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Model Studies on Acrylamide Generation from Glucose/Asparagine in Aqueous Glycerol

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Acrylamide formation from asparagine and glucose in different ratios in neutral glycerol/water mixtures was found to increase with decreasing water activity ($0.33 \leq a_w \leq 0.71$ investigated) and increasing temperature (120 °C $\leq T \leq$ 160 °C investigated). The initial rate of acrylamide formation was found to be approximately proportional to the asparagine concentration for an excess of asparagine, but less dependent on an excess of glucose. A steady-state concentration of acrylamide was established at 160 °C after 1 h for $a_w = 0.33$ (30 $\mu g \cdot L^{-1}$ for GLU:ASN = 10:1, 11 $\mu g \cdot L^{-1}$ for GLU:ASN = 1:1, and 130 $\mu g \cdot L^{-1}$ for GLU:ASN = 1:10) and for $a_w = 0.47$ (15 $\mu g \cdot L^{-1}$ for GLU:ASN = 10:1 and 80 $\mu g \cdot L^{-1}$ for GLU:ASN = 1:10), suggesting a protection by glucose against acrylamide degradation. The energy of activation, as estimated from the temperature dependence of the initial rate, increased with decreasing a_w despite a higher rate of formation of acrylamide at low a_w. For high a_w, water elimination from a reaction intermediate is suggested to be rate determining. For low a_w, the increase in energy of activation (and enthalpy of activation) is accordingly counteracted by a more positive entropy of activation, in agreement with decarboxylation as rate determining at low a_w.

KEYWORDS: Acrylamide; water activity; reaction mechanism; glucose; asparagine

INTRODUCTION

The carcinogenic compound acrylamide (2-propenamide), which is also a human neurotoxin, was recently found to be formed in foods high in carbohydrates (*1*). It is formed during thermal processing such as frying, baking, or roasting at temperatures of 120 °C or above. Several hypotheses concerning the formation of acrylamide in cooked foods have been suggested, but the Maillard browning reaction of a sugar and an amino acid is now considered to be the responsible mechanism with reducing sugars and asparagine as the major reactants in food (*2*−*5*). Acrylamide formation takes place in the early stages of the Maillard reaction and is highly interrelated with browning (*6*). In most foods acrylamide is formed in solid phases, but acrylamide is also found in beverages such as coffee originating from the roasting process (*7*). The Maillard reaction is highly complex, several factors such as the water activity (a_w) affect the many reaction steps involved, and a_w has also been found important for the rate of formation of acrylamide (*8*, *9*).

Several pathways and intermediates have been suggested for formation of acrylamide in the Maillard reactions. Among the proposed intermediates are Schiff bases (*5*, *10*, *11*), decarboxylated Amadori compounds (*10*, *11*), Strecker aldehydes (*3*), and the deamination product 3-aminopropionamide, which also generates acrylamide under aqueous conditions (*12*). One hypothesis proposed by Mottram and co-workers (*3*, *13*) emphasizes the importance of a pathway involving Strecker degradation. However, another hypothesis as proposed by Stadler et al. (*4*) includes glycoconjugates such as *N*-glycosides and related compounds formed in the early stage of the Maillard reaction as key intermediates.

The formation of acrylamide in heat-processed foods is affected by numerous factors, such as the initial concentration of the reducing sugar and amino acid and their ratio, pH, a_w, temperature, and time of processing (*6*). Apart from the reaction time and temperature, the major difference between thermal procedures found to result in high or low amounts of acrylamide from asparagine is the water content of the reaction system, which directly influences the physical state of the food. a_w is often critical for the shelf life of industrially processed dry food products sensitive to the Maillard reaction, and a_w may also influence formation of acrylamide. To limit acrylamide in various foods, it is accordingly important to understand the influence of a_w and temperature on the rate of formation of acrylamide.

In the present investigation the kinetics of formation of acrylamide from D-glucose and L-asparagine was followed at

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varying a_w values and temperatures to determine the activation energy involved in the rate-determining steps leading to acrylamide.

