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Self-Association of Glucagon. Equilibrium Studies*

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ABSTRACT: The self-association of glucagon at pH 10 (0.2 M phosphate buffer, 25°) has been investigated by means of sedimentation equilibrium, gel filtration, and concentration difference spectra over as wide a range of concentration as allowed by the solubility of glucagon (up to 0.3 g/dl).

The results are consistent with a monomer–dimer–hexamer mode of association, and equilibrium constants and changes in extinction coefficients have been calculated for dimerization and hexamerization. The equilibrium association constants are 3.3 $\times 10^3$ M^{-1} and 10.9 $\times 10^{17}$ M^{-5}, respectively. The large change in extinction coefficient near 250 mμ associated with dimerization suggests that this step involves a major conformational change in the glucagon molecule. Optical rotatory dispersion measurements of previous investigators indicate this conformational change probably involves a random coil to α-helix transition.

The pancreatic hormone glucagon is one of the smallest naturally occurring polypeptides capable of forming α-helical structure. It contains 29 amino acid residues in known sequence and has a molecular weight of 3500 (Behrens and Bromer, 1958). Gratzer *et al.* (1968) have shown that by a number of criteria, chiefly optical rotatory dispersion, the glucagon molecule appears to exist largely in a random coil form in dilute aqueous solutions at most pH values. However, a partly α-helical form of glucagon appears to be stable in 2-chloroethanol (Gratzer *et al.*, 1968) and in nearly saturated aqueous solutions above pH 8 (Blanchard and King, 1966; Gratzer *et al.*, 1967). Because helical structure is only observed at high concentrations, the suggestion has been made that the coil-to-helix transition is induced by an aggregation of glucagon molecules. Similar aggregation-induced conformational changes have been observed with synthetic polypeptides (*cf.* Hammes and Schullery, 1968). The study of aggregation-induced conformational changes represents an approach to the understanding of factors controlling the nonprimary structures of complex biological systems.

Previous studies of glucagon self-association, which were based on measurements of optical rotatory dispersion and concentration difference spectra, only permitted qualitative conclusions about the aggregation. In this work sedimentation equilibrium and gel filtration experiments on glucagon solutions at pH 10 were used to quantitatively study the aggregation, and the results were used as a basis for interpreting more extensive measurements of the concentration difference spectra. In the concentration range studied (which could be extended up to about 0.3 g/dl using supersaturated glucagon solutions) the data were consistent with an association of glucagon monomers to form dimers and hexamers. Furthermore the major portion of the spectral change, and therefore presumably the conformational change, occurs in the dimerization step of the association.

Experimental Section

The crystalline glucagon used for most of this work was obtained from Sigma Chemical Co. (batch no. 97B-0080). Some experiments were performed with material obtained from Calbiochem, and the initial sample was generously donated by Lilly Research Laboratories Ltd. The three samples appeared identical by polyacrylamide gel electrophoresis at pH 9.5, each exhibiting only a small trace of a faster migrating impurity, probably the desamido peptide (Staub *et al.*, 1955).

