Brown, S., Levinson, W., & Spudich, J. A. (1976) *J. Supramol. Struct. 5*, 119.
Brunette, D. M., & Till, J. E. (1971) *J. Membr. Biol. 5*, 215.
Brunk, V., Ericsson, J. L. E., Ponten, J., & Westermark, B. (1971) *Exp. Cell Res. 67*, 407.
Chen, W. T. (1977) *J. Cell Biol. 75*, 411a.
Cohen, D. M., Kalish, D. I., Jacobson, B. S., & Branton, D. (1977) *J. Cell Biol. 75*, 119.
Culp, L. A. (1974) *J. Cell Biol. 63*, 71.
Culp, L. A. (1975) *Exp. Cell Res. 92*, 467.
Culp, L. A. (1976a) *J. Supramol. Struct. 5*, 239.
Culp, L. A. (1976b) *Biochemistry 15*, 4094.
Culp, L. A. (1978) in *Current Topics in Membranes and Transport* (Juliano, R., & Rothstein, A., Eds.) Vol. II, pp 327–396, Academic Press, New York.
Culp, L. A., & Black, P. H. (1972a) *J. Virol. 9*, 611.
Culp, L. A., & Black, P. H. (1972b) *Biochemistry 11*, 2161.
Culp, L. A., & Buniel, J. F. (1976) *J. Cell. Physiol. 88*, 89.
Culp, L. A., Terry, A. H., & Buniel, J. F. (1975) *Biochemistry 14*, 406.
Cunningham, D. D. (1972) *J. Biol. Chem. 247*, 2464.
Curtis, A. S. G., Campbell, J., & Shaw, F. M. (1975a) *J. Cell Sci. 18*, 347.
Curtis, A. S. G., Shaw, F. M., & Spires, M. C. (1975b) *J. Cell Sci. 18*, 357.
Gail, M. H., & Boone, C. W. (1972) *Exp. Cell Res. 70*, 33.
Grinnell, F. (1974) *Arch. Biochem. Biophys. 160*, 304.
Hale, A. H., Pessin, J. E., Palmer, F., Weber, M. J., & Glaser, M. (1977) *J. Biol. Chem. 252*, 6190.
Harris, A. (1973) *Dev. Biol. 35*, 97.
Huang, R. T. C. (1977) *Z. Naturforsch., Teil C 32*, 656.
Hynes, R. O. (1976) *Biochim. Biophys. Acta 458*, 73.
Juliano, R. L., & Gagalang, E. (1977) *J. Cell. Physiol. 92*, 209.
Keski-Oja, J., Mosher, D. F., & Vaheri, A. (1976) *Cell 9*, 20.
Kulas, H. P., Marggraf, W. D., Koch, M. A., & Diringer, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1755.
Mapstone, T. B., & Culp, L. A. (1976) *J. Cell Sci. 20*, 479.
Marggraf, W. D., Diringer, H., Koch, M. A., & Anderer, F. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1761.
Nath, K., & Srere, P. A. (1977) *J. Cell. Physiol. 92*, 33.
Pagano, R. E., & Takeichi, M. (1977) *J. Cell Biol. 74*, 531.
Pessac, B., Alliot, F., & Girard, A. (1977) *J. Cell. Physiol. 90*, 15.
Portoukalian, J., Bugand, M., Zwingelstein, G., & Precausta, P. (1977) *Biochim. Biophys. Acta 489*, 106.
Revel, J. P., & Wolken, K. (1973) *Exp. Cell Res. 78*, 1.
Revel, J. P., Hoch, P., & Ho, D. (1974) *Exp. Cell Res. 84*, 207.
Rollins, B. J., & Culp, L. A. (1979) *Biochemistry 18*, 141.
Rosen, J. J., & Culp, L. A. (1977) *Exp. Cell Res. 107*, 139.
Sandra, A., & Pagano, E. (1978) *Biochemistry 17*, 332.
Sanford, K. K., Barker, B. E., Woods, M. W., Parshad, R., & Law, L. W. (1967) *J. Natl. Cancer Inst. 39*, 705.
Schenkein, I., Levy, M., & Uhr, J. W. (1972) *Cell. Immunol. 5*, 490.
Shields, R., & Pollock, K. (1974) *Cell 3*, 31.
Stamatoglou, S. C. (1977) *J. Ultrastruct. Res. 60*, 203.
Terry, A. H., & Culp, L. A. (1974) *Biochemistry 13*, 414.
Tsan, M. F., & Berlin, R. D. (1971) *J. Exp. Med. 134*, 1016.
Vessey, A. R. (1975) Ph.D. Thesis, Case Western Reserve University, Cleveland, OH.
Vessey, A. R., & Culp, L. A. (1978) *Virology 86*, 556.
Weber, M. J., Hale, A. H., & Lossasso, L. (1977) *Cell 10*, 45.

