonics.

2. Experimental

2.1. *Optical studies*

Intal was kindly provided by Fisons Ltd and was used without further purification. Ethidium bromide was purchased from Sigma. The dye solution (0·2% w/w) was made up and appropriate amounts of Intal were added to 5 ml samples of the dye and of water to make various samples of known concentrations. The mixtures were made homogeneous by heating, stirring and leaving overnight. The solutions were then pipetted on to microscope slides. A Vickers polarizing microscope was used with a Red Iλ plate in place so that the low birefringence colours could be seen more easily. A Mettler FP52 microscopy hot stage with a Mettler FP5 control unit was used to allow controlled variation of the temperature. A Zeiss ultraphot was used to obtain photographs of the optical textures observed.

Hot stage polarizing microscopy was used to investigate the systems and to identify the mesophases formed at various temperatures and concentrations. The phase boundaries were noted as samples were heated and cooled and phase diagrams for the Intal/water system (see figure 4) and for the same system with ethidium bromide (see figure 5) were drawn.

2.2. *Spectroscopic studies*

Metachromasy is the characteristic colour change which certain dyes exhibit when bound to particular substances or when concentrated in solution. This common phenomenon is seen as a *hypsochromic* shift and a *hypochromic* reduction of the peak. These metachromic effects are a direct result of dye aggregation whereby the dye molecules stack to form columns. This is also observed when dyes are absorbed into substrates with regularly spaced binding sites. The spacing of 3·4 Å of

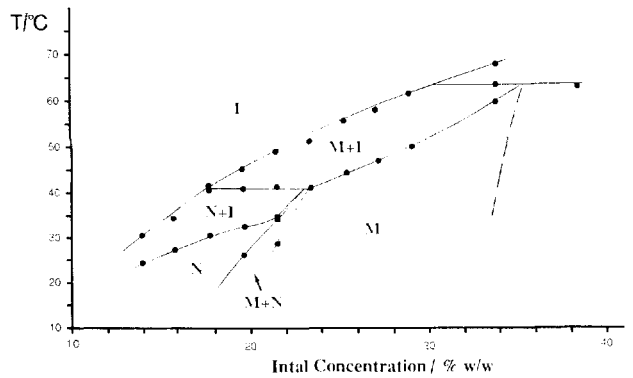

Figure 4. The Intal/water phase diagram. In addition to the isotropic solution (I) and the crystalline solid, there are two chromonic liquid crystal phases—the nematic N phase and the hexagonal M phase. Note the pronounced peritectic form of this phase diagram.

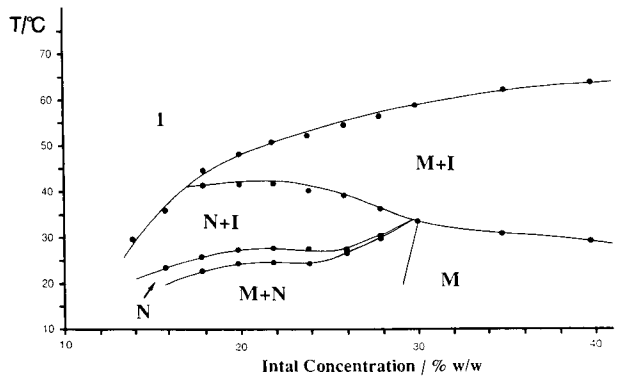

Figure 5. The Intal/water phase diagram after the addition of 0·2 per cent ethidium bromide.

chromonic mesophases is sufficient for interaction between the π clouds without causing overlapping. We are investigating whether this phenomenon is seen in chromonic systems as an indication of intercalation having taken place.

Spectroscopic studies were used to investigate changes in the absorption peak of EB on addition of Intal. The region covered by our investigations was 300–750 nm. A Pye UNICAM SP 8-100 scanning variable wavelength spectrophotometer was used for this purpose. The spectrum of EB was first recorded using water as a control. The dye was then added to samples of Intal to make up solutions of 1%, 10%, 15% and 20% w/w Intal. The spectra of the EB–Intal complexes were recorded using the corresponding Intal solutions as controls. The results can be seen in figure 6.

2.3. *Fluorescence*

Samples of EB with concentrated solutions of Intal and GMP (guanosine 5′-monophosphate) were made up.