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The buffer used throughout this work was 0.2 M Na_2HPO_4 adjusted to pH 10.0 with concentrated NaOH. Solutions of glucagon in this buffer were prepared by warming to about 50° (glucagon dissolves very slowly at room temperature under most conditions) and readjusting the pH to 10.0 after cooling to 25°. Glucagon concentrations were determined spectrophotometrically on the basis of an extinction coefficient of 23.0 dl cm^{-1} g^{-1} at 280 mμ (Kay and Marsh, 1959). Concentration difference spectra (see Results section) indicated that no significant error would be introduced by the assumption of a constant extinction coefficient at this wavelength. Absorbance measurements were made in a Zeiss Model PMQ II spectrophotometer; pH measurements were made with a Radiometer pH meter Model 26.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were carried out at 25° in a Spinco Model E ultracentrifuge, using the Rayleigh interference optical system and a 12-mm cell with sapphire windows. The procedures used were essentially those described by Richards *et al.* (1959, 1968). However, some modifications were necessitated by the low solubility of glucagon, which dictates the use of initial solute concentrations around 0.1 g/dl, and the low molecular weight of the glucagon monomer, which precludes dialysis. Under these conditions the initial concentration could not be determined reliably by direct refractometry in a synthetic boundary cell. Instead the concentration was determined spectrophotometrically in a 0.1-cm light-path cell, and converted into the required units of fringes, j, in the ultracentrifuge cell using the expression $j = kcl/\lambda$, where k is the refractive index increment for a 1-g/dl glucagon solution (calculated from Kay and Marsh (1959) to be 1.88×10^{-3} at 546 mμ), c is the glucagon concentration in grams per deciliter, l is the light path of the ultracentrifuge cell (found by micrometer measurement to be 1.202 cm when tightened), and λ is the wavelength of the refracted light, 546 mμ. Furthermore, since the glucagon concentration near the bottom of the cell approached saturation (approximately 0.2 g/dl at 25°) precipitation was always likely. Accordingly, the "initial concentration" required for the computation of absolute concentrations by the mass conservation principle was determined after the equilibrium run. Immediately on completion of the run the solution was remixed in the cell by inverting the rotor several times. Glucagon was found to dissolve so slowly at room temperature, even in an excess of buffer, that this procedure would be unlikely to redissolve any significant amount of precipitated material. The rotor was then accelerated again to the equilibrium speed at which time a "blank" photograph was taken to correct for any fringe displacements due to cell distortion. The rotor was then decelerated before appreciable sedimentation could occur, and a sample of solution was withdrawn from the cell for concentration measurement. Solution columns of 0.5–0.6 cm were used, and equilibrium was attained in 24–36 hr using the overspeeding and underspeeding technique (Richards *et al.*, 1968). Attainment of equilibrium was verified by comparing measurements on the final fringe pattern with those on photographs taken up to 12 hr before the end of the run. Rayleigh patterns were measured on a Gaertner two-dimensional comparator. Since at such low concentrations the total concentration change across the cell only amounted to five or six fringes, measurements of fringe number against radial distance were made at four different vertical levels on the photographic plate. Then from detailed measurements of fringe displacements near the meniscus the four sets of readings were combined to give 20–25 pairs of values for fringe number against distance. Blank corrections were then applied to these values.

Gel Filtration. A 12 × 1.0 cm Sephadex G-50 column at an ambient temperature of 22–25° was used for gel filtration. Glucagon solutions were applied in sufficient volume to produce a clearly defined plateau region in the elution profile. Fractions of about 0.7 ml were collected in tared tubes using a Gilson photoelectric fraction cutter and collector. Each tube was reweighed and the fraction volume calculated using a density of 1 g/ml. Fractions were analyzed spectrophotometrically at 280 mμ using a 1-cm path-length cell at low concentrations and a 0.1-cm path-length cell at high concentrations. From a graph of concentration *vs.* eluted volume, the elution volume corresponding to the equivalent sharp boundary for the leading edge of the plateau region (*cf.* Longsworth, 1943) was calculated by trapezoidal integration. Although trapezoidal integration was used, the observed boundaries were quite sharp and did not suggest the occurrence of a slow aggregation process.

Concentration difference spectra were measured at 25° in the Zeiss spectrophotometer. Rectangular tandem cells (Pyrocell Manufacturing Co.) having 4.4-mm path lengths for each compartment were used at high concentrations; similar cells of 10-mm path length were used for glucagon concentrations below 0.1 g/dl. The slit width was maintained at 0.2 mm except for a few qualitative measurements at wavelengths below 240 mμ. Each concentration difference spectrum was measured for a twofold dilution, *i.e.*, one set of tandem cells contained glucagon of concentration c in one compartment and buffer in the other while the second set contained glucagon of concentration $c/2$ in both compartments. By successive dilutions the entire accessible concentration range from 0.33 g/dl (a supersaturated solution at 25°) down to 0.01 g/dl was covered. The difference spectra were time independent, indicating equilibration of the solution occurs at least within a few minutes. Solutions for difference spectra were filtered through Millipore filters. Cell blank corrections were determined several times with buffer in all compartments; measurements at 360 mμ indicated that no corrections were necessary for differential light scattering.

