means, and it is likely that the results reported here can be explained at least partly on this basis.

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Aggregate Formation in Frozen Aqueous Solutions of Nucleic Acid Derivatives and Aromatic Amino Acids. Energy Transfer and Complex Formation

Thérèse Montenay-Garestier[1] and Claude Hélène*

The formation of aggregates of nucleic acid derivatives and aromatic amino acids is induced by freezing aqueous solutions of these molecules. Luminescence and absorption studies provide information about molecular interactions in these aggregates. Extensive migration of the excitation energy at the triplet level is demonstrated by phosphorescence quenching studies, indicating that molecules are stacked in the aggregates. Complex formation between two molecular species can be induced by freezing aqueous mixtures. Thus, tryptophan forms intermolecular complexes with nucleic acid bases which involve charge-transfer interactions. It has also been shown that neutral cytidine is able to form a charge-transfer complex with protonated cytidine. These results demonstrate the usefulness of aggregate formation in frozen aqueous solutions to study molecular interactions between biologically-important molecules.

Weak interactions between identical or different organic molecules in dilute aqueous solutions may be greatly enhanced by freezing these solutions. Rapid cooling of an aqueous solution induces the formation of a microcrystalline structure. Solute molecules are excluded from the growing ice crystals and accumulate in the interstices of solvent (ice) crystallites (Szent-Györgyi, 1960; Wang, 1960, 1961, 1965). This phenomenon has been previously reported by a number of workers. The formation of aggregates has been suggested to be responsible for such different phenomena as the photodimerization of thymine by uv radiation (Wang, 1961, 1965), which occurs to a large extent in frozen aqueous solution although it is completely inefficient in the fluid state (Beukers and Berends, 1960a,b), dipolar broadening of electron spin resonance spectra of paramagnetic cations (Mn^{2+}/Gd^{3+}) in frozen aqueous solutions (Ross, 1965), and enhancement of bimolecular reaction rate constants upon freezing or changes in kinetic order of reactions in the same medium (Bruice and Butler, 1965). Some colorless mixtures in a fluid medium, *e.g.*, quinone and indole derivatives (Szent-Györgyi, 1960; Stom, 1967), exhibit characteristic colors in frozen solutions.

All the foregoing results support the proposed concept that in the aggregates obtained in frozen aqueous solu-

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tions solute molecules are in close contact and their proximity permits electronic interactions.

The exact structure of aggregates is not known, but many arguments favor the idea of a microcrystalline state. The emission spectra of aromatic amino acids in powders and frozen aqueous solutions are quite similar (Nag-Chaudhuri and Augenstein, 1964). The photoproduct of thymine is the same whether thymine is irradiated in dry film or in frozen solution (Wang, 1965). Benzoquinone ground with hydroquinone in the solid state gives a purple substance which, on dissolving, becomes a colorless solution. When this solution is frozen, the purple color appears again (Slifkin, 1971). We observed that, upon lyophilization, the green color of frozen aqueous solutions containing Cu^{2+} ions and adenosine molecules remains unchanged, although the fluid solution is colorless.

Luminescence and reflectance techniques can provide information about the nature of molecular interactions in aggregates (Montenay-Garestier and Helene, 1971). Strong interactions between organic molecules in their ground state modify both absorption and emission properties. If interactions are induced after one of two molecules have absorbed a photon, only excited state properties of the system will be affected and such interactions will be detected in luminescence characteristics (spectra or decay times). Luminescence will thus be very sensitive to environmental modifications that perturb the electronic structure of organic molecules.

RESULTS

Electronic Interactions and Energy Transfer in Aggregates. At low temperature (77°K) in a rigid medium, most nucleic acid derivatives (bases, nucleosides, and nucleotides) emit fluorescence and phosphorescence. Previous studies on dinucleotides and frozen aqueous equimolecular mixtures of nucleosides (mixed aggregates) demonstrated that interactions of bases in aggregates are not very different from those observed between covalently bound residues. In mixed aggregates formed upon freezing an equimolar aqueous mixture of nucleosides and nucleotides, the fluorescence spectrum is often broad and red-shifted, as is also observed in many dinucleotides (Helene and Michelson, 1967). Phosphorescence is emitted by the base whose triplet level is lower both in mixed aggregates and in dinucleotides. The structure of polynucleotides and nucleic acids is determined both by direct interactions

