Research Article

Journal of Physical Organic Chemistry

Received: 4 May 2009, Revised: 22 June 2009, Accepted: 23 June 2009, Published online in Wiley InterScience: 3 August 2009

(www.interscience.wiley.com) DOI 10.1002/poc.1600

Reactivity of acrylamide as an alkylating agent: a kinetic approach

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Acrylamide (AA), an industrially produced reactive molecule, is used worldwide. The US EPA and IARC have classified this molecule as a probable human carcinogen. In this work, the alkylating potential of AA was investigated kinetically. The conclusions drawn are as follows: (i) AA shows alkylating ability on the nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents with nucleophilic characteristics similar to those of DNA bases; (ii) the rate equation for the NBP–AA adduct formation is as follows: $r = k_{alk}[AA][NBP]$; (ii) the thermoentropic term, $T\Delta^{\ddagger}S^{\theta}$, is the main term responsible for the lower reactivity of AA as an alkylating agent; (iii) the value of the Gibbs energy of activation, $\Delta^{\ddagger}G^{\theta}$, for the NBP alkylation reaction by AA is consistent with the conclusions of epidemiologic studies concerning the carcinogenicity of this substance; (iv) the results obtained here may be useful when working with hydrophilic/lipophilic media, such as in Food Science, since the dielectric constant of the medium, where alkylation occurs, influences the reaction rate, and alkylation can be inhibited by lowering the dielectric constant of the medium.

Keywords: acrylamide; alkylation reactions

INTRODUCTION

Acrylamide (AA), an industrially produced reactive molecule, is used worldwide to synthesize polyacrylamide. The monomeric form is widely used as an alkylating agent for the selective modification of protein SH groups and in fluorescence studies of tryptophan residues in proteins.

AA has two reactive sites: the amide group and the conjugated double bond (Fig. 1). The vinyl group is able to participate in nucleophilic reactions with active-hydrogen-bearing functional groups.[1] These include the SH of glutathione, the α-NH_2 of free amino acids, or the N-terminal amino acid residues of proteins and DNA.[2–4] Because of its low molecular weight and high water solubility, AA can easily pass through several biological membranes. In recent years, it has been discovered that AA is metabolized by cytochrome P450 2E1 to the epoxide glycidamide, which also possesses electrophilic reactivity.[5,6]

On the basis of the studies in rats and mice,[7,8] the IARC and EPA have classified AA as a probable human carcinogen.[9,10] Many authors have examined the neurotoxicity, genotoxicity, and developmental and reproductive effects of AA in humans and animals,[11–15] and have demonstrated that this molecule is neurotoxic and also has a low degree of genotoxicity. Regarding the carcinogenic potential of AA and its alkylating capacity, a report published in 2002 sparked a public debate about the correlation between human exposure to AA and cancer risk. Tareke *et al*. reported unexpectedly high levels of AA in a variety of baked and fried foods.[16] Since that time, many researchers have investigated the formation and elimination reactions of AA in several model reaction systems.[17–19] Moreover, the Maillard reaction (the main route of AA formation in foods),[20–23] and the main factors affecting that formation[24,25] have been investigated.

There are many biological and chemical methods available for determining the alkylating potential of mutagenic/carcinogenic substances, although they usually have some limitations such as high cost, use of animals, etc. Accordingly, *in vitro* experiments are now acquiring increased importance.

4-(*p*-Nitrobenzyl)pyridine (NBP), a trap for alkylating agents[26] with nucleophilic characteristics similar to those of DNA bases,[27] was previously used by us to measure the alkylating potential and the mechanism of the reaction, not only of highly electrophilic agents such as some lactones[28–30] and *N*-alkyl-*N*-nitrosoureas,[31] but also of low electrophilic agents such as sorbic acid[32] and its salts.[33] A correlation between the alkylating potential and carcinogenicity was found.

Although many aspects related to AA analysis have been very hot topics,[34] to the best of our knowledge, the alkylating potential of this molecule has not been investigated in quantitative kinetic terms. Here we were prompted to address this issue.