Results and Treatment of Data

Sedimentation Equilibrium. From the values of fringe number, j, determined at distances, x, from the axis of rotation, weight-average molecular weights, $\bar{M}_{wj}$, were evaluated from the expression

$$\bar{M}_{wj} = \frac{2RT(\mathrm{d} \ln j/\mathrm{d}(x^2))}{\omega^2(1 - \bar{v}\rho)} \qquad (1)$$

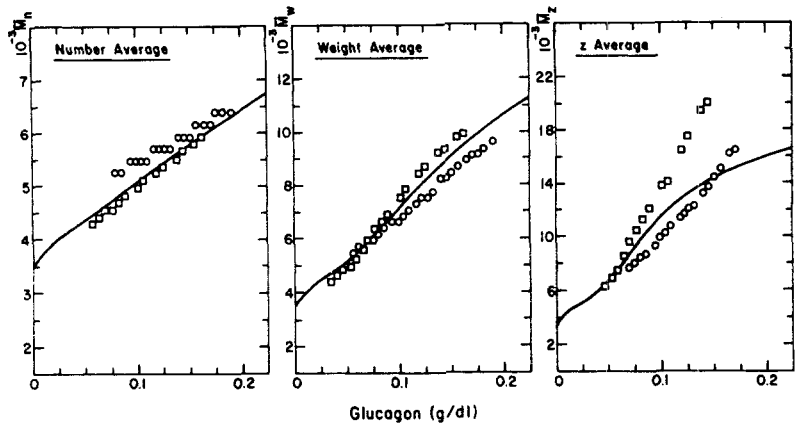

FIGURE 1: Plots of number-average, weight-average, and z-average molecular weights as a function of glucagon concentration obtained from sedimentation equilibrium in 0.2 M sodium phosphate (pH 10.0) at 25°. The circles show values obtained from an experiment where the initial glucagon concentration was 0.10 g/dl and the rotor speed 19,160 rpm; the squares show values obtained with an initial concentration of 0.067 g/dl and a rotor speed of 24,630 rpm. The solid lines are theoretical curves calculated for a monomer–dimer–hexamer association using the equilibrium constants given in Table I.

where R is the gas constant, T is the absolute temperature, ω is the angular velocity of the rotor in radians per second, $\bar{v}$ is the partial specific volume of glucagon (calculated from the amino acid composition (Behrens and Bromer, 1958) to be 0.71 cc/g), and ρ is the density of the solution. For such dilute protein solutions as we have used ρ may be taken as the density of the buffer which was determined by pycnometry to be 1.025 g/ml. In assuming that eq 1 gives the intrinsic weight-average molecular weight, we have neglected charge and nonideality effects. Such effects are, however, small for a system such as this where the protein concentration is less than 0.2 g/dl and a moderately concentrated buffer is used at a pH not far from the isoelectric point (Jeffrey and Coates, 1966). Furthermore, studies by Albright and Williams (1968) on a system exhibiting strong nonideality showed that at protein concentrations below 0.2 g/dl, corrections for nonideality had little effect on apparent molecular weights obtained from eq 1. The derivative d ln j/d(x^2) was evaluated by fitting the data to a local cubic equation using an iterative weighting procedure. A computer program for this calculation has been developed by Yphantis.[1] This computer program also provides number-average, $\bar{M}_n$, and z-average, $\bar{M}_z$, molecular weights using a modification of the procedure described by Yphantis (1964). Figure 1 shows the three types of average molecular weight plotted against glucagon concentration. The errors estimated as standard deviations within each of the individual experiments are about 2–3% in $\bar{M}_n$ and $\bar{M}_w$ and range up to 6% in $\bar{M}_z$. The noticeably high $\bar{M}_z$ values obtained near the bottom of the cell in the experiment at the higher speed have errors three to four times as large as the points at corresponding concentrations in the experiment at lower speed and were accordingly given less weight in the analysis. The reproducibility between runs at different initial concentrations and rotor speeds is not as good as might be expected from the internal precision within each run. Although this must to some extent reflect the difficulty of using the interferometric technique at such low solute concentrations, it is also a likely indication of heterogeneity, *i.e.*, the presence in the sample of a small amount of a species with differing association behavior.