MATERIALS AND METHODS

Chemicals. Acrylamide (2-propenamide) (>99.5%), L-asparagine (98%), D-(+)-glucose (99.5%), and glycerol (≥99.5%) were obtained from Sigma-Aldrich (St. Louis, MO). d_3-Acrylamide (>98%) was from Polymer Source (Dorval, Quebec, Canada). The SPE columns were Isolute Multimode 300 mg columns from International Sorbent Technology (Hengoed, Mid Glamorgan, U.K.). Mini UniPrep Teflon filter vials, 500 μL, filter pore size 0.45 μm, were from Whatman International Ltd. (Kent, U.K.). The water used was Milli-Q water (Millipore Corp., Bedford, MA). The acetonitrile was of HPLC grade from Rathburn Chemicals (Walkerburn, Scotland). Formic acid for the eluent (0.1% in water) was from Merck (Darmstadt, Germany). All stock solutions of acrylamide and d_3-acrylamide (1000 and 10 μg·mL^{-1}) as well as calibration standards (2−30 ng·mL^{-1}) were prepared in water and kept at −18 °C until use.

Preparation of Samples. The samples used to measure a_w and formation of melanoidines and acrylamide were prepared with three different ratios of glucose and asparagine. The glucose:asparagine ratios were 10:1 (0.02 mol·L^{-1}/0.002 mol·L^{-1}), 1:1 (0.02 mol·L^{-1}/0.02 mol·L^{-1}), and 1:10 (0.002 mol·L^{-1}/0.02 mol·L^{-1}). A 2 mL volume of the sample was heated in a Venticell heating oven (Buch & Holm, 2730 Herlev, Denmark) at 120, 140, and 160 °C in 4 mL sealed containers with a cap made of silicone/PTFE for 0, 20, 40, 60, 80, 100, and 120 min, and the containers were filled to have only a small headspace to prevent significant evaporation from the liquid sample. The heating times of the samples were calculated to be between 5 and 6 min using heat transferring data of the glass and oven. After heating, the samples were cooled on ice and kept in a freezer at −40 °C until measurement. Concentrations of glucose and asparagine were chosen as the highest possible to dissolve in the samples containing the highest amount of glycerol. pH in the reaction mixture was between 5 and 6 and was not affected by the reactant ratio. Experiments were all carried out in duplicate.

Measurement of a_w. Samples with four different water activities of 0.33 (10:90), 0.47 (20:80), 0.61 (30:70), and 0.71 (40:60) as measured at 25 °C were prepared by mixing water and glycerol prior to dissolution of glucose and asparagine. The water activities of the samples were measured by an Aqua-Lab CX2 (ADAB Analytical Devises AB, 11729 Stockholm, Sweden), and the standards used for calibration were saturated salt solutions: LiCl (a_w = 0.119), NaBr (a_w = 0.572), and NaCl (a_w = 0.736) (*14*).

Measurement of pH. The pH of the samples was measured at 25 °C by a Metrohm pH meter calibrated with standard buffers of pH 4.01 and 7.00 (Bie & Berntsen, 2610 Rødovre, Denmark).

Measurement of Melanoidines. Quantification of melanoidines was performed by measuring the absorbance at 470 nm on a Cintra 40 UV−vis spectrometer (GBC Scientific Equipment Pty Ltd., 3175 Victoria, Australia). The concentration of melanoidines was calculated using the Lambert−Beer equation with an extinction coefficient of 282 L·(mol·cm)$^{-1}$, a value derived for melanoidines formed from glucose and asparagine (*15*).

Sample Preparation for LC−MS/MS. For LC−MS/MS, 200 μL of the heated sample was added to 1.80 mL of Milli-Q water and 10 μL of d_3-acrylamide (10 μg·mL^{-1}) as the internal standard. The cleanup was done on 300 mg Isolute Multimode SPE columns (IST), using an ASPECTM XLi automatic SPE cleanup system (Gilson Inc., Middleton, WI). The SPE columns were conditioned with acetonitrile (1 mL) and subsequently with 2.2 mL of water. A 500 μL volume of sample was applied to the SPE column and pushed through the column by 2 mL of air at a flow of 6 mL min^{-1} to waste. The next 400 μL sample pushed through the SPE column (by 4 mL of air at a flow of 6 mL·min^{-1}) was collected directly in Mini UniPrep Teflon filter HPLC vials.

Analysis of Acrylamide by LC−MS/MS. The content of the acrylamide and asparagine was measured according to the procedure described by Granby and Fagt (*7*).