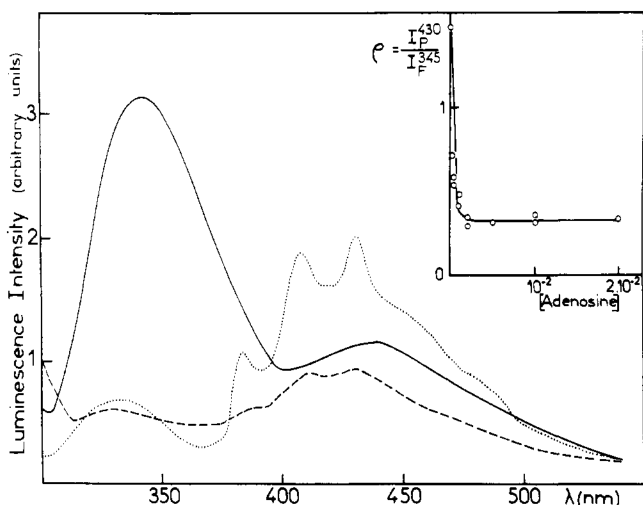

Figure 1. Total luminescence spectra of adenosine at 77°K. 10^{-3} *M* in frozen aqueous solution (——); 10^{-5} *M* in frozen aqueous solution (- - - -); 10^{-3} *M* in a water-propylene glycol glass (1 v/1 v) (· · ·); inset: variation with adenosine concentration of the ratio of phosphorescence and fluorescence intensities measured at 430 and 345 nm, respectively.

between bases and by the allowed conformations of the ribose phosphate backbone. The study of mixed aggregates containing two different bases may help one to understand the nature of molecular interactions between noncovalently bound bases and, to a certain extent, it may allow the determination of the influence of the backbone upon base interactions in polynucleotides and nucleic acids. We have focused our attention on the study of adenosine aggregates in order to compare electronic interactions of adenine residues in these aggregates with those observed in the corresponding biologically important polymers [poly(rA) and poly(dA)]. The emission properties of frozen aqueous solutions of adenosine depend on the concentration of solute molecules. At low concentration (10^{-5} *M*) (Figure 1) the emission spectrum is similar to that emitted by adenosine molecules dispersed in a glassy medium at 77°K (WPG: water-propylene glycol 1:1). When the concentration increases, the luminescence spectrum of frozen aqueous solutions of adenosine changes markedly (Figure 1). At concentrations higher than 2.10^{-3} *M*, emission properties no longer vary and the luminescence spectrum exhibits broad, red-shifted fluorescence and phosphorescence bands without any vibrational structure. The phosphorescence decay cannot be fitted to a single exponential curve (as observed in glasses) and two decay times (Kleinwächter, 1972) are necessary to describe this decay, a long one similar to that observed in glassy solutions (2.3 sec) and a shorter one (0.4–0.5 sec).

As already noticed (Wang, 1965; Ross, 1965), addition of organic solvents (ethanol or propylene glycol) prevents aggregate formation. Strong hydrogen bonding makes ice structure very reluctant to include foreign molecules. Freezing induces the separation of two phases, solute molecules in the organic solvent form "puddles" (Wang, 1961, 1965) surrounded by ice crystallites. This is supported by the observation that, upon increasing the concentration of the organic solvent, the ratio ρ of phosphorescence and fluorescence intensities measured at two given wavelengths very rapidly reaches the value obtained for the solute molecules in the pure organic solvent.

The addition of mineral salts to aqueous solutions before freezing also modifies electronic interactions in the aggregates. The influence of these salts on the structure of the frozen sample is rather complicated. It depends on the solute concentration, the salt concentration, and the nature of the salt and the solute molecules (Taborsky, 1970). When the salt concentration increases, the phosphorescence intensity of adenosine is enhanced more than the fluorescence intensity, but the ratio of phosphorescence and fluorescence quantum yields never reaches the value obtained for dispersed molecules in a glassy organic solvent. The formation of new phases (hydrate or eutectics) may be involved in the structural changes responsible for modifications of electronic interactions.