Reversible association is clearly indicated by the increase in all the molecular weight averages with increasing solute concentration. The association behavior was analyzed from equations formulated by Adams (1965, 1967) which, with the assumption of ideal solution behavior, can be written as follows in terms of the various apparent average molecular weights obtained experimentally.

$$c = \sum_i K_i c_1^i \tag{2}$$

$$cM_1/\bar{M}_n = \sum_i (K_i c_1^i/i) \tag{3}$$

$$c\bar{M}_w/M_1 = \sum_i iK_i c_1^i \tag{4}$$

$$c\bar{M}_w\bar{M}_z/M_1^2 = \sum_i i^2 K_i c_1^i \tag{5}$$

where M_1 is the molecular weight of the glucagon monomer (3500), c is the total glucagon concentration, c_1 is the concentration of glucagon monomer, i is the number of monomer molecules combined in a particular aggregate, and K_i is the equilibrium constant for the association of i molecules of *monomer* to that aggregate, *i.e.*, $K_i = c_i/c_1^i$. For as many as four aggregated species (including the monomer) these four equations can be combined to give a value of c_1 at any c. The best equilibrium constants for a particular association scheme under consideration can then be obtained from plots of the appropriate functions of c_1 against c. These equilibrium constants were used to compute theoretical curves for $\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$ as a function of c. Small vari-

[1] We are greatly indebted to Professor D. A. Yphantis for processing our data with his computer program.

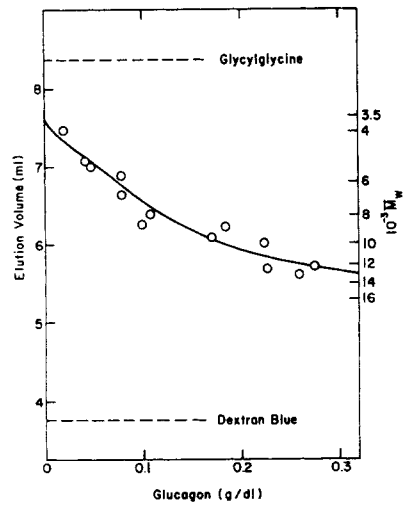

FIGURE 2: Variation of elution volume with concentration for glucagon in 0.2 M phosphate buffer (pH 10.0) on a Sephadex G-50 column. The weight-average molecular weight scale on the right is calculated using the empirical relation given by eq 6; the solid line represents values calculated from this expression using theoretical $\bar{M}_w$ values for a monomer–dimer–hexamer equilibrium with the association constants given in Table I.

ations in the K's were then made in order to obtain the best possible fit to the weight-average molecular weight curve. The number-average and z-average molecular weights are less directly related to the primary experimental data and should thus be given considerably less weight. Equations 2–5 are derived on the basis of a weight concentration scale rather than a molar concentration scale; therefore the calculations can be conveniently carried out in terms of concentrations expressed as grams per deciliter, and the equilibrium constants can afterwards be converted to the usual molar concentration units.

The simplest association scheme giving a satisfactory fit to the data was one in which the glucagon monomer associates to form dimers and then hexamers of the 3500 molecular weight unit. Figure 1 shows the theoretical molecular weight curves calculated for a monomer–dimer–hexamer scheme with the association constants given in Table I. From the effect of small variations in K on the behavior of the theoretical curves the error in each K is estimated to be about $\pm 15\%$. A monomer–trimer–hexamer scheme was found to be inconsistent with the data, and the inclusion of trimers or tetramers in the monomer–dimer–hexamer scheme could not be made to improve the fit. In fact a noticeably worse fit was obtained if the choice of equilibrium constants was such as to make the weight proportions of trimer or tetramer more than 10% anywhere in this concentration range.