Thermodynamics of Glucagon Aggregation†

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ABSTRACT: Heats of dilution of concentrated glucagon solutions have been measured calorimetrically at 10 and 25 °C in 0.2 M potassium phosphate buffer of pH 10.6. Analysis of the data in terms of a monomer–trimer equilibrium gives the following thermodynamic parameters for the association reaction at 25 °C: $\Delta G°$ = −7.34 kcal/mol of trimer, $\Delta H°$ = −31.2 kcal/mol, $\Delta S°$ = −80 cal/(K mol), ΔC_p = −430 cal/(K mol). The sensitivity of heat of dilution data to the association constant and stoichiometry of the reaction is discussed.

There has been interest in recent years in the characterization of peptide-hormone receptor interactions, especially the determination of the relative importance of hydrophobic bonding in these interactions. Glucagon, a 29 amino acid peptide hormone, has received considerable attention. It has been implicated in the pathology of diabetes (Unger & Orci, 1975, 1977), its crystal structure has been determined at high resolution (Sasaki et al., 1975), its liver plasma membrane receptor has been characterized (Rodbell et al., 1975; Welton et al., 1977), and a wide variety of structure/function studies have appeared (Hruby et al., 1976; Epand et al., 1976). In these studies, the hydrophobic properties of glucagon have been implicated as important to the biophysical and biological properties of the hormone.

Edelhoch and his collaborators published recently a series of papers on the trimerization of glucagon, a process known to involve hydrophobic interactions in the crystal (Sasaki et al., 1975). Formisano et al. (1977) have presented a van't Hoff analysis of the effect of temperature on the concentration-dependent circular dichroism of glucagon in solution, from which they conclude that the formation of trimers involves a large decrease in enthalpy and a large decrease in entropy. For model compounds, in contrast, hydrophobic associations are typically characterized by small changes in enthalpy and large

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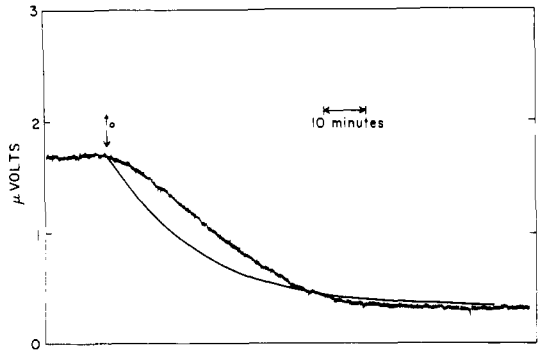

FIGURE 1: Heat of dilution of glucagon at 10 °C and pH 10.6. The wide line is a tracing of the voltage signal; the thin line is the signal calculated for the heat of dilution being directly proportional to glucagon concentration. The plateau before t_0 reflects the heat of dilution of the stock solution; t_0 indicates the beginning of the exponential gradient in glucagon concentration, having $t_{1/2}$ = 18 min. The rounding at the beginning of the calculated curve reflects the response time of the calorimeter, for which correction is made during data reduction.

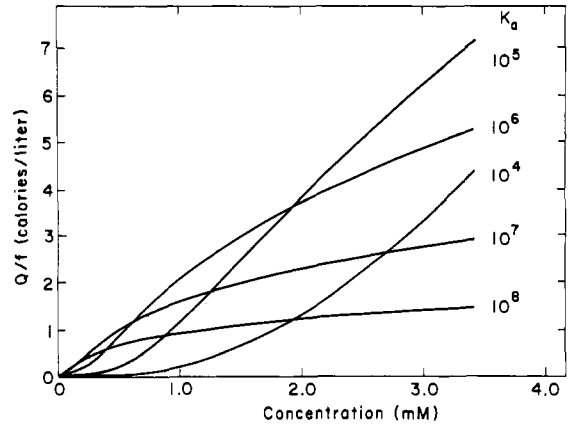

FIGURE 2: Heat of dilution calculated for a monomer–trimer equilibrium and varied association constant (K_a). Fixed parameter values were ΔH = 30 kcal/mol of trimer, dilution factor = 0.456, flow rate in = 2.285 μL/s.

increases in entropy (Kauzmann, 1959). Formisano et al. (1977) attribute the thermodynamic values they observed for glucagon association to α-helix formation being part of the association process and suggest that the heat capacity change upon association would be a much better test for the involvement of hydrophobic bonds than either the enthalpy or entropy changes.