Taborsky (1970) noted that with multicomponent systems reciprocal effects of the concentration of one solute on the other are observed. In this case also organic solvents and mineral salts added to the solutions prevent mixed aggregate formation. Upon freezing colorless mixtures such as quinone–indole derivatives or Cu^{2+}–adenosine a coloration appears; small amounts of ethanol or sodium chloride prevent complex formation and the frozen solutions are then colorless.

Modifications of molecular excited states by electronic interactions may induce changes in luminescence spectra or decay time, but also excitation energy transfer may be observed. This transfer may occur from molecules in their lowest excited singlet or triplet states. Energy transfer from an excited singlet state (long-range transfer) depends on the relative orientation of the transition dipoles of the donor and acceptor molecules, on their distance, and on the overlap between the fluorescence spectrum of the

donor and the absorption spectrum of the acceptor (Förster, 1965). The efficiency of energy transfer from a molecule in its lowest triplet state depends essentially on an electron exchange between donor and acceptor molecules (Dexter, 1953). Partial overlap of the electronic clouds of both partners is required. To fulfill this condition, donor and acceptor molecules have to be stacked. Such a transfer has been studied in helical polyriboadenylic acid [poly(rA)] using paramagnetic metal cations as energy traps (Bersohn and Isenberg, 1964; Eisinger and Shulman, 1966). Experiments performed on adenosine aggregates with metal cations (Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}) demonstrate that energy delocalization does exist. In the absence of any energy transfer, one would expect one cation to quench only the phosphorescence of the neighboring molecules. As shown in Figure 2, one paramagnetic cation is able to quench the phosphorescence of a large number of adenosine molecules. This number depends on the initial adenosine concentration supporting the previous luminescence studies showing that aggregate length varies with initial solute concentration.

Quantitative data analysis leads to the distinguishing of two different cases. If the extent of energy migration is limited by the aggregate size, the phosphorescence intensity (I^P) will decrease according to eq 1.

$$I^P/I^P{}_0 = (1 - r)^n \qquad (1)$$

where r is the ratio of metal cation and nucleoside concentrations and n is the average number of adenosine residues in the aggregate. A plot of log $I^P/I^P{}_0$ *vs.* log $(1 - r)$ will give a straight line whose slope yields the value of n. Figure 2 shows that eq 1 is obeyed when the adenosine concentration is less than 2.10^{-3} *M*.

If the extent of transfer (number of residues involved in energy transfer) is not limited by the size of the aggregate but by the transfer process itself, the experimental results can be analyzed according to eq 2 (Bersohn and Isenberg, 1964; Levinson, 1962)

$$I^P/I^P{}_0 = 1 - \alpha^2 \int_0^\infty e^{-\alpha u} \tanh u \, du \qquad (2)$$

where $\alpha = 2r(\tau_p/\tau_t)^{1/2}$, τ_p is the triplet state lifetime, and τ_t is the transfer time between two residues. The adjustable parameter α/r gives the average number of adenosine residues whose phosphorescence is quenched by a single cation (Figure 2c). The large number (100–300) of residues on which energy migrates requires a hopping of triplet excitation energy from molecule to molecule until a trap is reached. This result proves that adenosine molecules are stacked in aggregates and that one aggregate contains at least between 150 and 300 stacked molecules. Similar results are obtained with 9-ethyladenine and adenosine monophosphate, showing that neither the ribose nor the phosphate groups prevent energy migration along stacks of adenine residues. The data published on poly(rA) (Eisinger and Shulman, 1966) are similar to our results; one paramagnetic divalent cation is able to quench the phosphorescence of 120 residues, showing that triplet energy migrates over about 60 adenine rings.

In the previous investigations we used nonluminescent traps (paramagnetic cations) to provide evidence for energy transfer. Luminescent molecules or ions may be used instead and their emission is sensitized by energy transfer if their energy levels are lower than the lowest triplet level of adenosine. Thymidine (Helene and Montenay-Garestier, 1968), tryptophan (Montenay-Garestier and Helene, 1971), or Eu^{3+} ions (Montenay-Garestier, 1972) fulfill this condition. Triplet energy migration may also be proved by observation of delayed fluorescence by annihilation of two triplet excitations. This emission has been detected both in the case of poly(rA) and adenosine aggregates (Hélène and Longworth, 1972).