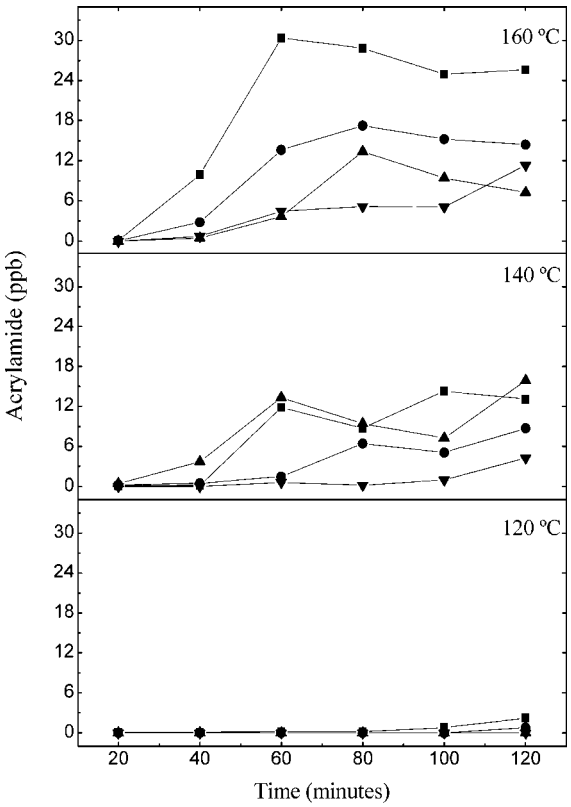

Figure 1. Formation of acrylamide in glycerol/water mixtures containing glucose/asparagine in a molar ratio of 10:1 investigated at 160 °C (top), 140 °C (middle), and 120 °C (bottom). Water activities are 0.33 (■), 0.47 (●), 0.61 (▲), and 0.71 (▼).

Statistical Analysis. Analyses of variance were performed to find significant effects of a_w, pH, and temperature by a two-way analysis of variance, including the main factors a_w and replicates. All the statistical analyses were done in the Analyst application within the SAS system statistical software, 8.02 (SAS Institute, Cary, NC). The analysis was carried out using Microcal Origin, version 6.0 (Microcal Software, Inc., Massachusetts). The rate constants were further analyzed according to the Arrhenius equation by linear regression to estimate the activation energy, E_a, for formation of acrylamide.

RESULTS

For all experimental conditions used, the concentration of acrylamide in the model mixtures increased with increasing temperature and decreasing water activity. The concentration of acrylamide formed was thus highest in the glycerol/water mixtures with the lowest a_w, i.e., 0.33, for all three molar ratios of asparagine and glucose and at any of the three temperatures investigated (**Figures 1−3**). For higher a_w and lower T, a lag phase was observed prior to the formation of acrylamide. An increased heating temperature resulted in a rapid formation of acrylamide, which under these conditions occurred without any lag phase.

At 140 and 160 °C, the concentration of acrylamide in the reaction mixture initially increased, but this increase with or without a lag phase was followed by a plateau with only minor changes in the concentration of acrylamide with time. This is in accordance with establishment of a steady state where formation and degradation of acrylamide approach the same rate. This is most clearly seen at the highest temperature of 160 °C for each of the three reactant ratios. Formation of acrylamide was initiated faster for an excess of asparagine (GLU:ASN = 1:10, **Figure 3**) and reached a higher steady-state concentration

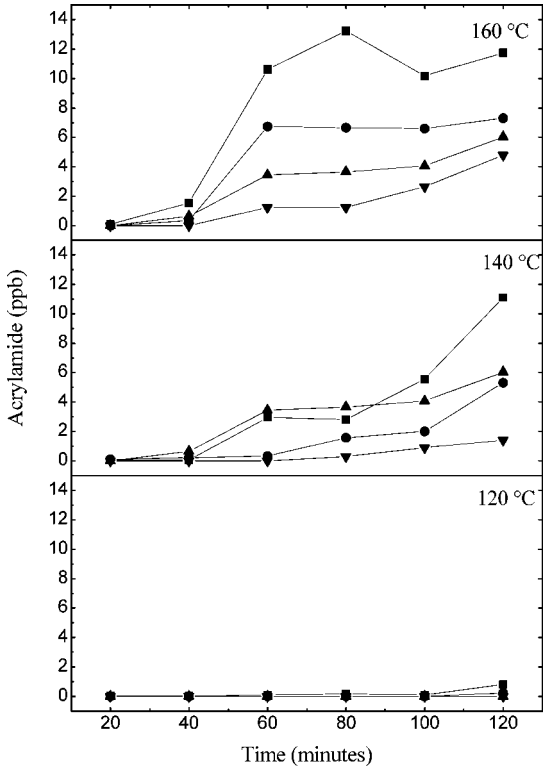

Figure 2. Formation of acrylamide in glycerol/water mixtures containing glucose/asparagine in a molar ratio of 1:1 investigated at 160 °C (top), 140 °C (middle), and 120 °C (bottom). Water activities are 0.33 (■), 0.47 (●), 0.61 (▲), and 0.71 (▼).