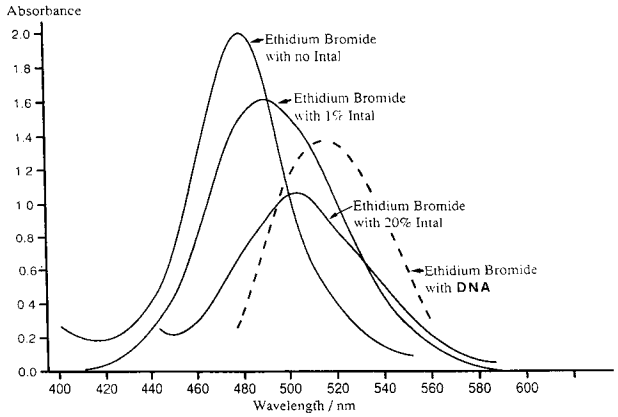

Figure 6. The UV/visible spectra of ethidium bromide dissolved in varying concentrations of Intal. The dashed line represents the spectrum of ethidium bromide dissolved in DNA [24].

These were illuminated with UV light alongside a sample of EB alone. Any fluorescence seen would indicate that intercalation had occurred as EB is known to fluoresce only in the intercalated form.

3. Discussion

3.1. *Phase diagram*

With reference to figures 4 and 5 it can be seen that the quantitative features of the phase diagram were appreciably altered by the presence of EB, even in small quantities (0·2 per cent), but the qualitative pattern of phases remains similar. This implies that the host phase is accepting EB as a similar chromonic molecule. The major changes observed were: (i) The M+N/M phase boundary is moved to a higher temperature. (ii) The N phase region is narrowed and extends further across the phase diagram. (iii) The M/M+I boundary occurs at a lower temperature and causes the M + I region to be extended. (iv) The clearing points remain virtually unchanged.

An interesting feature of the new phase diagram is the way in which the line dividing the M + I and N + I slopes downwards from left to right. According to the phase rule, for a two component system, if there are three phases present simultaneously (as there are at the boundary of the M + I and N + I regions), the system has only one degree of freedom. Thus, if the pressure is fixed, the temperature must be constant, i.e. the boundary line must be strictly horizontal. We have repeated these observations a number of times and we are convinced that the observed slope is too pronounced to be explained in terms of experimental errors. A possible explanation is that with the addition of even such a small quantity of EB, and although the overall concentration of EB was constant, the system can no longer be treated as having two components. Presumably, if the partition of EB between the various phases is not constant, the constraints of the phase rule for a three-component system rather than a two-component system should apply.

Underlying the phase diagrams (of the type shown in figures 4 and 5) are the free energy curves of the various phases. The phase or phases which occur at a particular set of conditions are those with the lowest free energy. Thus, phases are formed by default and one cannot discuss individual features—such as the position of a particular phase boundary, in isolation.

Bearing this in mind, the changes observed can be explained in terms of the following picture: (i) The free energy curve of the isotropic phase is relatively unaffected by the addition of EB. (ii) There is a moderate shift of the minimum in the free energy curve of the N phase to higher concentrations. (iii) There is an appreciable shift of the minimum in the free energy curve of the M phase towards higher concentration.

We are therefore drawn to the not unreasonable conclusion that the more ordered the phase, the more profound is the effect of the intercalated molecules.

The phase boundaries were also much more poorly defined with EB present than without it, and the temperatures of the phase transitions were harder to determine by optical microscopy. This is a phenomenon seen in mesophase systems of some dyes with a more circular cross-sectional area. We believe that the bulky side chain of EB is effectively making the columns more cylindrical and less blade-like and therefore the transitions are less sharply defined.

Table 1. Peak heights and positions of EB with varying amounts of Intal.

Intal concentration/wt %	Peak position/nm	Peak height/Abs units	Phase
0	478	2·0	Isotropic
1	498	1·6	Isotropic
10	504	1·6	Isotropic
15	506	1·1	N
20	507	1·0	N

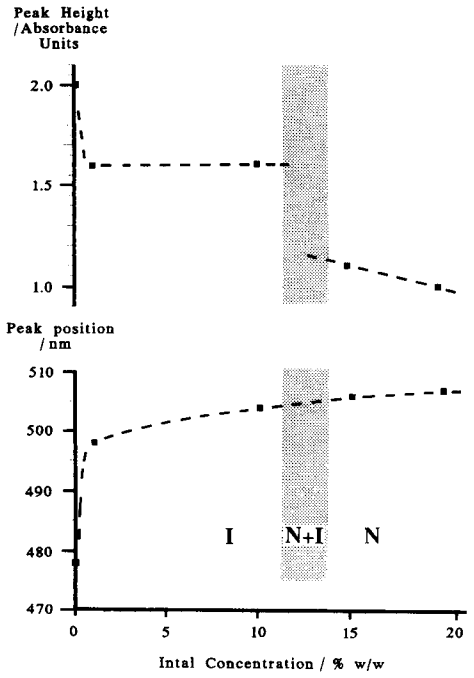

Figure 7. The optical properties of an intercalated dye. Graph showing the relationship between the peak height and the wavelength of maximum absorption for 0·002 per cent ethidium bromide in different concentrations of Intal. Note the way in which the abrupt change in the chromonic environment at the I/N phase change is reflected in the discontinuity in the peak height trace.