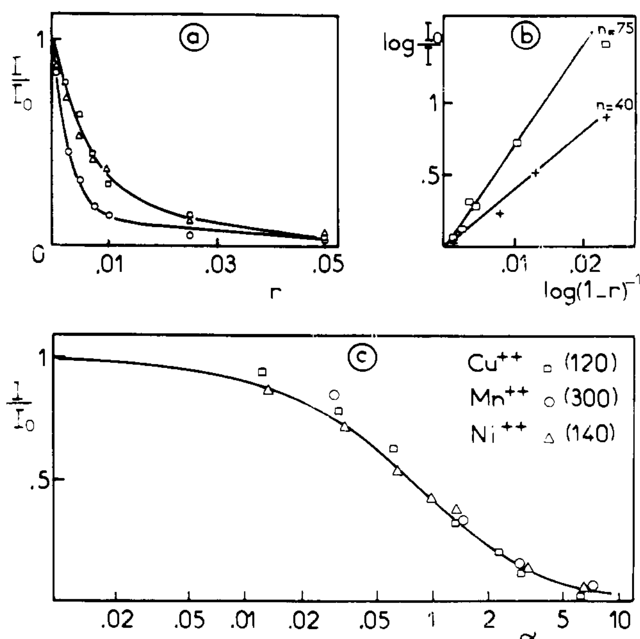

Figure 2. (a). Variation of the ratio I/I_0 of phosphorescence intensities measured at 440 nm of 10^{-3} *M* adenosine frozen aqueous solutions *vs.* *r*. The number *r* is the ratio of metal cations and adenosine concentrations. O, Mn^{2+}; Δ, Ni^{2+}; □, Cu^{2+}. (b). Analysis following eq 1 of phosphorescence quenching data of a frozen aqueous solution of adenosine at two different concentrations by Cu^{2+} cations. +, 5.10^{-4} *M*; □, 2.10^{-3} *M*. (c). Analysis following eq 2 of phosphorescence quenching data of a frozen aqueous solution of adenosine 5.10^{-3} *M* by Cu^{2+}, Ni^{2+}, and Mn^{2+} cations. The numbers in brackets are the average numbers of adenosine residues on which excitation energy migrates (see text).

The high similarity of results obtained with poly(rA) and adenosine aggregates demonstrates that the phosphodiester backbone is not determinant in the nature of interactions occurring between monomeric units in this polynucleotide. However, poly(rA) and poly(dA), which differ only in their sugar chemical structure, exhibit different luminescence behavior, demonstrating that the backbone configuration may modify electronic interactions between bases. Thus, aggregates are useful as models in the study of nonchemically bound molecules.

Complex Formation in Mixed Aggregates. Observation of complex formation between two species is another application of the study of mixed aggregates obtained upon freezing a two-component aqueous solution.

Interaction between Cytidine and Its Cation. In the following case, the two species are two different ionic forms of the same molecule. Fluorescence and phosphorescence titration curves of cytidine molecules dispersed in WPG glasses have a classical sigmoidal shape. The apparent p*K* values of the lowest singlet and triplet states of cytidine deduced from luminescence titration curves in these WPG glasses are found to be very close to that of the ground state. The rigidity of the medium and the short lifetime of the excited species can prevent the protonation equilibrium from being established in the excited state (especially in the fluorescent singlet state whose lifetime is in the nanosecond range). Luminescence titration curves of cytidine or cytosine in frozen aqueous solutions exhibit an anomalous behavior in the vicinity of the ground state p*K* (4.15) of the cytidine molecule (Figure 3) (Montenay-Garestier and Helene, 1970). The fluorescence is red-shifted and markedly broadened. The phosphorescence yield is fivefold higher than that of the neutral or protonated forms. Neutral and protonated species are equally concentrated when pH = p*K*. Therefore, it seems very likely that the