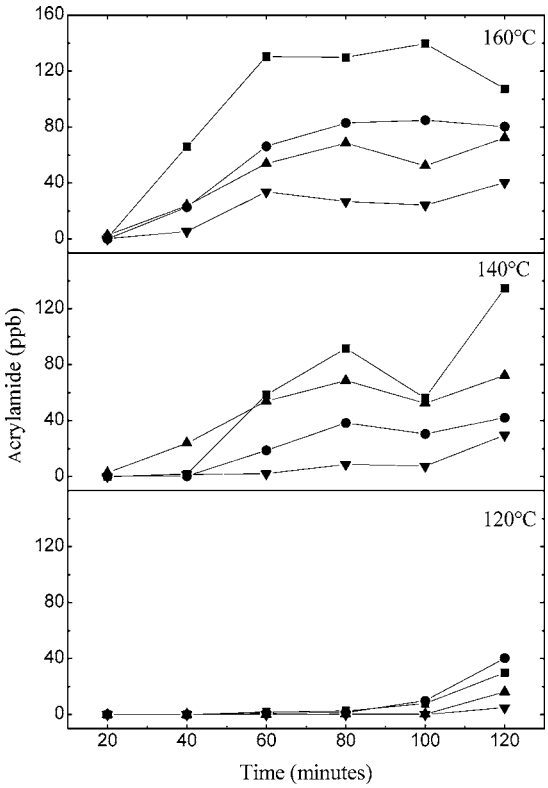

Figure 3. Formation of acrylamide in glycerol/water mixtures containing glucose/asparagine in a molar ratio of 1:10 investigated at 160 °C (top), 140 °C (middle), and 120 °C (bottom). Water activities are 0.33 (■), 0.47 (●), 0.61 (▲), and 0.71 (▼).

of acrylamide than for the samples with an excess of glucose (GLU:ASN = 10:1, **Figure 1**), or with equimolar concentrations of glucose and asparagine (**Figure 2**), independent of temperature and a_w. For all experimental conditions used only a small fraction of asparagine was converted to acrylamide. Less than 0.1% asparagine was converted to acrylamide as a steady-state concentration under conditions resulting in maximum formation of acrylamide (GLU:ASN = 1:10, a_w = 0.33, T = 160 °C for 100 min).

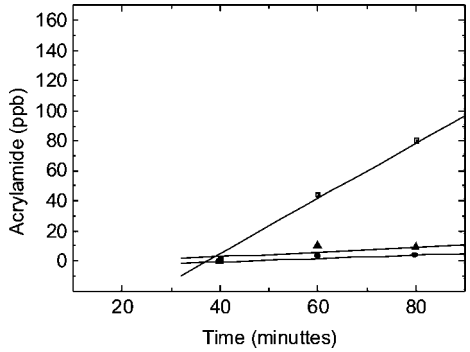

Figure 4. Estimation of the rate [(μg·L^{-1})·min^{-1}] for initial acrylamide formation from the linear part of the acrylamide concentration/time plot for an a_w of 0.33 at 140 °C. The molar ratios are GLU:ASN = 1:10 (■), GLU:ASN = 1:1 (●), and GLU:ASN = 10:1 (▲).

Table 1. Initial Rate [(μg·L^{-1})·min^{-1}] with Standard Deviation of Acrylamide Formation from Glucose and Asparagine in Glycerol/Water Mixtures[a]

a_w	molar ratio GLU:ASN	120 °C	140 °C	160 °C
0.33	1:10	0.32 ± 0.05	1.16 ± 0.1	3.71 ± 0.2
	1:1	0.0065 ± 0.0008	0.052 ± 0.003	0.35 ± 0.005
	10:1	0.020 ± 0.0007	0.14 ± 0.005	0.85 ± 0.08
0.47	1:10	0.48 ± 0.08	0.60 ± 0.02	0.76 ± 0.2
	1:1	0.016 ± 0.001	0.043 ± 0.003	0.11 ± 0.02
	10:1	0.037 ± 0.0002	0.11 ± 0.003	0.16 ± 0.2
0.61	1:10	0.46 ± 0.01	0.60 ± 0.1	0.78 ± 0.3
	1:1	0.0087 ± 0.005	0.022 ± 0.008	0.054 ± 0.006
	10:1	0.0098 ± 0.003	0.037 ± 0.005	0.12 ± 0.002
0.71	1:10	0.27 ± 0.02	0.38 ± 0.06	0.55 ± 0.2
	1:1	0.017 ± 0.004	0.032 ± 0.004	0.058 ± 0.0003
	10:1	0.020 ± 0.0003	0.042 ± 0.007	0.085 ± 0.03

[a] All experiments were made in duplicate.