Table 2. The effect of other small molecules on the position of the maximum in the spectra of ethidium bromide.

Dopant (1% w/w)	Peak position/nm
No dopant (pure EB)	478
Acid (HCl)	450
Alkali (NaOH)	478
Ionic salt (NaCl)	478
Small organic molecule (urea)	478
Chromonic drug (Intal)	498

3.2. *Spectroscopic results*

The spectra for various concentrations of Intal with 0·002 per cent EB are seen in figure 6. On addition of Intal to EB, there is a marked shift in the maximum wavelength. There is a visible change from orange to deep red, seen by the naked eye even when small amounts of Intal are added to a solution of EB. This change is similar to that seen when EB is intercalated into the chromonic stack of base pairs in DNA. These results are summarized in table 1. We see that on crossing the I→N phase boundary (see figure 7) there is also a marked hypochromic shift which would be expected, bearing in mind the sudden increase in molecular ordering and aggregation.

Other compounds were added to the EB solution to be sure that the spectroscopic effect seen was not due to some other interaction between the dye molecule and the compound. An acid (HCl), an alkali (NaOH), an ionic salt (NaCl) and a small-molecule organic compound (urea) were all tested for a change to the position of the peak. The results can be seen in table 2. The only compound to affect the position of the peak was the acid, but this resulted in a shift in the opposite direction to that seen for Intal.

We conclude that the change in colour seen is therefore a consequence of the EB molecules being in an environment where their π clouds are able to interact with neighbouring molecules and not due to any ionic or covalent chemical reaction between the Intal and EB. We believe that the EB molecules are therefore intercalated into the chromonic stack of Intal and that this would account for such interactions.

3.3. *Fluorescence*

On illuminating the samples with UV light, no fluorescence was seen in a sample of EB alone. On addition of EB to a 20% w/w solution of Intal, fluorescence was clearly exhibited by the solution—another indication that the EB was indeed intercalating into the chromonic column of Intal. We repeated the experiment using a concentrated solution of one of the phosphorylated bases found in DNA, guanosine 5′-monophosphate (GMP). Again, fluorescence was seen and this was also indicative of the self assembly of the GMP in solution, which is

further evidence to suggest the self assembly of nucleic acid bases in solution.

4. Conclusions

We regard these studies as being highly significant. EB is the archetypal nucleic acid intercalator. It binds with high affinity and there is a pronounced change is the optical properties, most notably, the fluorescence of the stained material.

We have demonstrated that the pattern of interaction of EB with a chromonic drug/water mesophase is analogous to its interaction with DNA. The dye is intercalated into the chromonic columns and there is a parallel change in the optical properties. The fact that in DNA the stacked bases are covalently linked by sugar-phosphate chains appears to be of minor relevance.

This reinforces our view that the chromonic stack is an effective model for the dynamic state of the central column of bases in DNA, in the same way that a single molecular bilayer of the lamellar phase is a model for the biological membrane.

We draw an analogy between the liquid crystalline state of the bases in DNA and the liquid crystalline state of the monomer units in a side chain liquid crystal polymer. In a liquid crystalline side chain polymer, the pendant mesogenic monomers are more or less free to behave as if they were in a small molecule mesophase; the covalently bonded main chain prevents them from drifting away, but it leaves the short range thermal motion largely unhindered. We regard the thermal state of the DNA in the chromosome at biological temperatures as being analogous.

The stack of bases behaves as if it were a column in a chromonic mesophase and although the sugar–phosphate chain holds the bases in order, it is wound loosely enough to allow the bases an appreciable degree of movement. The various physical/chemical properties of nucleic acids: flexibility, tolerance of intercalation, and the ease with which the molecules can slide and tilt giving a range of phase structures (known as A, B, C, L, and Z forms), can all be regarded as aspects of the liquid crystalline nature of the chromonic DNA stack.

The characteristic properties of liquid crystalline systems, spontaneous ordering, flexibility and miscibility, appear to be essential ingredients for the functioning of the biological machinery.

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