In order to confirm the values of the thermodynamic parameters given by Formisano et al. (1977) and to determine the change in heat capacity for glucagon association, we have measured the heats of dilution of glucagon solutions in a flow microcalorimeter.

Experimental Procedures

Crystalline salt-free glucagon was obtained from Elanco. A concentrated solution of glucagon was prepared for each experiment by dissolving the dry glucagon in 0.2 M K_2HPO_4 buffer solution of pH 10.6, adjusting pH to 10.6 with concentrated KOH, and clarifying by Millipore filtration. Concentrations were determined by absorbance measured at pH 10.2, using ϵ_{278} = 8260 (Gratzer & Beaven, 1969).

The heat of dilution was measured continuously as a function of glucagon concentration by establishing an exponential gradient in glucagon concentration with a half-time of approximately 18 min. The gradient was generated by use of an open mixing vessel with an effective volume of about 6 mL. Solution was pumped from the gradient vessel into an LKB flow microcalorimeter, where it was diluted approximately 2:1 with buffer (see figure captions for precise dilution factors). Mountcastle et al. (1976) give a complete description of the gradients and of how they are made. Figure 1 is a tracing of the strip-chart recording of the calorimeter voltage signal for one experiment. The thin line in Figure 1 is the signal that would be expected if the heat of dilution were directly proportional to glucagon concentration. The shape and magnitude of the experimental signal reflect the stoichiometry of the glucagon association process, its free energy, and its enthalpy.

Immediately after each experiment the gradient system was calibrated by measuring the heat of mixing of solution from an exponential gradient in HCl concentration with excess NaOH solution. The HCl dilution data could be fit to a single exponential with an error of less than 1% at all HCl concentrations. The calorimeter was calibrated using either the internal heater or the heat of dilution of sucrose (Gucker et al., 1939). Flow rates were determined by weighing the amount of water pumped in 1 h.

Calorimeter voltage was monitored with an A-to-D converter; the signal was recorded every 5.31 s on paper tape. Approximately 1500 data points were collected during an experiment. These were corrected for the response time of the calorimeter (see Mountcastle et al., 1976) and then converted, using the time constant for the exponential gradient determined from the HCl dilution, to an array of glucagon concentrations and corresponding heats of dilution.

For analysis of the data, approximately 100 eight-point averages extracted from the full set of data were fitted to a monomer–trimer model, using the equations

$$C_t = C_m + 3K_aC_m^3 \quad (1)$$

$$q = \Delta H(C_{m,out}f_{out} - C_{m,in}f_{in}) \quad (2)$$

where C_m is the monomer concentration (mol/L), C_t is the total concentration of subunits (mol/L), q is the measured heat flow (cal/s), and f is the flow rate (1/s); the subscripts "in" and "out" refer to the concentrations and flow rates before and after dilution, i.e., into and out of the calorimeter, respectively. The ratio f_{in}/f_{out} is the dilution factor, which is about 0.5 in this work. A nonlinear least-squares fitting routine based on the Nelder-Mead algorithm (Nelder & Mead, 1965) was used to determine the values of K_a and ΔH, the association constant and enthalpy for trimerization, respectively, that give the best fit to the data. In this routine, the Newton–Raphson method was used to solve eq 1 for C_m.

A convenient way of presenting heat of dilution data from a flow system is to divide the signal (q; cal/s) by the flow rate (f_{in}; 1/s), giving the heat produced per unit volume of the solution diluted in the calorimeter. Because the signal is a linear function of the flow rate, use of the ratio q/f allows data obtained at different flow rates to be compared or combined. We recommend that q/f not be divided by the protein concentration, because although this operation gives the more usual units, cal/mol, it is unnecessary and it includes in the dependent variable the uncertainty in an independent variable, the concentration. Figures 2–5 are plots of q/f (cal/mol) vs. the protein concentration of the solution at the point of dilution in the calorimeter, C_t (mol subunits/L). In the experiments of Figures 4–5, the protein concentration was varied continuously using the exponential gradient device; the plotted value of C_t is the concentration of the solution at the instant it is mixed with the diluent in the calorimeter. The values of q/f are for finite dilution, from C_t to C_tf_{in}/f_{out}. Plots of q/f