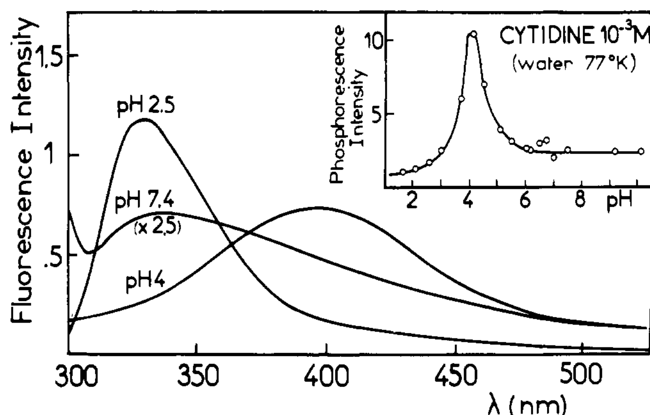

Figure 3. Fluorescence spectra of cytidine (10^{-3} *M*) frozen aqueous solutions at pH 2.5, 4, and 7.4 (77°K). Inset: variation of phosphorescence intensity measured at 500 nm *vs.* pH.

anomalous behavior of the titration curves in cytidine aggregates is due to an interaction between these two species. This might be attributed either to stacking or to hydrogen-bond interactions between two molecules in the same plane. Polycytidylic acid [poly(C)] presents luminescence titration curves quite similar to those of cytidine aggregates. This polynucleotide adopts a double-helical structure at acid pH when half of the bases are protonated. In WPG glasses, the dinucleoside phosphate cytidylyl-5′-cytidine whose bases are stacked but which does not form a double-stranded structure in this medium exhibits the same behavior. This leads to the conclusion that the interactions responsible for the anomalous luminescence titration curves occur between stacked molecules. Such interactions also modify the absorption spectrum. The opacity of frozen samples precludes the use of standard absorption spectrophotometry. Absorption spectra are deduced from reflectance spectra obtained with frozen samples at 253°K. The absorption spectrum at pH 4.2 extends to much longer wavelengths than those of cytidine and its cation. This new absorption can also be observed with frozen solutions of poly(C) at pH values lower than 5.

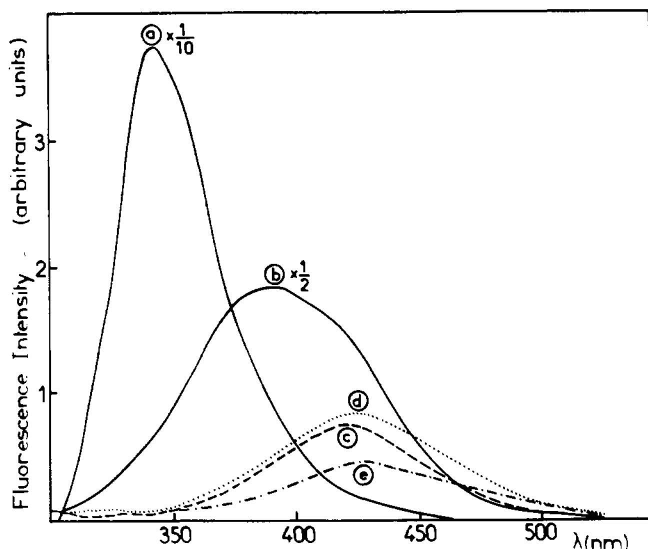

Figure 4. Fluorescence spectra of tryptophan (5.10^{-3} *M*) (a) and equimolar frozen aqueous mixtures of tryptophan and nucleosides (5.10^{-3} *M* each) at 77°K. (b) Tryptophan and adenosine (——). (c) Tryptophan and cytidine (- - -). (d) Tryptophan and thymidine (· · · ·). (e) Tryptophan and uridine (– · – · –). Intensities in arbitrary units are multiplied by the numbers indicated on the spectra.