The rate of formation of acrylamide under the different conditions was for each set of a_w and temperature conditions estimated as the slope of the linear part of the acrylamide concentration/time plot as shown for varying GLU:ASN ratios in **Figure 4**. The initial rate of formation of acrylamide following the lag phase was selected to avoid complication from subsequent reactions of acrylamide, leading to the observed steady-state concentration under some of the reaction conditions. Notably, it was not possible to convert observed rates to rate constants, but on the basis of the Arrhenius equation, the energy of activation may still be estimated. The estimated rate of acrylamide formation (**Table 1**) showed a temperature dependence which is strongly dependent on both a_w and the reactant ratio. The rate of formation was highest for the samples with an excess of asparagine. At an a_w of 0.33 at 160 °C, the rate of formation of acrylamide for a GLU:ASN ratio of 1:10 was thus 10 times higher than the rates for a GLU:ASN molar ratio of 1:1 and 4 times higher than the rates for a molar ratio of 10:1. A similar pattern was seen for the other conditions, although some deviations were noted especially for low temperature and high a_w, where the experimental uncertainties were higher due

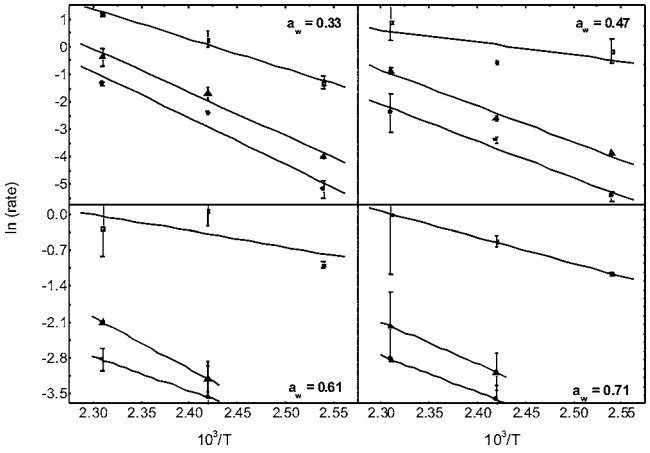

Figure 5. Arrhenius plot for formation of acrylamide from glucose and asparagine for GLU:ASN reactant molar ratios of 1:10 (■), 1:1 (●), and 10:1 (▲) for $a_w = 0.33$, 0.47, 0.61, and 0.71 as estimated from the initial rate of acrylamide formation [(μg·L^{-1})·min^{-1}]; see **Figure 4**. E_a calculated as $E_a = (-\text{slope})R$ is given in **Table 2**.

Table 2. Activation Energy, E_a, Based on Estimated Rates for the Initial Acrylamide Formation from Glucose and Asparagine in Glycerol/Water Mixtures (see **Figure 5**)

	E_a[a] (kJ/mol)			
molar ratio GLU:ASN	$a_w = 0.33$	$a_w = 0.47$	$a_w = 0.61$	$a_w = 0.71$
10:1 (0.02 and 0.002 mol/L)	129 a	73 bc	83 c	58 b
1:1 (0.002 and 0.002 mol/L)	137 a	75 bc	55 c	51 b
1:10 (0.002 and 0.02 mol/L)	88*** a	25*** bc	26** c	35 b

[a] Asterisks indicate significant difference from other values in the same column at a (***) 0.001% level, a (**) 0.01% level, and a (*) 0.05% level. Different letters indicate significant difference from other values in the same row at a 0.01% level.

to less formation of acrylamide. However, it is clear that the rate of formation of acrylamide is more dependent on the concentration of (excess) asparagine than on the concentration of (excess) glucose.

E_a was estimated for each of the 12 different reaction conditions investigated (three different reactant ratios and four water activities) from the slope ($-E_a/R$) of the straight line derived from plotting ln(rate) versus $1/T$ assuming an Arrhenius temperature dependence (see **Figure 5**). The activation energy showed a large variation depending on the actual reaction conditions. The activation energies changed with a_w (**Table 2**), were significantly higher at $a_w = 0.33$, and decreased with increasing a_w. Significantly lower activation energy is noted under conditions of an excess of asparagine compared to conditions of equal molar concentrations and with an excess of glucose, independent of a_w.

