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Some solution properties of pentagastrin and angiotensin: aggregation of pentagastrin

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Pentagastrin and angiotensin have been studied in the presence and absence of urea by light-scattering, viscosity, dialysis, surface tension and other physicochemical techniques for evidence of peptide aggregation. Pentagastrin forms large linear, flexible aggregates (molecular weight 40 000) in phosphate buffer above a concentration of $\sim 0{\cdot}1$ g dl^{-1} and in solutions shows time-dependent flow behaviour. Angiotensin shows no evidence of aggregation beyond dimers. In 1M urea the pentagastrin is in the form of dimers.

Pentagastrin (butyloxycarbonyl-β-alanyl-L-tryptophanyl-L-methionyl-L-aspartyl-L-phenylalaninamide) and the linear octapeptide angiotensin ([Asn^1, Val^5]-angiotensin II) are potent pharmacologically active peptides. The solution conformation of angiotensin II has been investigated by several techniques which have provided evidence of conformational restraints in the peptide. At concentrations as great as *ca* 25×10^{-3}M, angiotensin is monomeric from pH 2·5 to 8·6 (Paiva, Paiva & Scheraga, 1963; Fermandjian, Fromageot & others, 1972). At pH 5·6 the molecule apparently exists in a coiled form which increases in size with increase in pH (Craig, Harpenist & Paladini, 1964). The biological activity of angiotensin is also pH dependent (Franze de Fernandez, Delius & Paladini, 1968; Needleman, Freer & Marshall, 1972) but the increase in activity observed at high pH seems mainly attributable to a titratable group on the receptor (Vine, Brueckner & others, 1973). Evidence has been adduced for preferred solution conformations of angiotensin from thin-film dialysis (Ferreira, Hampe & Paiva, 1969), ^{1}H-nmr (Glickson, Cunningham & Marshall, 1972), ^{19}F-nmr, infrared and Raman spectroscopy (Fermandjian & others, 1972) and CD (Fermandjian & others, 1971); these preferred conformations are, however, dependent upon experimental conditions. The conformation of angiotensin at the receptor is a matter of speculation and both conformational transitions (Marshall, Bosshard & others, 1973; Fermandjian, Morgat & Fromageot, 1971) and aggregation phenomena have been invoked to explain the action of angiotensin at the molecular level. Inactivation of angiotensin by urea (Bumpus, Khairallah & others, 1961), which disrupts both hydrogen and hydrophobic bonds, lends credence to these views.

The physical properties of pentagastrin have, in comparison, received little attention. During experimental work on aqueous solutions of these peptides it was found that some pentagastrin solutions gelled on standing overnight. The aggregation tendencies of both peptides were investigated further by light-scattering, and the viscosity, surface-activity and dialysability of both compounds are reported here.

MATERIALS AND METHODS

Pentagastrin was a gift from ICI Pharmaceuticals used without further treatment; *Angiotensin-II-amide* was a gift from Ciba-Geigy Ltd. used as received.

Rotational viscosity measurements were made using an Epprecht-Rheomat 15 instrument (Contraves Industrial Products Ltd., Middlesex). Solutions of pentagastrin were filtered through a No. 3 sinter immediately after preparation and viscosity measurements were made at intervals up to 24 h after preparation without further filtration.

Capillary viscometry was carried out using a suspended level dilution viscometer. Solutions were filtered through Millipore or sintered glass filters.

Light scattering measurements were made over a limited concentration range (in the case of pentagastrin up to 0·2%) at 25° using a Fica 42000 photogoniodiffusiometer (A.R.L. Ltd.) at a wavelength of 546 nm after filtration of solutions through Millipore filters. Using a differential refractometer at 546 nm a refractive index increment of 0·180 ml g^{-1} was obtained for pentagastrin in buffer and a value of 0·143 ml g^{-1} was obtained for angiotensin.

Surface tensions were measured by a drop volume technique using the correction tables of Lando & Oakley (1967).

Dialysis rates were determined using a Perspex cell. One compartment containing 4 ml of pure solvent was separated from a second containing 0·5 ml of the solution under examination, by a Visking membrane with a surface area of approximately 4 cm^2. Assay of the solvent side was carried out spectrophotometrically at intervals of time using the absorbance maximum at 275 nm for angiotensin and that at 285 nm for pentagastrin.

Densities were measured in a Lipkin pycnometer of 2 ml capacity equilibrated in a thermostatted bath (±0·01°).