Complex formation of the charge-transfer type between neutral and protonated cytidine in equal quantities when pH = p*K* can explain the preceding results. The red-shift of the fluorescence spectrum is much more important than that of the absorption spectrum. This indicates that charge-transfer interactions are stronger in the lowest excited singlet state than in the ground state. In the complex, neutral cytidine molecules behave as electron donors and protonated molecules behave as electron acceptors. This has been recently supported by photochemical studies of cytidine in ice (Rhoades and Wang, 1971). Cytidine can react photochemically with its cation and this very likely results from the charge-transfer interaction that we have described. The phenomenon of complex formation between different ionic forms of a same molecule has also been observed with adenosine in the vicinity of its p*K* value (3.5) both in aggregates and in poly(A), and with thymidine for p*K* values close to its p*K* (9.8). In this case, neutral thymidine would act as an electron acceptor and the deprotonated molecule would behave as an electron donor (Montenay-Garestier, 1972).

Complex Formation between Aromatic Amino Acids and Nucleic Acid Bases. When an aqueous solution containing an equimolar mixture (10^{-3} *M* each) of L-tryptophan and one of the nucleosides (A, G, T, U, C) is frozen to 77°K, a new absorption and a new fluorescence appear at longer wavelengths that are not observed with either of the two separated components under the same conditions (Figure 4, Table I) (Montenay-Garestier and Helene, 1971). This indicates that interactions between the two molecular species do occur in mixed aggregates formed upon freezing. At the same concentration (10^{-3} *M*), interactions in the fluid medium are too weak to be observed. Much higher concentrations are needed to observe complex formation either by absorbance or by proton magnetic resonance measurements (Dimicoli and Helene, 1971). Fluorescence and absorption red-shifts are much more important with pyrimidine than with purine derivatives. Fluorescence is always much more affected than absorption. A 1:1 stoichiometry is determined for the complexes at pH 7 from absorbance measurements at a wavelength where neither of the two components absorbs light. The stoichiometry appears to be 2:1 (tryptophan–nucleoside) in the acidic medium (pH 2). The fluorescence maximum is at longer wavelengths for mixtures of tryptophan with pyrimidines than for mixtures with purines (Table I). The fluorescence quantum yield of the equimolar mixtures is much lower than that of free tryptophan and the quenching is larger with pyrimidines than with purines. The phosphorescence is rather similar to that of tryptophan alone, except for thymidine-containing mixtures.

To determine the respective role of the indole ring and the amino acid chain in complex formation, we have investigated a number of derivatives where various functions have been chemically substituted or removed. Most of the compounds tested containing the indole ring give luminescence and reflectance results similar to those obtained with tryptophan. An aliphatic amino acid such as glycine does not compete with tryptophan for complex

Table I. Fluorescence Maximum Wavelength in Nanometers for Complexes of Tryptophan and Nucleosides (5.10^{-3}*M* Each) in Frozen Aqueous Solutions at 77°K

	Tryptophan	Adenosine	Cytidine	Thymidine	Uridine
Separated components	342	347	345	340	340
Equimolar mixture with tryptophan		390	420	425	428

formation. Only small differences are observed when the nucleoside is replaced by the corresponding base or nucleotide. This indicates that neither the ribose nor the phosphate is required for the interaction with tryptophan. Protonation of the base moiety at acidic pH's shifts the absorption and the luminescence spectra to longer wavelengths. The complexes with adenosine and cytidine cations do not exhibit any fluorescence, only phosphorescence.

All the foregoing results are consistent with the involvement of the indole ring and the base moiety in complex formation. The spectroscopic behavior of the complex is characteristic of weak charge-transfer interactions. The indole ring is the electron donor and the base moiety is the acceptor. Charge-transfer contribution is enhanced in the excited state and the fluorescence spectrum is expected to be much more affected than the absorption spectrum. From the value of the fluorescence maximum wavelength (Table I) and from the red-edge of the reflectance spectra, one can deduce that electronic affinities of the nucleosides increase in the order G < A < C, U, T < GH^+ < AH^+ < CH^+. It is important to emphasize that this order has been confirmed by detailed nmr and absorption studies of complex formation between tryptophan and nucleic acid derivatives in concentrated fluid aqueous solutions (Dimicoli and Helene, 1971). When studies in frozen aqueous mixtures provide evidence for interactions, interactions of the same nature are demonstrated in the fluid medium. Due to the property of frozen aqueous solutions of inducing the formation of aggregates, much lower concentrations are required as compared with those required for fluid solutions.