EXPERIMENTAL

To monitor the alkylation reaction of NBP by AA (Scheme 1), 2.4 ml aliquots of the alkylation mixture (AA+NBP) were removed at different times and added to a cuvette containing 0.6 ml of triethylamine reagent to stop the alkylation process, after which

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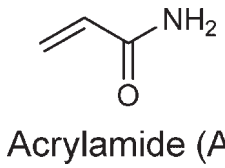


Acrylamide (AA)

Figure 1. Structure of acrylamide

the absorbances at the wavelength of maximum absorption of the adduct (AD) were followed ($\lambda = 570$ nm). To render NBP soluble, the alkylation mixtures were prepared at three different water/dioxane ratios: 7:3, 6:4, and 5:5 (vol). Detailed reaction conditions are given in the figure and table legends.

A Shimadzu UV-2401-PC spectrophotometer with a thermoelectric six-cell holder temperature control system (±0.1 °C) was used.

Reaction temperatures were kept constant (±0.05 °C) with a Lauda Ecoline RE120 thermostat.

Positive-mode electrospray ionization mass spectra were recorded on a Waters ZQ4000 spectrometer, by direct injection with methanol as solvent.

Water was deionized with a MilliQ-Gradient (Millipore). AA was obtained from Fluka (≥ 98%); dioxane (99%) was purchased from Panreac. Triethylamine (99%) and NBP (98%) were from Sigma Aldrich.

All kinetic runs were performed in triplicate. Numerical treatment of the data was performed, using SigmaPlot software, Version 10.0.

RESULTS AND DISCUSSION

Kinetics of the alkylation reaction

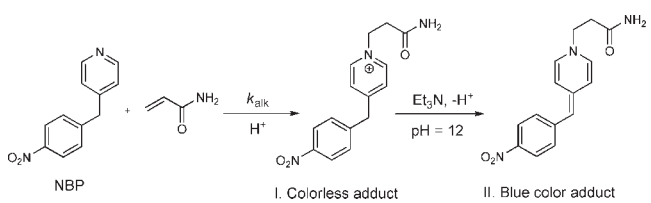
The blue AA–NBP adduct (AD) shows maximum absorption at $\lambda = 570$ nm. To check its structure, a positive-mode electrospray ionization mass spectrum was obtained with a mass/charge ratio = 286.2, which is coherent with the suggested structure for the adduct (Scheme 1).

Figure 2 shows the increase in absorption caused by the formation of the adduct with time. As AA was in excess compared to NBP, it may be assumed that the total amount of NBP was converted into adduct.

Figure 3 shows a typical kinetic run for the alkylation of NBP by AA. As the time for reaching the plateau was very long, the initial rate method (IRM) was used.[35] In this a way, some possible reactions different from NBP alkylation were also circumvented (approximately two months after the initiation of the NBP alkylation reaction, a second reaction emerged).

Formation of the NBP–AA adduct is according to the rate Eqn (1).

$$r = \frac{d[AD]}{dt} = k_{alk}[AA][NBP] \quad (1)$$



Scheme 1. Method for monitoring the NBP alkylation by acrylamide

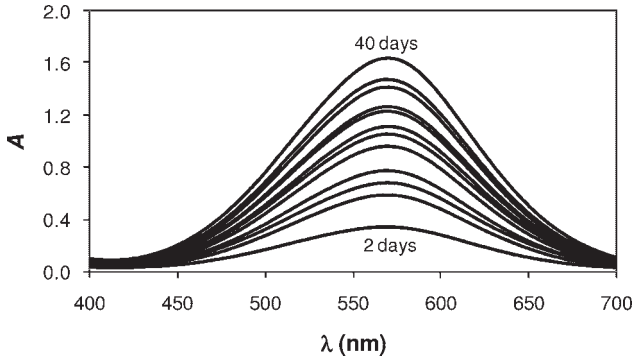


Figure 2. Spectrograms showing the formation of the acrylamide–NBP adduct with time in a 7:3 water/dioxane medium, over the 2–40 day interval. $[NBP]_o = 3 \times 10^{-4}$ M; $[AA]_o = 0.1$ M; $T = 37.5$ °C

The initial rate (v_o) can be written as follows:

$$v_o = \frac{1}{(\varepsilon l)} \frac{\Delta A}{\Delta t} \quad (2)$$

where ε, l, and ΔA represent the molar absorption coefficient of the adduct, the light path, and the variation of absorbance with time, respectively.