Table I: Thermodynamics of Association of Glucagon into Trimers at pH 10.6 in 0.2 M Potassium Phosphate Buffer

T (C°)	K_a (M^{-2})	ΔH (kcal/mol)	ΔC_p [cal/(K mol)]	F^a	note
10	$(2.8 \pm 0.1) \times 10^{6c}$	-24.8 ± 0.2^c		1.0	best fit; both K_a and ΔH free in the fitting
	6.8×10^{6b}	-28.2 ± 0.3^c		6.0	K_a from Formisano et al. (1977); ΔH free in the fitting
	6.8×10^{6b}	-16.9^b	-540^b	101.0	ΔH and K_a from Formisano et al. (1977)
25	$(2.4 \pm 0.1) \times 10^{5c}$	-31.2 ± 0.1^c		5.4	best fit; both K_a and ΔH free in the fitting
	1.03×10^{6b}	-34.3 ± 0.6^c		148	K_a from Formisano et al. (1977); ΔH free in the fitting
	1.03×10^{6b}	-25.5^b	-611^b	525	ΔH and K_a from Formisano et al. (1977)
10–25			-427		this work

[a] F is a measure of the deviation of the fit from the data and is minimized during the fitting procedure. [b] Calculated from eq 3–6 and Figure 3 of Formisano et al. (1977). [c] The indicated error is the standard error of the parameter. The true error is five times greater, estimated from the uncertainty in the calorimeter signal compared to the sensitivity of the function of K_a and ΔH in the low concentration region.

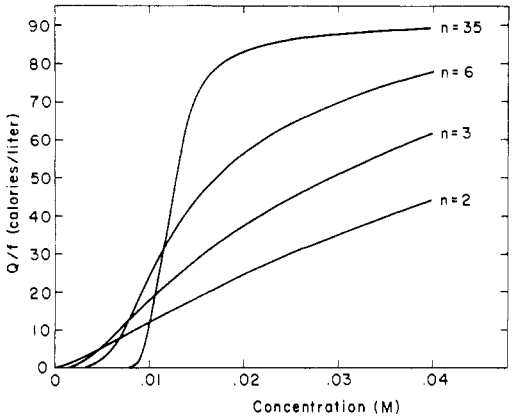

FIGURE 3: Heats of dilution, calculated for a monomer–$n$mer equilibrium. Parameter values are $K_a = 1/n(0.01)^{n-1}$, $\Delta H = 6n$ kcal/mol of $n$mer, dilution factor = 0.629, flow rate in = 2.59 μL/s. K_a values are set so that when $C_t = 0.02$ M, $C_m = 0.01$ M.

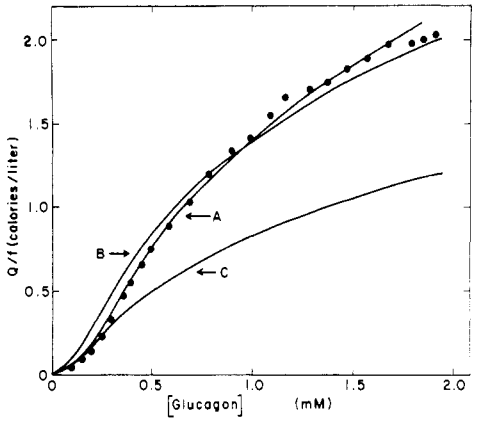

FIGURE 4: Heats of dilution of glucagon in 0.2 M potassium phosphate buffer solution of pH 10.6 and at 10 °C; the flow rate in = 2.493 μL/s and dilution factor = 0.500. The highest glucagon concentration, at the start of the exponential gradient, was 0.00208 M. Plotted points are eight-point averages of reduced data and are representative of the 100 points used in the computer fits. (A) Best least-squares fit of the model to the data, with K_a and ΔH free in the fitting program. (B) Same as A, except K_a fixed at the value found by Formisano et al. (1977). (C) Both K_a and ΔH fixed at the values found by Formisano et al. (1977).