The concentration of melanoidines in the reaction mixtures was near the detection limit at 120 and 140 °C and showed no correlation with temperature, heating time, or acrylamide concentration. At 160 °C the concentration of melanoidines increased with heating time and the acrylamide concentration (**Figure 6**). However, a_w had less influence on browning than on acrylamide formation, suggesting that acrylamide formation is occurring in a reaction step preceding browning. This is further confirmed by the observation that reaction conditions with an excess of glucose favored formation of melanoidines in marked contrast to what was seen for acrylamide formation, where an excess of asparagine is more important. In the samples with a GLU:ASN ratio of 10:1 melanoidine formation (**Figure 6**) was seen already after 40 min at 160 °C, whereas browning started at 80 min for a 1:10 molar ratio.

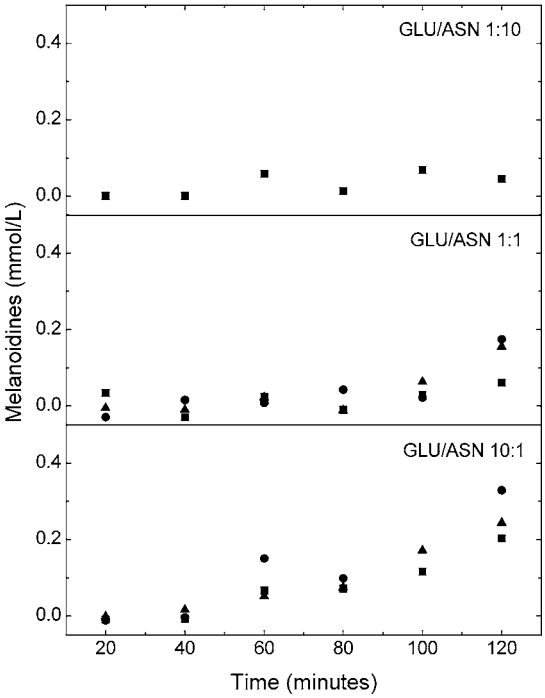

Figure 6. Formation of melanoidines at 160 °C measured as absorbance at 470 nm in glycerol/water mixtures and at $a_w = 0.47$ (■), 0.61 (●), and 0.71 (▲).

DISCUSSION

Formation of acrylamide from glucose and asparagine was found to be strongly affected by a_w in the mixtures of glycerol and water used as a model reaction medium. The rate of formation and the steady-state concentration of acrylamide increased with decreasing a_w, and both longer reaction time and higher temperature led to an increased content of acrylamide at constant water activity in the three molar ratios of glucose and asparagine examined.

The rate of formation of acrylamide appears to be of zeroth order with respect to glucose and first order with respect to asparagine, which indicates that a simple collision of the two precursor molecules asparagine and glucose is not rate determining. This is in marked contrast to the browning reaction quantified as the melanoidine content, the rate of which was highly affected by the concentration of glucose. The browning of the reaction mixture was rather modest when formation of acrylamide became significant, and browning appeared after approximately 1 h at 160 °C for all three reaction ratios investigated. An excess of glucose increased browning (**Figure 6**) but decreased the acrylamide steady-state concentration. Acrylamide is accordingly concluded to be formed in a reaction step prior to browning. The browning of the present model system is concluded to be due to Maillard reactions and not caramelization, since caramelization under the present nonalkaline conditions has been found to be negotiable and also requires longer heating times (*21*). A steady-state concentration of acrylamide was established at 160 °C to 1 h for the two lowest a_w values investigated. For an a_w of 0.33 the steady-state concentration of acrylamide was approximately 130 μg·L^{-1} for a GLU:ASN ratio of 1:10, approximately 13 μg·L^{-1} for a GLU:ASN ratio of 1:1, and 30 μg·L^{-1} for a GLU:ASN ratio of 10:1. At an a_w of 0.47 the steady-state concentration of acrylamide