The same experiments have been performed with tyrosine and nucleoside equimolecular mixtures frozen to 77°K (Helene *et al.*, 1971a). The fluorescence emissions of both tyrosine and pyrimidine nucleosides are completely quenched (Figure 5). The quenching is ascribed to an interaction of charge-transfer type between the two aromatic rings. When the hydroxyl group of tyrosine is substituted (as in *p*-methoxyphenylalanine), quenching of both fluorescence emissions is still observed in equimolar mixtures. The new fluorescence which appears at long wavelengths in mixtures of pyrimidines with *p*-methoxyphenylalanine has a higher quantum yield than that observed with tyrosine. The equimolar mixtures of purine derivatives with both tyrosine or *p*-methoxyphenylalanine are characterized by a quenching of the phenyl ring fluorescence, while the fluorescence of the purine is not markedly affected. It must be noticed that purine derivatives have much higher extinction coefficients than tyrosine in the same wavelength range, so that their screening effect may contribute greatly to the apparent quenching of tyrosine fluorescence.

Equimolar mixtures of phenylalanine with cytidine in acidic conditions (pH 2) where cytidine is protonated exhibit a slight red-shift of the cytidine fluorescence. In other cases, whatever the nucleoside and the conditions (neutral or acidic medium), we have not been able to detect any fluorescence change in the equimolar mixture as compared to the separated components. This does not mean that phenylalanine and bases are not interacting, but does mean only that the interaction does not lead to any change in spectroscopic properties.

Histidine and pyrimidine mixed aggregates exhibit an interaction at pH values where the imidazole ring is not protonated and can act as electron donor (Montenay-Garestier, 1972).

From the previous data, clear-cut conclusions can be drawn from the investigations reviewed here. Charge-transfer interactions between aromatic amino acids and nucleosides occur in mixed aggregates. The aromatic amino acid ring is usually the electron donor; the base moiety of the nucleosides acts as an electron acceptor. Pyrimidine derivatives are better electron acceptors than purine derivatives. Comparison of absorbance and fluorescence modifications shows that charge transfer is more important in the lowest excited singlet state than in the ground state.

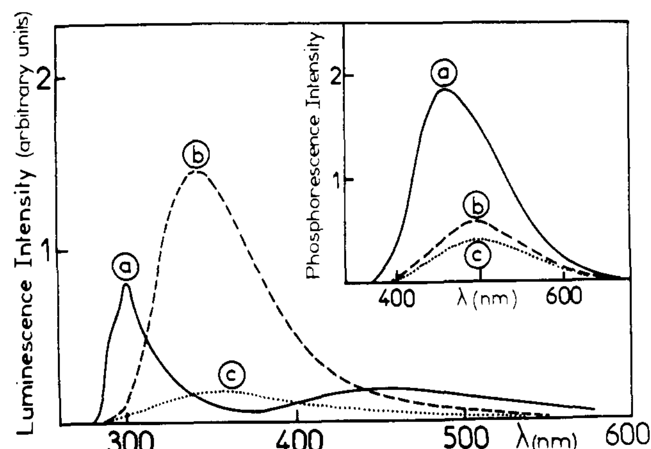

Figure 5. Total luminescence spectra of tyrosine (a), thymidine (b), and their equimolar mixture (c) (10^{-3} *M* each) in frozen aqueous solutions at 77°K. Inset: phosphorescence spectra of the same solutions.

CONCLUSION

The results presented in the first part of this review show that the freezing of an aqueous solution of aromatic molecules such as nucleic acid derivatives induces the formation of aggregates. The size of aggregates depends on the initial concentration of the solute molecules. Excitation energy transfer at the triplet level demonstrates that aromatic rings are stacked in these aggregates.

The formation of mixed aggregates containing two types of solute molecules can be used to provide evidence for the formation of complexes which are not easily detected in the fluid medium or which are destroyed as a result of solvation effects. It is usually observed that the same types of complexes are formed in highly concentrated fluid solutions. High concentrations are required due to the weakness of the interactions. The freezing of dilute solutions can thus be thought of as an artificial means to increase locally (in aggregates) the concentrations of both reactants.