From Eqs (1) and (2), the alkylation rate constant can be written as follows:

$$k_{alk} = \frac{v_o}{[AA][NBP]} \quad (3)$$

Table 1 presents the values of k_{alk} in different water/dioxane media at several temperatures.

To follow the formation of the AA–NBP adduct kinetically, its absorption coefficient ($\lambda = 570$ nm) must be known, and hence it was determined. Several experiments were performed using $[AA]_o = 0.1$ M and six NBP concentrations in the $(0.5–5.0) \times 10^{-4}$ M range. When absorbance reaches a plateau, it may be assumed that the reaction of NBP with AA is completed. Figure 4 and Table 2 show the results obtained for different media.

Figure 5 shows the good fit of the k_{alk} values to the Eyring–Wynne-Jones equation:[36]

$$k = \frac{\mathbf{k}T}{h} e^{-\frac{\Delta^{\ddagger}G^{\theta}}{RT}} = \frac{\mathbf{k}T}{h} e^{-\frac{\Delta^{\ddagger}H^{\theta}}{RT}} e^{\frac{\Delta^{\ddagger}S^{\theta}}{RT}} \quad (4)$$

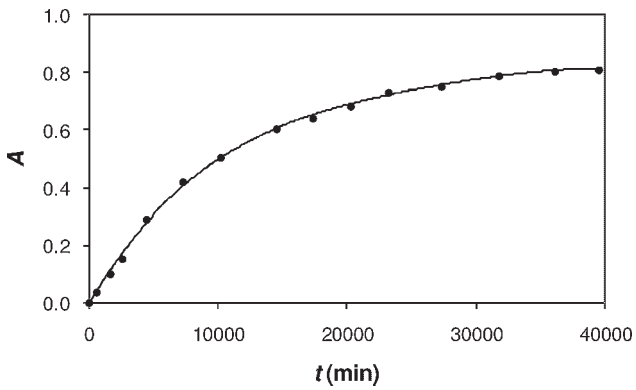


Figure 3. Formation of the acrylamide–NBP adduct in a 7:3 water/dioxane medium; variation in absorbance ($\lambda = 570$ nm) with time. $[NBP]_o = 1.5 \times 10^{-4}$ M; $[AA]_o = 0.1$ M; $T = 37.5$ °C

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Table 1. Rate constants of NBP alkylation by AA as a function of temperature and medium composition

	$10^6\ k_{alk}$ ($M^{-1}\ s^{-1}$)[a]					
Water/dioxane (v/v)	25.0 °C	27.5 °C	30.0 °C	32.5 °C	35.0 °C	37.5 °C
7:3	6.10	7.20	9.00	10.1	11.0	13.7
6:4	1.83	2.05	2.60	3.17	3.83	4.69
5:5	0.70	0.81	0.94	1.30	1.55	1.98

[a] Values of the rate constants are reproducible to 5%.

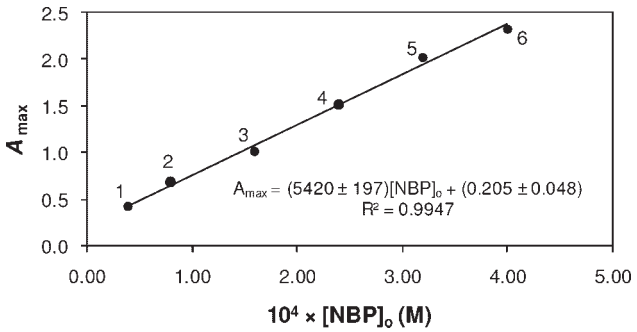

Figure 4. Determination of the mean absorption coefficient value of the AA–NBP adduct in a 7:3 water/dioxane medium. $[AA]_o = 0.1$ M; $[NBP]_o = 0.4 \times 10^{-4}$ M (1), $[NBP]_o = 0.8 \times 10^{-4}$ M (2), $[NBP]_o = 1.6 \times 10^{-4}$ M (3), $[NBP]_o = 2.4 \times 10^{-4}$ M (4), $[NBP]_o = 3.2 \times 10^{-4}$ M (5), $[NBP]_o = 4.0 \times 10^{-4}$ M (6); $\lambda = 570$ nm; $T = 37.5$ °C