Gel Filtration. The low solubility of glucagon unfortunately precludes the determination of molecular weight by sedimentation equilibrium at concentrations greater than 0.2 g/dl. Glucagon, however, readily forms supersaturated solutions which are stable for at least 1 hr at 25° at concentrations up to about 0.3 g/dl. This provides enough time to conduct a gel filtration chromatography experiment on a short column. We have therefore used gel filtration as a guide to the association behavior of glucagon in supersaturated solutions in order to provide a further basis for the interpretation of concentration difference spectra. Figure 2 is a graph of elution volume determined as described in the previous section against concentration of glucagon in the plateau region of the elution profile. Elution volumes are also shown for dextran blue, a large molecule which should be completely excluded from the gel particles, and glycylglycine, a small molecule which should be completely included.

TABLE I: Equilibrium Constants for Association of Glucagon Monomers to Dimers and Hexamers at 25° in 0.2 M Phosphate Buffer (pH 10.0).

Assocn Reaction	K
$2G \rightleftharpoons G_2$	$3.3 \times 10^3\ M^{-1}$
$6G \rightleftharpoons G_6$	$10.9 \times 10^{17}\ M^{-5}$

Molecular weights on nonassociating proteins can be obtained from gel filtration data making use of an empirical linear relationship which usually exists between elution volume and the logarithm of the molecular weight. Marker proteins of known molecular weight are used to calibrate the column. However, Winzor and Scheraga (1963, 1964) have shown that this method fails when applied to reversibly associating proteins, since in these cases elution volume depends upon other factors, as well as upon molecular weight. These investigators found that empirical expressions could be used to relate weight-average molecular weight and elution volume for such systems over quite wide concentration ranges provided calibration was done not by marker proteins but by molecular weight measurements on the particular protein. For glucagon, where we have both molecular weights and elution volumes over the concentration range 0.04–0.18 g/dl, it was found that weight-average molecular weight, $\bar{M}_w$, and elution volume, V_e, were related within the experimental error (± 0.2 ml in V_e and ± 500 in $\bar{M}_w$) by the expression

$$V_e = 20.3 - 3.57 \log \bar{M}_w \qquad (6)$$

This expression was used together with $\bar{M}_w$ values calculated for the monomer–dimer–hexamer system described by the parameters in Table I to calculate a curve of V_e against concentration. The resultant curve is shown in Figure 2 from which it is seen that the data are satisfactorily fit up to a concentration of 0.28 g/dl. Thus the monomer–dimer–hexamer association scheme appears to be sufficient to describe the behavior of glucagon in supersaturated solutions under these conditions.

Concentration Difference Spectra. Typical concentration difference spectra observed with glucagon are shown in Figure 3. The outstanding feature is a large

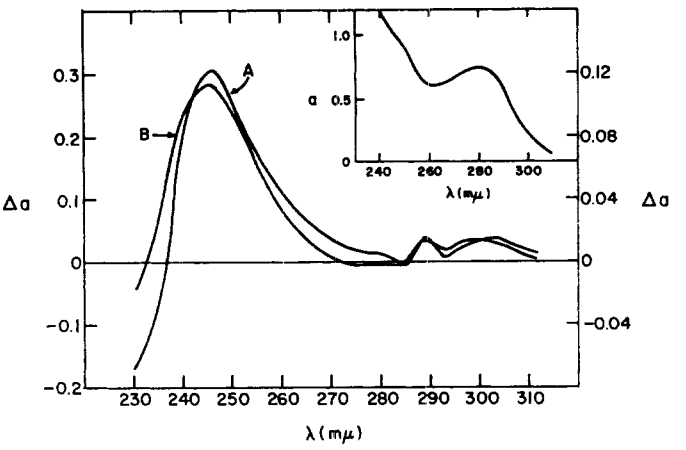

FIGURE 3: Concentration difference spectra of glucagon in 0.2 M phosphate buffer (pH 10.0) and 25°. Spectrum A (absorbance scale on left): 0.105-g/dl solution measured against 0.210-g/dl solution as reference using 4.4-mm path-length tandem cells. Spectrum B (absorbance scale on right): 0.0276-g/dl solution measured against 0.0552-g/dl solution as reference using 10-mm path-length tandem cells. The top right inset shows the ultraviolet spectrum of glucagon (0.033 g/dl in a 10-mm cell) under these conditions.

