Minimization of Asymmetry Potential in ETH 129-Based Calcium-Selective Membrane Electrodes

Sung Bae Park*, **Sarah Chung**, **Geun Sig Cha**†, **and Hai Dong Kim**‡

Department of Chemistry and Research Institute of Basic Sciences, Inje University, Kimhae 621-749, Korea
†*Department of Chemistry and Research Institute of Basic Sciences, Kwangwoon University, Seoul 139-701, Korea*
‡*Research Institute of Basic Sciences and Department of Chemistry, Kyunghee University, Seoul 130-701, Korea*
Received June 7, 1995

PVC-based calcium-selective electrodes doped with ETH 129 usually suffer from a shift in the standard potential when they are in contact with protein-containing solutions (e.g. blood serum) after being calibrated with aqueous standards. The shift is due to the development of asymmetry potential in inherently symmetric PVC membranes through the contamination of outer membrane surface by proteins in the biological samples. Membranes prepared with polyurethane showed much reduced shifts in terms of standard potential. This study was performed with a flow-injection system following a protocol designed to observe minor shifts in baseline potential. Other electrochemical properties of the system, including selectivity and response slope, were similar to those obtained with regular PVC-based ones. PVC-based calcium selective membrane electrodes, doped with commonly used ETH 1001, were also tested to compare their electrochemical performances.

Introduction

Among various ion-selective electrodes (ISE's), liquid/polymeric membrane electrodes have become routine tools in clinical chemistry laboratories.[1~4] Up to now, most of related papers have reported electrochemical properties including selectivity in conjunction with poly(vinyl chloride) (PVC)-based membrane electrodes.[5] Indeed most commercial analyzers use PVC as a matrix of the polymeric membrane electrodes for the measurement of ions. This is due to the well studied behavior of the polymer system in terms of electrochemical properties (including specific resistance), physical robustness, manufacturability, etc. However, it has been observed that such sensors could suffer from a shift in the standard cell potential, Eo, when their ion-selective membranes are in contact with sample solutions containing proteins after being calibrated with aqueous standards. This effect has been shown to be caused by non-specific adsorption of serum proteins on the PVC-based membrane surface.[6,7]

Two different approaches to solve this problem can be considered. First, if the Eo shift remained constant and sample-independent, one-point or two-point calibration can be performed after each serum exposure to compensate the new offset. Second, modification to the membranes to make them more biocompatible for the less adsorption of protein can be attempted. By attaining protein-compatibility, the membrane should exhibit minimized or eliminated serum bias which results in systematic errors (usually positive errors). In fact, the Eo precision is quite poor (Eo shift up to 2 mV) depending on serum samples.[4,8] For example, after overnight idling period, data obtained with PVC-based membrane electrode can show significantly different readings in a consecutive measurement of an ion in a serum sample. Therefore it is thought that protein adsorption is not completely irreversible. In addition, longer calibration stability (24 hours or longer) is a common goal in the development of clinical autoanalyzers, because this can significantly reduce the time-cycle of measurements by eliminating the recalibration step. Because of these reasons, the first approach has not been very successful.

Naturally, studies on the second approach to solve the problem have been reported more frequently. One of the earlier reports showed that a sensor coated with a cellulose acetate membrane exhibited improved reproducibility of standard potential at the cost of prolonged response time.[8] In an independent study, Simon *et al.* has reported the use of vinyl chloride-vinyl alcohol copolymers.[6] Although the report showed a significantly reduced protein bias, reasons including poor robustness of the membrane seem to make them less viable. Asymmetric membrane systems employing cellulose triacetate were also reported.[9,10] The heterogeneous polymeric system, whose outer surface was hydroxylated to make it more hydrophilic, has proven successful in reducing protein bias. However, it seems that more studies are needed to improve their manufacturability, especially when they are applied on top of the solid electrode surface such as graphite rods. On the other hand, polyurethane (PU) has been reported as a membrane matrix of good biocompatibility.[7] P. D'Orazio has also suggested a reference method for the detection of ionized calcium using a PU-based membrane doped with ETH 1001 as the calcium ionophore.[11,12]

ETH 129, a Ca ionophore available commercially from Fluka, has proven very selective toward calcium over sodium and potassium.[13] However, seemingly poor protein compatibility has prevented it from being used despite of the better selectivity and lower price than ETH 1001. In the present study, ETH 129-based PU membrane electrodes were prepared. Then their electrochemical properties including selectivity and biocompatibility were compared with ETH 1001- and ETH 129-based PVC membrane electrodes.