The studies of aggregates have proved to be very useful in allowing for the first observation of charge-transfer interactions between two different ionic forms of the same molecule (cytidine and its cation). The observation of complex formation between aromatic amino acids and nucleic acid bases might shed some light on the role that could be played by these amino acid residues in the interaction between enzymes or proteins and nucleic acids. Investigations of complex formation of aromatic amines and oligopeptides containing aromatic residues with nucleic acids are now developed to elucidate the role of these residues in the recognition process (Helene *et al.*, 1971a,b).

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Influence of Frozen State Reactions on Freeze-Dried Foods

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The quality of freeze-dried foods depends strongly on chemical reactions and on physical phenomena which occur during freezing and during the maintenance of frozen state before and during vacuum dehydration. The following specific aspects are discussed: effects of freezing conditions on water binding by freeze-dried foods; effects of freezing on drying behavior; rates of chemical reactions in frozen foods; and effects of freezing and freeze-drying on retention of flavor in foods.

The quality of freeze-dried foods is potentially superior because the process is characterized by a low drying temperature, the preservation of food's original shape and appearance, and substantial flavor retention. These desirable characteristics depend on the maintenance of macroscopic, microscopic, and molecular morphology since low drying temperature is only practical if the rate of sublimation is substantial, even at low temperatures and vapor pressures of ice. The porosity of the dry layer must therefore be high, and there must be no excessive impedance to vapor flow due to collapsing pores and capillaries.

Preservation of shape, texture, and appearance requires that internal structure be maintained.

Flavor retention depends on the formation of microregions where flavor compounds are locked within a matrix of solids (Flink and Karel, 1970a). When this matrix is disrupted, flavor loss increases.

During freezing events occur which develop the structure and therefore predetermine the properties of subsequently dried material. Ideally, the thermal history of freeze-dried materials (Figure 1) is such that while moisture content is high, the temperature is low enough to prevent mobility and structural changes, and conversely that when the temperature finally rises, the moisture content has become low enough to achieve the same result. Freezing variables, including rate, minimum temperature during freezing, and the temperature of the frozen layer during drying, affect reactions in subsequent stages of product life.

STATE OF WATER AND OF SOLUTES IN FROZEN AND FREEZE-DRIED SYSTEMS

In foods some of the total water is bound to hydrophilic compounds and does not crystallize. Most of the water, however, does separate out during freezing in crystals of pure ice. During freeze-drying in aqueous systems containing hydrophilic polymers and smaller solutes the water partitions into three states; water bound to the polymer, ice crystals, and concentrated solution.

Among systems with this characteristic behavior is muscle, which contains proteins, water, and electrolytes. Other protein- or polysaccharide-containing foods behave similarly. In these systems, the electrolytes and other solutes may also appear in the following forms: bound to the polymer; in concentrated solutions; (or upon removal of water) in amorphous precipitates or in crystalline form. Gal (1969) studied the casein–water–NaCl system and found NaCl to separate into different forms (Table I).

The state of the water bound to polymers has been frequently investigated using various physical and physicochemical methods of analysis. In frozen systems that portion of water which remains unfrozen, while less "free" than water in solutions, is more mobile and therefore more "free" than water in ice crystals. This conclusion is supported by most recent nuclear magnetic resonance (Dehl, 1970; Kuntz *et al.*, 1969) and infrared work (Falk *et al.*, 1970).

Energy of binding of unfreezable water has also been studied. Up to 0.5 g of water per g of proteins and of other hydrophilic polymers does not freeze, but calorimetric studies on water sorbed on proteins and polymers show that only less than 0.1 g per g has a significant heat of adsorption, and that this heat of adsorption is usually less than the latent heat of fusion. There are, however, a few reported heats of adsorption, usually involving polyelectrolytes which exceeded heats of fusion (Amberg, 1957).

The thermodynamic confirmation of the greater mobility of the nonfreezing water in muscle-derived foods compared to water in ice is also shown by Riedel (1966), who studied the apparent specific heat of frozen beef. The specific heat for "bound" water (unfreezable water) calculated by Riedel was intermediate between that for ice and

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