maximum at 245 mμ. To characterize this spectral change in terms of the proposed association mechanism, detailed calculations of the change in extinction coefficient were made for the measurements at 250 and 260 mμ. The changes are larger at 250 mμ, but at 260 mμ, where the absorption by glucagon is less, measurements can be made at higher concentrations before stray light errors occur. The change in extinction coefficient at any wavelength, $\Delta\epsilon_d$, for each dilution is given by the expression $\Delta\epsilon_d = \Delta a/cl$ (Fisher and Cross, 1965), where Δa is the measured absorbance difference, c is the concentration of either solution, and l is the corresponding path length. Dilutions from the highest to the lowest concentration in each series were made in four twofold steps. Thus by summing the $\Delta\epsilon_d$ values, the extinction coefficient change at any concentration was obtained relative to the lowest concentration in that series. Several series of dilutions were made from initial solutions of different concentrations, and since the concentration ranges overlapped, the extinction coefficient changes at all concentrations can be plotted on a single graph. Figure 4 shows the graphs obtained from the data at both wavelengths. Initially, the extinction coefficient changes could only be expressed as $\Delta\epsilon'$, the change in ϵ relative to that at some fixed concentration (the lowest was chosen in order to make all $\Delta\epsilon'$ values of the same sign). Extrapolation to zero concentration must be based on a postulated mechanism as shown below. The values of $\Delta\epsilon_d$ from the two tandem cells of different path length agree well up to a concentration of 0.15 g/dl at 260 mμ, and up to 0.10 g/dl at 250 mμ. At higher concentrations, absorbances measured in the 10-mm cells were low, indicating a stray light error. Absorbances measured in the 4.4-mm cells should therefore be reliable up to 10.0/4.4 times these concentrations, *i.e.*, about 0.34 g/dl at 260 mμ and 0.23 g/dl at 250 mμ.

Assuming any glucagon solution to be an equilibrium mixture of monomer, dimer, and hexamer, the absorbance is given by

$$a = (\epsilon_1 c_1 + \epsilon_2 c_2 + \epsilon_6 c_6)l$$

where ϵ_i and c_i denote the extinction coefficient and concentration of the aggregate containing i molecules of monomer. The total concentration, c, on a gram-per-deciliter scale is the sum of c_1, c_2, and c_6, so that

$$a = (\epsilon_1 c - \Delta\epsilon_2 c_2 - \Delta\epsilon_6 c_6)l$$

where $\Delta\epsilon_2 = \epsilon_1 - \epsilon_2$ and $\Delta\epsilon_6 = \epsilon_1 - \epsilon_6$ (the experimentally observed spectra indicate that the monomer would be the species of highest extinction coefficient on a weight basis). An analogous equation can be written for the fixed concentration, c^*, so that $\Delta\epsilon'$, the difference between apparent extinction coefficients at concentrations c and c^*, is given by

$$\Delta\epsilon' = a^*/c^*l^* - a/cl$$

$$= \Delta\epsilon_2 c_2/c + \Delta\epsilon_6 c_6/c - (\Delta\epsilon_2 c_2^*/c^* + \Delta\epsilon_6 c_6^*/c^*)$$

The term in brackets on the right is a constant which can be designated $\Delta\epsilon^*$, and c_2 and c_6 are related to c_1 by the equilibrium equations. Hence

$$\Delta\epsilon' = K_2\Delta\epsilon_2 c_1^2/c + K_6\Delta\epsilon_6 c_1^6/c - \Delta\epsilon^* \qquad (7)$$