Table 2. Absorption coefficients of the AA–NBP adduct in different water/dioxane media

Water/dioxane (vol)	ε_{570} ($\times 10^{-3}\ M^{-1}\ cm^{-1}$)[a]
7:3	5.4 ± 0.2
6:4	5.8 ± 0.2
5:5	5.9 ± 0.3

[a] Values are given within a 95% confidence interval.

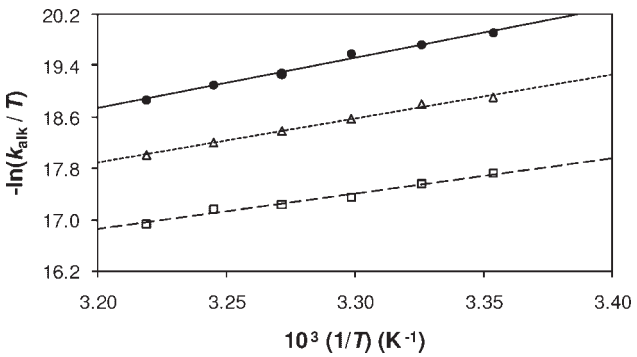

Figure 5. Eyring plot for the alkylation of NBP by acrylamide in different water/dioxane media: 7:3 (●), 6:4 (Δ), 5:5 (□) (vol)

Table 3. Initial rate of formation of the NBP–AA adduct as a function of the composition of the medium

Water/dioxane (v/v)	v_o ($\times 10^9$ M min^{-1})[a]
7:3	9.80 ± 0.40
6:4	3.90 ± 0.06
5:5	1.20 ± 0.04

[a] Values are given within a 95% confidence interval.

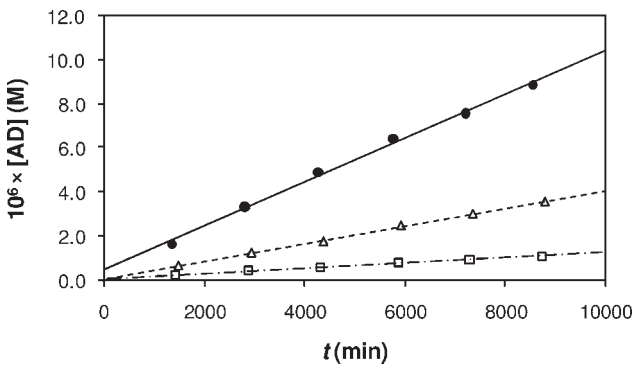

Figure 6. Variation in the initial rate of formation of the NBP–AA adduct (AD) in different water/dioxane media: 7:3 (●), 6:4 (Δ), 5:5 (□) (vol)

Table 3 and Fig. 6 show the variation in the initial rate of formation of the NBP–AA adduct in different water/dioxane media.

Table 4 reports the values of the activation parameters for NBP alkylation.

Table 4. Activation parameters for NBP alkylation by AA in different water/dioxane media

	Water/dioxane (v/v)		
Activation parameter	7:3	6:4	5:5
$\Delta^{\ddagger}H^{\theta}$ (kJ mol^{-1})[a]	45 ± 2	57 ± 2	65 ± 2
$-\Delta^{\ddagger}S^{\theta}$ (J mol^{-1} K^{-1})[a]	193 ± 9	163 ± 8	145 ± 7
$\Delta^{\ddagger}G^{\theta}$ (35 °C) (kJ mol^{-1})[a]	104 ± 2	107 ± 2	110 ± 2

[a] Values are given within a 95% confidence interval.