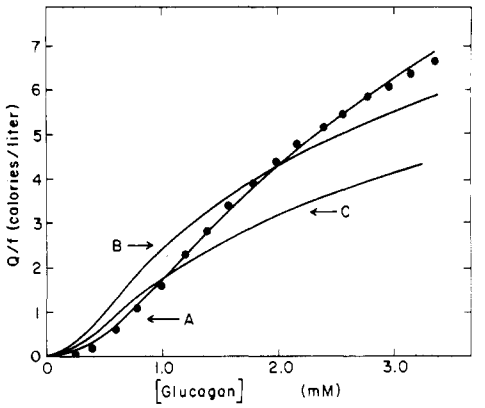

FIGURE 5: Heat of dilution of glucagon in 0.2 M potassium phosphate buffer solution of pH 10.6 and at 25 °C; the flow rate in = 2.29 μL/s and dilution factor = 0.456. The highest glucagon concentration was 0.00355 M. See legend of Figure 4 for further explanation.

vs. concentration may seem unfamiliar since they are not obviously related to a saturation function, but they are a direct method of presenting heat of dilution data without introducing bias from assuming a model. Plots of this kind are useful displays of data only if the dilution factor (f_{in}/f_{out}) is approximately constant for all measurements of a set. Data obtained using a variable dilution factor are better displayed after correction of the finite dilution measurements to the value estimated for infinite dilution, which requires a somewhat arbitrary choice of the method of performing this correction. Figures 2 and 3, which give calculated values of q/f vs. the concentration of solute before dilution, illustrate the sensitivity of the shape of this function to the value of the association constant and to the size of the cooperative unit (n). In the case of glucagon it can be assumed that $n = 3$ because the trimer has been found both in the crystal (Sasaki et al., 1975) and in solution (Gratzer et al., 1972). To summarize, heat of dilution data are effectively and most correctly displayed in plots of q/f vs. C_t, provided the dilution factor is constant. The fitting of parameters (e.g., K_a and ΔH) is independent of the method of display. In order to allow direct comparison with the results of Formisano et al. (1977), all pH values were measured at 24 °C and the heats of dilution are uncorrected for changes in protons bound, i.e., for the heat of protonation of the phosphate buffer. The heat of protonation of phosphate buffer is small compared to the heat of aggregation of glucagon.

Results and Discussion

Representative selections of data from experiments at 10 and 25 °C are plotted in Figures 4 and 5, with curves calculated for three different choices of the parameters K_a and ΔH: (1) the best fit values of both ΔH and K_a, (2) the best fit value of ΔH for K_a fixed at the value found by Formisano et al. (1977), and (3) both ΔH and K_a fixed at the values of Formisano et al. (1977). The parameter values used to generate the six theoretical curves in Figures 4 and 5 are listed in Table I.

The values given in Table I provide an accurate thermodynamic characterization of glucagon aggregation. The most compelling evidence in support of this statement is the self-

consistency of the data at 10 and 25 °C. The change in K_a between these temperatures corresponds to a van't Hoff ΔH that is in close agreement with the average of the calorimetric ΔH values (27.6 vs. 28.0 kcal, respectively). In general, an error in the data or in the model will result in the enthalpy derived calorimetrically being different from that calculated by the change in equilibrium constant with temperature. This comparison has been used, for example, as a test for the validity of the two-state assumption in the thermal denaturation of proteins (Lumry et al., 1966).

The possibility that glucagon trimers interact in some weak, nonspecific way at high glucagon concentrations was tested by use of a monomer–trimer–hexamer model for the fitting. The "best fit" K_a values for trimer formation were for this model not significantly different from the values reported in Table I. Furthermore, it has been found with proteins that do not polymerize at high concentration, such as oxidized lysozyme (Banerjee et al., 1975) and malate dehydrogenase (Johnson and Rupley, unpublished observations), that there is no significant nonspecific heat of dilution at concentrations well above those used in this study.