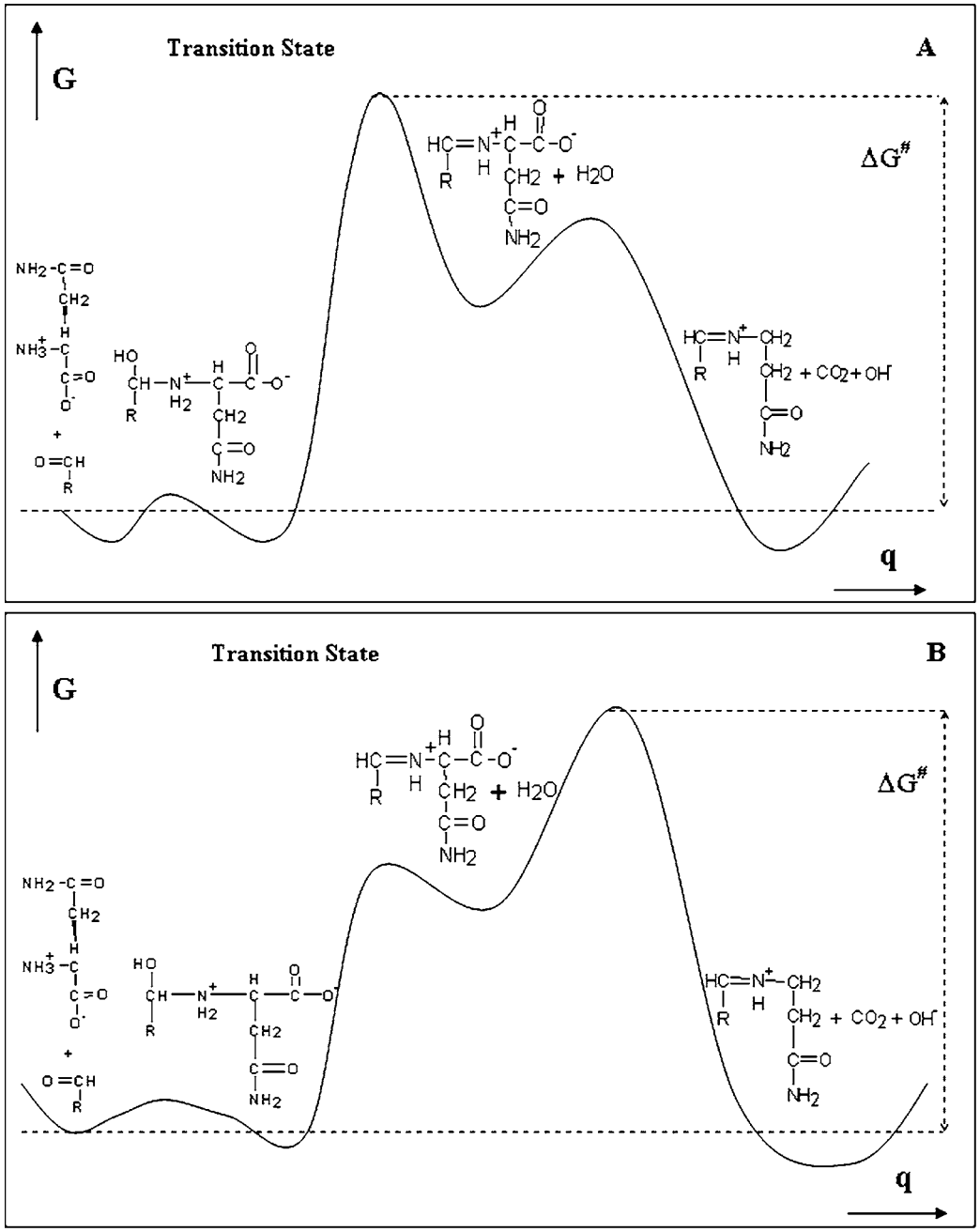

Figure 7. Energy profile of the reaction between glucose and asparagine at high a_w (**A**) and low a_w (**B**) in glycerol/water mixtures with Schiff base reaction intermediates.

was approximately 80 $\mu g \cdot L^{-1}$ for a GLU:ASN ratio of 1:10 and approximately 7 $\mu g \cdot L^{-1}$ for a GLU:ASN ratio of 10:1. Notably, the higher concentration of acrylamide was found for reaction conditions with an excess of asparagine. Degradation of acrylamide could involve reaction with other acrylamide molecules, leading to polymerization, or reaction with either the precursors or other Maillard reaction products. The dependence of the steady-state concentration on the reactant concentration is different from the dependence observed for the rate and may suggest some protection against degradation of formed acrylamide by glucose or a reaction product of glucose.