At low concentrations the c_1^2/c term should greatly outweigh the c_1^6/c term (*i.e.*, very little hexamer is present). Thus eq 7 predicts that a plot of $\Delta\epsilon'$ *vs.* c_1^2/c should be almost linear at low concentrations and curve upwards at higher concentrations as the c_1^6/c term becomes important. The slope of the linear portion should be $K_2\Delta\epsilon_2$ and the intercept at $c_1^2/c = 0$ (*i.e.*, zero concentration) should be $-\Delta\epsilon^*$. A graph of $(\Delta\epsilon' - K_2\Delta\epsilon_2 c_1^2/c + \Delta\epsilon^*)$ *vs.* c_1^6/c should then be linear with slope $K_6\Delta\epsilon_6$. This procedure suggested by eq 7 was applied to the spectral data. The $\Delta\epsilon'$ values were read from a smooth curve drawn through the points and pairs of values of c_1 and c were calculated from eq 2 using the equilibrium constants found from the molecular weight measurements previously discussed. Figure 5 shows the two plots for the 250-mμ data, and illustrates that eq 7 describes the data very well. In particular, the good linearity of the c_1^6/c plot provides a convincing confirmation of the monomer–dimer–hexamer association scheme. The theoretical curves shown in Figure 4 were then computed from eq 7 using c_1 and c values found from eq 2. The $\Delta\epsilon$ plotted in Figure 4 is $(\Delta\epsilon' + \Delta\epsilon^*)$ which is, of

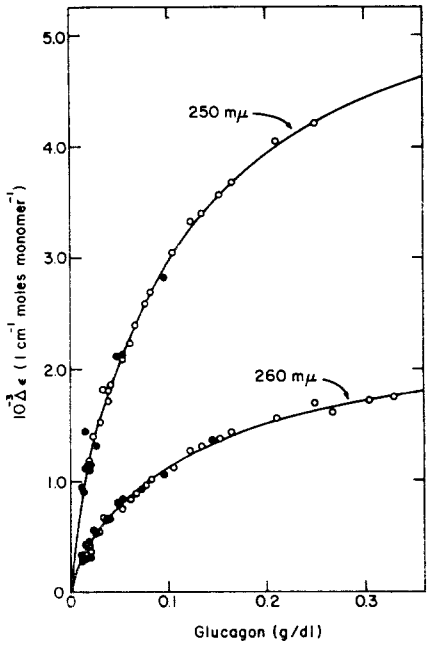

FIGURE 4: Change in extinction coefficient of glucagon from that at zero concentration *vs.* concentration of glucagon in 0.2 M phosphate buffer (pH 10.0) at 25°. $\Delta\epsilon$ is calculated in terms of the molar concentration of glucagon monomer. The solid lines are theoretical curves calculated as described in the text for an assumed monomer–dimer–hexamer association. Open circles: 4.4-mm path-length tandem cells; filled circles: 10-mm path-length tandem cells.

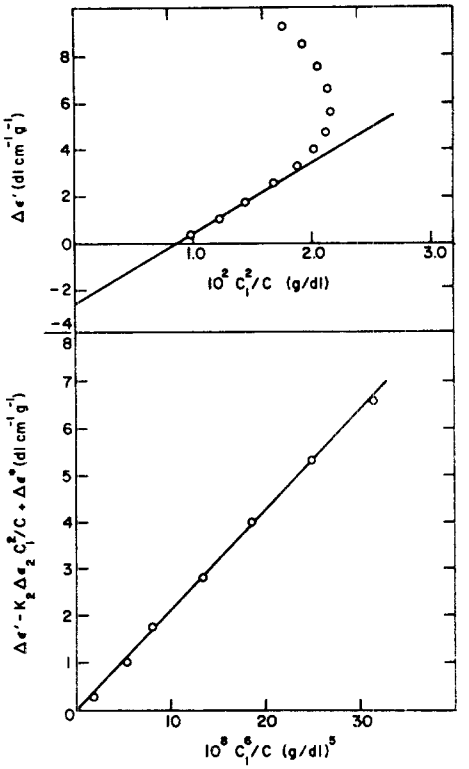

FIGURE 5: Upper: Plot of $\Delta\epsilon'$, the difference in ϵ from that at one fixed concentration *vs.* (monomer concentration)^2/total concentration. Lower: Plot of the deviations of $\Delta\epsilon'$ from the linear part of the upper figure *vs.* (monomer concentration)^6/total concentration. The slopes of the lines are related according to eq 7 to the equilibrium constants and extinction coefficient changes for dimerization and hexamerization.