In doing the protein compatibility studies, various methods have been reported: The methods have used specially designed flow-cell systems, radiotracer protein adsorption, or whole blood clotting time measurements (6, 7, 12). While

*To whom correspondence should be addressed

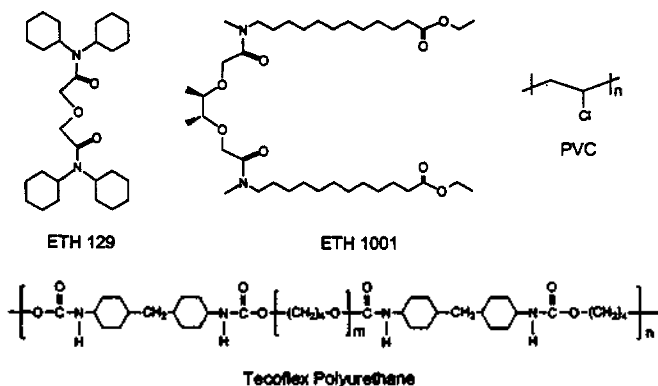

Figure 1. Structures of ionophores (ETH 129 and ETH 1001) and polymer matrices (PVC and Tecoflex polyurethane) for preparing potentiometric Ca^{2+} sensors.

the concepts of flow-cell systems used in these studies are similar to those of commercially available ones, their specific designs (geometry, sample volume, etc.) and measurements schemes (e.g. measurement timing, sample/diluent ratio, etc.) are different design to design. In addition, radiotracer and clotting time studies are not very suitable to visually observe the adsorption-desorption phenomena of proteins on the membranes. We therefore have employed a flow-injection analysis (FIA) mode to study aforementioned problems.

Experimental

Reagents. Ca-selective ionophores, N,N,N',N'-tetracyclohexyl-3-oxapentanediamide (ETH 129) and (−)-(R,R)-N,N'-[bis(11-ethoxycarbonyl)undecyl]-N,N',4,5- tetramethyl-3,6-dioxaoctanediamide (ETH 1001) were purchased from Fluka (Ronkonkoma, NY). PVC, *o*-nitrophenyl octyl ether (NPOE) as well as the anionic additive, potassium tetrakis (*p*-chlorophenyl)borate (KTpClPB) from Fluka were used to cast the ISE membranes. PU (Tecoflex SG-80A) were obtained from Thermedics Inc. (Woburn, MA). Some of these structures are shown in Figure 1. Tetrahydrofuran (THF) was obtained from Aldrich (Milwaukee, WI) and purified over sodium before use. A tris buffer solution consisting of 0.05 M tris[hydroxymethyl]aminomethane (Trizma Base) adjusted with HCl solution to pH 7.4 was used as the working buffer for the potentiometric measurements. Trizma-Base and bovine serum albumin (BSA) were products of Sigma Chemical Co. (St. Louis, MO). Before use, the BSA was first dialyzed against the tris buffer for three days with fresh buffer changes every 12 h. All other chemicals used were analytical-reagent grade. The buffer and standard solutions were prepared with deionized water.

Preparation of Polymer Membranes and Electrodes. PVC and PU ISE membranes were prepared by the method of Simon and co-workers.[14] The membrane compositions studied in this study are listed in Table 1. After allowing the membranes to cure, 5.5 mm diameter disks were cut out and placed in Phillips electrode bodies (ISE-561, Glasblaserei Moller, Zurich). As an internal filling solution, 0.1 M NaCl was used for all electrodes.