The energy of activation of formation of acrylamide, E_a, was found to increase with decreasing a_w and varied from 137 to 25 kJ/mol depending on the water activity and molar ratio of glucose and asparagine. E_a for acrylamide formation has previously been found to have the value 161 kJ/mol in a system of asparagine and glucose in the temperature range 140−200 °C at constant water content (*22*). E_a was lowest at the highest a_w (0.71) compared to the lower a_w (0.33). The rate of formation of acrylamide surprisingly increased with increasing E_a and was highest at the lowest a_w, suggesting a change in the rate-determining step. The unusual relation between the rate and E_a for varying a_w implies a dramatic change in the entropy of activation of the reaction. Since the exact rate law has not been established, numerical values cannot be calculated for $\Delta S^{\ddagger}$, but the ordering is $\Delta S^{\ddagger}$ (low a_w) > $\Delta S^{\ddagger}$ (high a_w). At high a_w, where the entropy of activation $\Delta S^{\ddagger}$ must be lowest and probably negative, elimination of water is suggested to be the rate-determining step in the cascade of reactions leading to acrylamide. In contrast, for conditions of low a_w with a much larger E_a and accordingly higher $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ must, to counteract the high enthalpy barrier, be more positive, and we suggest that a decarboxylation (see **Figure 7**) becomes the rate-determining step. The proposed energy profile of a simplified reaction

scheme may be seen in **Figure 7**. Our suggestions for rate-determining steps under different conditions of water activity are further supported by mechanistic considerations. For a high water activity the elimination of a water molecule is likely to be more difficult than for low water activity. Elimination of a water molecule in an aqueous environment is furthermore not expected to be characterized by large entropy effects. In contrast, for low a_w, water elimination becomes easier and a large positive $\Delta S^{\#}$ characterizes decarboxylation, generating a gaseous reaction product. It should also be noted that none of these steps as rate determining would lead to simple second-order kinetics in agreement with the observed dependence of glucose and asparagine concentrations.

The influence of a_w on formation of acrylamide in a homogeneous liquid reaction system allows identification of rate-determining steps better than for the amorphous/crystalline reaction systems previously investigated for which the physical state is becoming an important factor. Roberts et al. (*16*) found no significant difference in acrylamide formation in amorphous/crystalline model systems with water activities in the range of 0.07−0.22, and they could not correlate the formation of acrylamide with a_w. For the present investigation, the use of higher water activities and a homogeneous liquid system allowed us to identify an affect of a_w on the formation of acrylamide. Glycerol has previously been used as a humectant for investigations of Maillard reactions and as a solvent for water at lower a_w to ensure an aqueous reactant mobility at much lower moisture content than would be expected solely from the water content alone (*17*, *18*). It has been found that both liquid and solid model systems containing glycerol had a maximum nonenzymatic browning rate in the a_w range 0.41−0.55 (*17*, *18*), and maximum browning in most foods occurs in the a_w range 0.3−0.7 depending on the type of food and its physical state (*17*). Maximum acrylamide formation was in the present investigation found to occur at the lowest a_w of 0.33, which notably is in the range of Maillard browning in food. Clearly, a_w has an effect on formation of acrylamide, and in systems based on silica gels an effect of the water content was also noted. The yield of acrylamide decreased when the water content decreased from 25% to 0%, indicating that formation of acrylamide needs at least a certain amount of water to generate distinct transients able to release acrylamide on thermal treatment (*19*). Blank et al. (*20*) have also shown that the amount of acrylamide formed in binary mixtures of asparagine and glucose (0.2 mmol) was dependent on the water content in different physical states.

Temperature affects the water activity, and a_w increases for most systems as temperature increases for a constant moisture content. In general, the temperature dependence on increasing a_w at constant moisture content is greatest at the lower to intermediate water activities. The a_w values of the reaction mixtures used in the present investigation were all measured at room temperature prior to heating to 120, 140, or 160 °C for reaction. The a_w of the actual reaction mixtures at the higher temperature where acrylamide is formed is higher than measured at room temperature. The change in a_w between 120 and 160 °C is as an equilibrium effect expected to be small compared to the effect on the rate, and no correction was attempted. Part of the deviation from linearity seen, for example, in the Arrhenius plots may, however, be ascribed to this uncertainty (**Figure 5**).

The formation of acrylamide was found to be affected by temperature, heating time, and a_w. In conclusion, the results together showed that a change in a_w caused a change in the rate-determining step for acrylamide formation, further resulting in a marked change in both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ as observed in a different temperature dependence of the reaction at different a_w values.

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