course, the decrease in extinction coefficient relative to that at *zero* concentration, *i.e.*, to that of the monomer. In addition, $\Delta\epsilon$ values are calculated for a molar concentration scale. The parameters $\Delta\epsilon_2$ and $\Delta\epsilon_6$ are readily obtained from the graphs based on eq 7 and the previous determined equilibrium constants. Calculated for a 1 M solution of glucagon monomer, the values are $\Delta\epsilon_2^{260}$ $(= \epsilon_1 - {}^1/_2\epsilon_2) = 2.0 \times 10^3$, $\Delta\epsilon_6^{260}$ $(= \epsilon_1 - {}^1/_6\epsilon_6) = 2.4 \times 10^3$, and $\Delta\epsilon_2^{250} = 5.4 \times 10^3$, $\Delta\epsilon_6^{250} = 6.0 \times 10^3$ cm^{-1} M^{-1} where ϵ_1, ϵ_2, and ϵ_6 are molar extinction coefficients. In view of the estimated 15% error in the K's, these $\Delta\epsilon$ parameters can only be considered accurate to within ±20%. Nevertheless, it seems clear that the results from concentration difference spectra are quantitatively accounted for by the monomer–dimer–hexamer association hypothesis and indicate that most of the spectral change is associated with the dimerization step.

Discussion

Although a monomer–dimer–hexamer mechanism is consistent with the molecular weight data, the presence of aggregates larger than hexamer and/or intermediate between dimer and hexamer cannot be ruled out because of the difficulty in obtaining molecular weights over a sufficiently wide concentration range. However, the fact that the more precise spectral data also quantitatively fit a monomer–dimer–hexamer mechanism affords strong support to this scheme. The formation of a hexameric aggregate in solution is not inconsistent with the results of X-ray studies of glucagon crystals (King, 1959, 1965) which have shown a threefold symmetry axis. However, the results presented here do not support the suggestion by Blanchard and King (1966) that a glucagon trimer might exist in concentrated solutions.

The spectral change occurring on aggregation of glucagon is much larger and predominates at wavelengths considerably shorter than reported by Blanchard and King (1966). The small side bands at wavelengths longer than 280 mμ (Figure 3) were the only features observed by these investigators. A stray light error resulting from the use of moderately concentrated solutions in cells of long path length may have obscured the spectral features at shorter wavelength in their experiments. The large magnitude of the spectral effect which we have observed around 245 mμ is well illustrated by comparison of the $\Delta\epsilon$ values with the extinction coefficients of the monomer at corresponding wavelengths. The latter are readily calculated from the measured extinction coefficients at some concentration and the $\Delta\epsilon$ values given in Figure 4. The extinction coefficients of the monomer are 7.2×10^3 cm^{-1} M^{-1} at 260 mμ and 11.0×10^3 cm^{-1} M^{-1} at 250 mμ. Comparison with the $\Delta\epsilon$ values in the previous section shows that the association of monomer to hexamer is accompanied by a decrease in extinction coefficient of over 30% at 260 mμ and over 50% at 250 mμ. Moreover, about 90% of this change occurs in the

monomer to dimer step of the aggregation. In this wavelength region difference spectra of proteins reflect changes in the conformation of the polypeptide backbone as well as in the environment of aromatic chromophoric residues. The latter are almost the entire cause of spectral changes above 280 mμ but such effects are scarcely of comparable magnitude in the concentration difference spectra of glucagon. In contrast to this, concentration difference spectra of insulin are most prominent in the 270–300-mμ wavelength region and appear to reflect a change in the environment of side-chain chromophores on dimerization (Rupley *et al.*, 1967).

These results, therefore, confirm previous findings that a major conformational change occurs in the self-association of glucagon. As mentioned previously, studies of the optical rotatory dispersion have suggested that glucagon changes from a random coil form in dilute solution to a partly α-helical form in concentrated solution. The results presented here indicate that this conformational change is involved mainly with formation of a dimer and that the dimerized helices then aggregate to form hexamers. The mechanistic details of the aggregation processes cannot be obtained from equilibrium studies. However the quite large associated spectral changes might make possible kinetic studies which could provide further insight into the mechanism

Acknowledgments

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