RESULTS

Pentagastrin

Capillary viscometry indicated that pentagastrin exists in an aggregated form (Fig. 1) at concentrations higher than 0·1 g $litre^{-1}$ and that deaggregation occurs below this concentration. Correction of the viscosity findings for the "monomer"

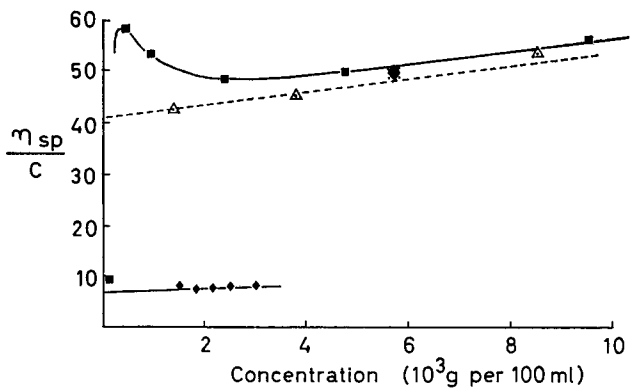

FIG. 1. Plots of reduced specific viscosity of pentagastrin in buffer pH 7·4 (■) and in 1M urea buffer mixtures (◆). The dotted line (– – –) represents the reduced specific viscosity corrected assuming monomer concentrations of 0·1%. Large symbol on upper line is from rotational viscosity measurements to show agreement.

concentration (taken to be 0·1 %) results in a modified intrinsic viscosity of 45 ml g^{-1} but the Huggins constant (K_H) calculated from the equation (Tanford, 1962)

$$\eta_{sp}/C = [\eta] + K_H[\eta]^2C$$

increases from 0·66 to 0·94. For solid uncharged spheres K_H is approximately 2, while for flexible polymers in good solvents K_H is often near 0·35. Higher values occur in poor solvents. The results are therefore compatible with pentagastrin behaving as a flexible "polymer" in a solvent which causes it to aggregate. Urea (1M) breaks down the aggregates, the intrinsic viscosity falling from ~45 ml g^{-1} to 7·7 ml g^{-1} (Fig. 1). Because of the time-dependent nature of the pentagastrin aggregation and the high intercepts obtained, rotational viscosity measurements were made as a function of time and shear rate. Fig. 2 shows the change in flow behaviour for a 0·57 % solution (the solubility limit of pentagastrin is approximately 0·97 % in ammonia solution). At 1 h the solution behaves as a Newtonian system and gives a reduced specific viscosity in agreement with capillary viscosity values (see Fig. 1). After 2–3 h the solution becomes thixotropic and exhibits shear-thinning phenomena. After 24 h the solution shows plastic flow behaviour with a yield stress of 35 dyne cm^{-2} (Fig. 2). In the presence of urea (1M), confirming the capillary viscometry, the solution retains its Newtonian flow properties with no significant viscosity change after keeping for 24 h. Increasing temperature had little effect on the size of the pentagastrin aggregates (as measured by the reduced specific viscosity) although at 34° gelling could not be observed in the chamber of our nmr instrument.

Light-scattering measurements on pentagastrin in buffer and pentagastrin in buffer +urea affirm the above results. In buffer there is an apparent aggregation con-

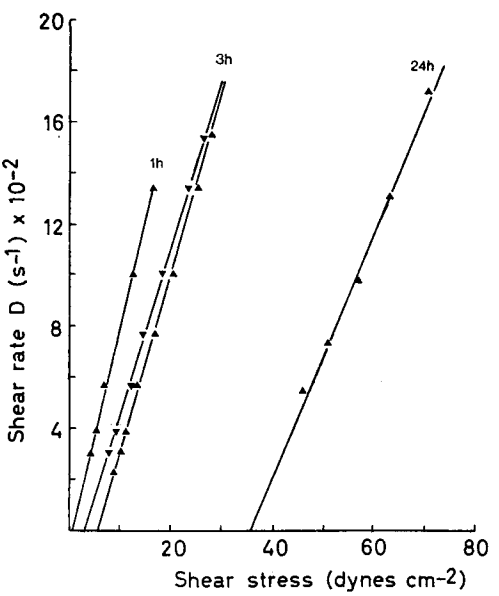

FIG. 2. Flow behaviour (shear rate *vs* shear stress) with time for a 0·57 % w/v solution of pentagastrin in buffer. At 1 h the solution exhibits Newtonian flow characteristics with a relative viscosity in agreement with values obtained using capillary viscometry. After 2–3 h the solution becomes thixotropic and exhibits shear thinning. After 24 h the solution shows plastic flow properties, with a yield stress of 35 dyne cm^{-2}.

centration at 0·093% at which there is a rapid rise in scattered light intensity (Fig. 3). The molecular weights, calculated in the normal manner, were 40 000 in the absence of urea and 1500 in 1M urea. These molecular weights are equivalent to aggregation numbers of 56 and 2 respectively. Filtration procedures for light-scattering had to be thorough, and as filtration through Millipore filters has some effect on intrinsic viscosity, the absolute aggregation number of the pentagastrin in buffer may be higher than 56. In addition the experiments were confined to concentrations below 0·2% thus any concentration-dependency of the molecular weight could not be estimated.