Table 5. Chemical reactivity of some alkylating agents

Alkylating agent	$\Delta^{\ddagger}H^{\theta}$ (kJ mol^{-1})[a]	$-\Delta^{\ddagger}S^{\theta}$ (J mol^{-1} K^{-1})[a]	$\Delta^{\ddagger}G^{\theta}$ (35 °C) (kJ mol^{-1})[a]
β-Propiolactone (7/3)[b]	41 ± 2	148 ± 6	87 ± 2
β-Butyrolactone (7/3)[b]	47 ± 2	148 ± 6	93 ± 2
Potassium sorbate (7/3)[c]	78 ± 7	70 ± 12	99 ± 6
Acrylamide (7/3)[d]	45 ± 2	193 ± 9	104 ± 2

[a] Values are given within the 95% confidence interval.
[b] Values are taken from Reference[28].
[c] Values are taken from Reference[32].
[d] This work.

Since comparison of the Gibbs energy of activation, $\Delta^{\ddagger}G^{\theta}$, for NBP alkylation by different alkylating agents can be useful, these values were also calculated. Table 5 reports the values obtained for some alkylating agents studied by us previously. These values reveal the following:

(1) The existence of a correlation between chemical reactivity and carcinogenic potential: while β-propiolactone and β-butyrolactone are classified as possible human carcinogens, sorbate has a low genotoxic capacity. The higher value of $\Delta^{\ddagger}G^{\theta}$ in the case of AA permits its low carcinogenic capacity to be understood.
(2) While the $\Delta^{\ddagger}H^{\theta}$ value for the alkylation reaction by AA is almost the same as that of β-propiolactone (one of the most potent carcinogens), the thermoentropic term, $T\Delta^{\ddagger}S^{\theta}$, seems to be the main term responsible for the lower reactivity of AA as an alkylating agent.

The values of the activation parameters (Table 4) help to rationalize the fact that NBP alkylation by AA in different water/dioxane media is inhibited by the increase in organic component in the reaction medium (Table 1). The rise – in absolute value – of the activation entropy due to an increase in the amount of water in the reaction medium and the simultaneous decrease in $\Delta^{\ddagger}H^{\theta}$ suggest an active polar complex, similar to the resulting adduct.[37]

As an increase in the dioxane percentage in the reaction medium implies a decrease in its dielectric constant ($\varepsilon = 48.54$ in 7:3 water/dioxane),[38] it should be possible to inhibit the alkylating capacity of AA via a decrease in the dielectric constant of the reaction medium.

CONCLUSIONS

From the obtained results, the following conclusions may be drawn:

(i) AA shows alkylating capacity on the nucleophile NBP, a trap for alkylating agents with nucleophilic characteristics similar to those of DNA bases.
(ii) The rate equation for the formation of the NBP–AA adduct is as follows:

$$r = \frac{d[AD]}{dt} = k_{alk}[AA][NBP]$$

(iii) The thermoentropic term $T\Delta^{\ddagger}S^{\theta}$, is the main term responsible for the low reactivity of AA as an alkylating agent.
(iv) The values of the Gibbs energy of activation, $\Delta^{\ddagger}G^{\theta}$, or NBP alkylation reactions by different alkylating agents reveal the existence of some correlation between chemical reactivity and carcinogenic potential. The high value of $\Delta^{\ddagger}G^{\theta}$ in the case of AA is consistent with the conclusions of several epidemiological studies addressing the carcinogenicity of this substance.
(v) The results obtained may be useful when working with hydrophilic/lipophilic media, as is frequent in Food Science. Since the dielectric constant of the medium, where NBP alkylation occurs, influences the reaction rate, the alkylation reaction can be strongly inhibited by lowering the dielectric constant of the medium. Thus, the simultaneous presence of mixtures of alcoholic spirits and foods containing vegetable oils, such as salads or tinned foods, could inhibit the alkylating potential of the AA ingested with them.

Acknowledgements

We thank the Spanish Ministerio de Ciencia e Innovación (CTQ2007-63263), as well as the Spanish Junta de Castilla y León (Grant SA040 A08) for supporting the research reported in this article. I.F.C.C. thanks the Spanish Ministerio de Asuntos Exteriores y de Cooperación (MAEC-AECID) for Ph.D. grant. R.G.B. thanks the Ministerio de Ciencia e Innovación and M.G.P. thanks the Junta de Castilla y León for Ph.D. grants. We also thank the referees for their valuable comments.

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