The heat of dilution measurements give the following values of the thermodynamic parameters at 25 °C: $K_a = 2.4 \times 10^5$ M^{-2}, $\Delta G° = -7.34$ kcal/mol of trimer, $\Delta H° = -31.2$ kcal/mol, $\Delta S° = -80$ cal/(K mol), $\Delta C_p = -430$ cal/(K mol). The value for K_a is significantly smaller and the value for ΔH significantly larger than those reported by Formisano et al. (1977) ($K_a = 1.03 \times 10^6$ M^{-2}; $\Delta H° = -25.5$ kcal/mol). The agreement between the values for ΔH from this work and that of Formisano et al. (1977) is surprisingly good, in view of the inherent limitations of van't Hoff analysis, but the difference in K_a cannot be explained simply. The family of curves given in Figure 2 illustrates how the shape of the plot of q/f vs. C_t is sensitive to the association constant. It is clear that the data plotted in Figures 4 and 5 cannot be fit by a curve of the shape required by the K_a of Formisano et al. (1977). The choice of ΔH affects the amplitude on the q/f axis without affecting the shape of the curve. Analysis of the dilution experiments indicates an allowed range of K_a values no greater than ±25% of the values given in Table I; the values of Formisano et al. (1977) are 2.4 at 10 °C and 4.3 at 25 °C times the values found in this work. With regard to this difference, it should be noted that the heat of dilution measurements extend to 2 to 3.5 times higher concentration than the measurements of Formisano et al. (1977) and that the heat measurements require no significant assumptions about the temperature dependence of the properties of the monomer and oligomer. In view of these comments, we believe that the thermodynamic parameters in this work accurately characterize glucagon trimerization.

It must be emphasized that the general conclusions of Formisano et al. (1977) are correct: the enthalpy of aggregation of glucagon is large and negative, but the heat capacity has the sign expected for a hydrophobic interaction. However, the sign and magnitude of the heat capacity change, as for the enthalpy change, may not be an adequate diagnostic of the extent of the contribution from hydrophobic interactions. Sturtevant (1977) has listed the factors that determine the heat capacity change in a protein reaction. Sturtevant (1977) emphasized, in addition to the widely recognized contribution of hydrophobic bonding, the coupling of an association reaction to other processes, such as large amplitude motions in proteins. The coupling of association to the uptake or release of protons can make a major contribution to changes in heat capacity. If the extent of coupling (i.e., the number of moles of protons taken up or released) changes with temperature, the apparent heat capacity of binding will be pH and buffer dependent. Since the trimerization of glucagon involves a significant proton uptake at pH 10.6 (Gratzer & Beaven, 1969), the heat capacity value reported in this paper undoubtedly contains a contribution from this effect. A contribution of as much as 140 cal/(K mol) can be expected per ionizable group of high pK (Johnson & Rupley, unpublished calculations). The contribution of changes in proton binding can be proportionately greater for the heat capacity than for the enthalpy.

Because of the possible importance of these and other effects, the use of heat capacities of binding as a measure of hydrophobic interactions should be approached with caution.

References

Banerjee, S. K., Pogolotti, A., Jr., & Rupley, J. A. (1975) *J. Biol. Chem. 250*, 8260–8266.

Epand, R. M., Cote T. E., Hoa, D. H. B., Rosselin, G., & Schreier, J. (1976) *Metabolism, Suppl. 25*, 1317–1318, and reference therein.

Formisano, S., Johnson, M. L., & Edelhoch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3340–3344.

Gratzer, W. B., & Beaven, G. H. (1969) *J. Biol. Chem. 244*, 6675–6679.

Gratzer, W. B., Creeth, J. M., & Beaven, G. H. (1972) *Eur. J. Biochem. 31*, 505–509.

Gucker, F. T., Pickard, H. B., & Planck, R. W. (1939) *J. Am. Chem. Soc. 61*, 459–470.

Hruby, V. J., Wright, D. E., Lin, M. C., & Rodbell, M. (1976) *Metabolism, Suppl. 25*, 1323–1325, and references therein.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.

Lumry, R., Biltonen, R., & Brandts, J. (1966) *Biopolymers 4*, 917–944.

Mountcastle, D. B., Freire, E., & Biltonen, R. L. (1976) *Biopolymers 15*, 355–371.

Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308–313.

Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., & Berman, M. (1975) *Adv. Cyclic Nucleotide Res. 5*, 3–29, and references therein.

Sasaki, K., Dockerill, S., Adamiak, D., Tickel, I. J., & Blundell, T. (1975) *Nature (London) 257*, 751–757.

Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.

Unger, R. H., & Orci, L. (1975) *Lancet 1*, 14–16.

Unger, R. H., & Orci, L. (1977) *Annu. Rev. Med. 28*, 119–130.

Welton, A. F., Lad, P. M., Newby, A. C., Yamamura, H., Nicosia, S., & Rodbell, M. (1977) *J. Biol. Chem. 252*, 5947–5950, and references therein.