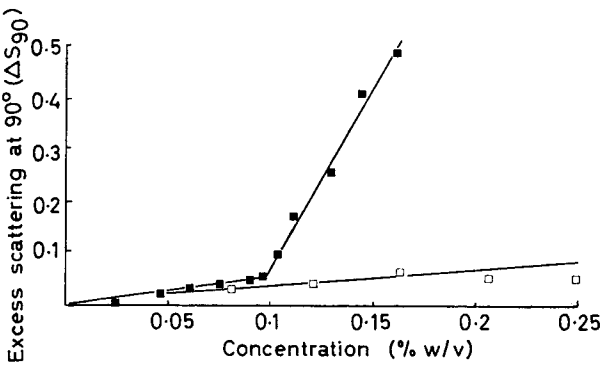

FIG. 3. Pentagastrin light-scattering (ΔS_{90}) plots vs solution concentration in buffer (■) and (□) 1M urea solution. Pronounced changes occur at 0·1% in buffer.

The sharp break in the light-scattering ΔS_{90} *vs* concentration plot is reminiscent of the behaviour of classical amphipathic surfactant molecules (Elworthy, Florence & Macfarlane, 1968). The pentagastrin molecule is not obviously amphipathic in nature, although many proteins and peptides are highly surface-active molecules (Pearson & Alexander, 1968). Surface tension measurements show that pentagastrin is surface-active (Fig. 4), but there is no typical inflection in the surface tension curve at the concentration which from light-scattering one might have inferred to be a critical micelle concentration. Using the normal form of the Gibbs' adsorption

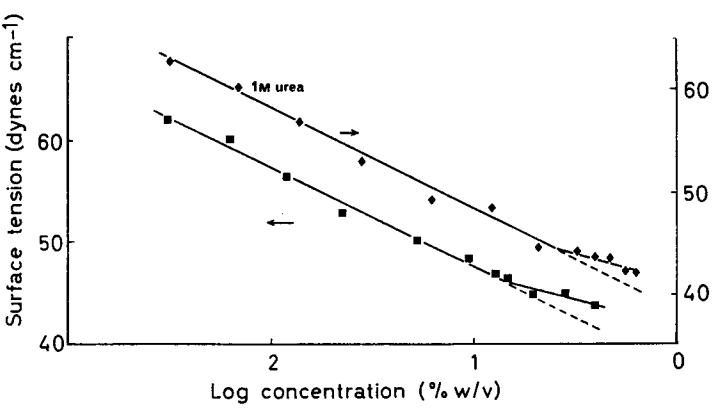

FIG. 4. Surface-tension-log (concentration) plots for pentagastrin: lower plot in phosphate buffer (left hand ordinate) (■), upper plot in 1M urea (◆) with values displaced, (right hand ordinate). An apparent break occurs at about 0·14% in buffer and at higher concentration in urea.

equation, an area per adsorbed molecule of 97Å^2 was obtained. This is not affected by urea (100Å^2).

Density measurements on pentagastrin solution showed a maximum at about 0·1 %, illustrating the abnormal behaviour of these solutions.

Fig. 5 shows the change in rate of dialysis of pentagastrin as a function of its concentration. Solutions had been freshly prepared for these measurements and the most concentrated had not gelled. A decrease in rate of dialysis can be perceived around 0·1 % suggesting an increase in the size of the kinetic units.

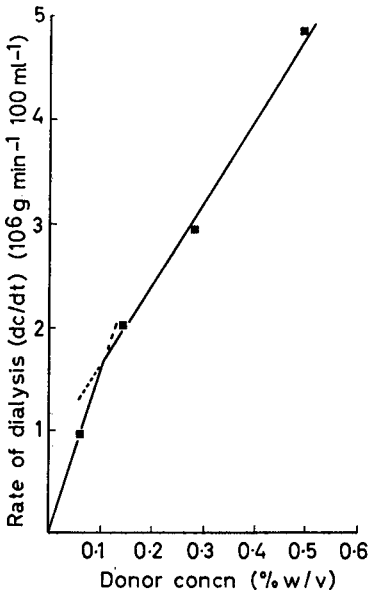

FIG. 5. Pentagastrin dialysis result: rate of dialysis of pentagastrin as a function of solution concentration to donor cell.

The final corroborative evidence of the aggregation tendencies of pentagastrin was the concentration dependence of absorbance (Fig. 6) at 280·5 and 287 nm. On aggregation the environment of the groups responsible for absorbing in this region (that is the tryptophanyl and phenylalanyl residues) evidently changes. As both of these groups are likely to be involved in hydrophobic associations this is not surprising.

Angiotensin shows none of the tendencies to aggregate that pentagastrin does. At most, the molecule forms dimers. Light-scattering results indicate a molecular weight of $2{\cdot}4 \times 10^3$ but are not sufficiently accurate to permit an unequivocal statement. An intrinsic viscosity of 3·8 in buffer and 3·0 in buffer/urea mixtures (Fig. 7) may indicate dissociation of a dimer or else a conformational change. The partial specific volume increases on addition of urea. Dialysis and ultraviolet spectrophotometry give no indication of a concentration-dependent aggregation phenomenon. Solutions of angiotensin are, however, surface-active, a 0·4 % w/v solution having a surface tension of 50 mN m^{-1} but solutions show no break which might indicate aggregation. It is possible, however, that our experimental methods are insufficiently sensitive to distinguish monomer-dimer equilibria, especially if this occurs at low concentrations.

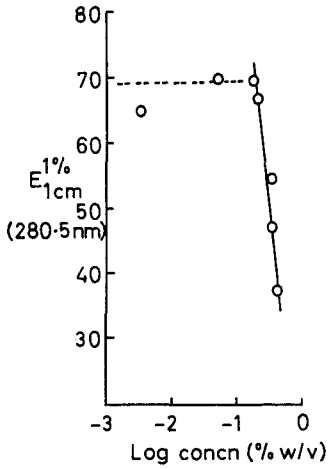

FIG. 6. E (1%, 1cm) values for pentagastrin solution in buffer as a function of the logarithm of the solution concentration, showing pronounced decrease in value above 0·14%.

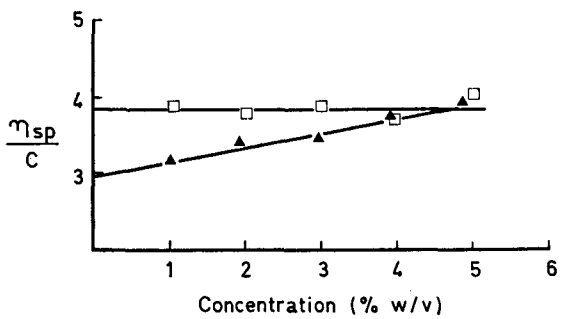

FIG. 7. Reduced specific viscosity of angiotensin in buffer (□) and in 1M urea (▲) indicating near spherical units in solution.

DISCUSSION

The overall aggregation of haemoglobin leading to its gelation has been regarded as a two-step process. Linear aggregation of the protomers to rod-like oligomers precedes lateral association of the formed rods (Minton, 1973). Similar processes must be occurring in pentagastrin solutions. The pentagastrin molecule has a molecular weight of 768. The axial ratio of the aggregated species is about 19 as calculated from the shape factor ν in Oncley's equation (Tanford, 1962), viz.

$$[\eta] = \nu[\bar{V}_2 + \omega\bar{V}] \text{ when } \bar{V}_2 = 0{\cdot}8 \text{ and } \omega = 0{\cdot}2\text{g g}^{-1}$$

while the axial ratio of the monomer in urea is approximately 4. That solutions of pentagastrin gel at low concentration (volume fraction $\emptyset < 0{\cdot}01$) is indicative of linear associations. If the association occurs as shown in Fig. 8, 56 monomers assembled in this way would have an axial ratio of 14, very close to that determined experimentally.

The low intrinsic viscosity of angiotensin II is in agreement with the conclusions of Craig & others (1964) that angiotensin is a compact molecule of "near minimal

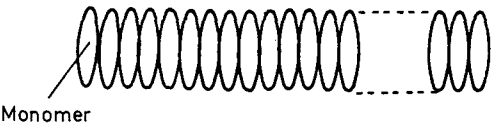

FIG. 8. Diagrammatic representation of pentagastrin monomers in a linear aggregate.

axial ratio". Increase in partial specific volume on the addition of 1M urea indicates a weakening of hydrogen-bonded structures rather than a breakdown of hydrophobic bonds. The latter should result in a decrease in volume if the newly exposed hydrophobic residues return to an aqueous environment.

It would be essential to carry out similar measurements on active and inactive analogues of pentagastrin before the biological significance of these results could be suggested. If the active species of either pentagastrin or angiotensin were some multimer then those residues responsible for the association would be critical in determining the activity of the peptide. Paiva & others (1963) concluded that at the concentrations at which angiotensin exerted its physiological actions the monomer is probably the species that interacts with the cellular receptor. As it is only at physiologically unrealistic concentrations that pentagastrin forms aggregates, it is likely that the association has no physiological significance except in so far as it indicates the propensity of the molecule to participate in associative hydrophobic interactions with other molecules.

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