Static Mode Potentiometric Measurements. Cell potentials were measured at ambient temperature (18 ℃). The potentiometric cell used was as follows: Ag/AgCl(s)/4 M KCl saturated with AgCl/tris buffer/sample solution/ion-selective membrane/0.1 M NaCl/AgCl(s)/Ag. The ion-selective and Ag/AgCl reference electrodes were connected through a high impedance amplifier to an IBM AT-type computer equipped with an A/D converter with a sampling rate of 0.5 Hz. This apparatus was used to determine the selectivity of each membrane toward Ca^{2+} over other cations in the static mode. Selectivity coefficients, $k^{pot}_{Ca/X}$, were obtained by using the fixed interference method.[15]

Table 1. Calcium membrane formulation studied in the present study

Material	Weight %		
	A	B	C
ETH 129	1.0		1.0
ETH 1001		1.0	
PVC	32.8	34.5	
PU			32.8
NPOE	65.6	64.0	65.6
KTpClPB	0.6	0.5	0.6

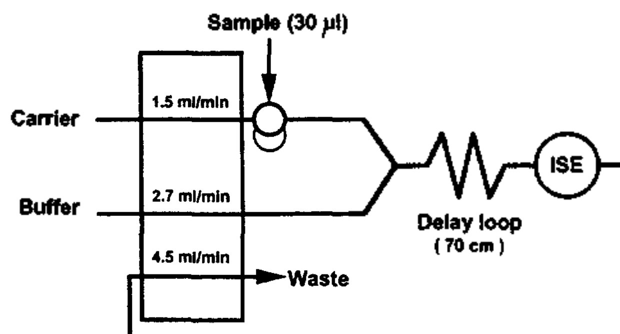

Figure 2. Schematic of the membrane electrode-based flow-injection system used for the study of minimization of asymmetry potential.

Flow-Injection System. The arrangement shown in Figure 2 was used to evaluate the performance of the PVC- and PU-based membrane electrodes in the FIA mode. A Phillips electrode body was fitted with a wall-jet type flow-through cap similar to that described previously.[16] The flow cap was connected to the injection valve by teflon tubing (0.012 in. i.d.). A peristaltic pump (Ismatech SA, Zurich) and a Rheodyne four-way rotary Teflon valve (Model 7125) equipped with a 30 μL sample loop completed the flow-injection setup. The tris buffer was used as the carrier and the buffer stream unless otherwise indicated. The calcium-selective and Ag/AgCl reference electrode were connected as described above. Sampling rate was adjusted to 2 Hz to observe delicate changes in the mV response. To examine the shift of baseline potentials of the electrodes, measurements were made following the injection protocol described in Table 2. To increase the S/N of obtained data, the moving average technique was employed using every 5 data points.[17]

Table 2. Time table used in the flow-injection tests for the alternate injection of aqueous calcium standards and 7 wt.% albumin solution

Time (sec)	Injected
10	0.5 mM Ca standard
80	5 mM Ca standard
150	7 wt.% dialyzed BSA in (carrier+buffer flow) for 180 sec; stop data aquisition for this period, then start to flow the tris buffer in (carrier+buffer flow) for 100 sec to wait for baseline return.
250	5 mM Ca standard
320	5 mM Ca standard
390	0.5 mM Ca standard
460	0.5 mM Ca standard
530	5 mM Ca standard
600	5 mM Ca standard
670	5 mM Ca standard

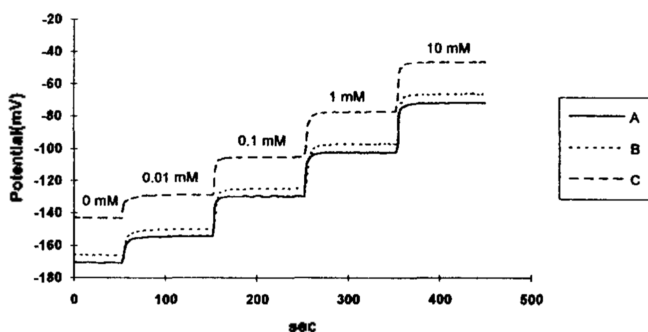

Figure 3. Potentiometric response of the ETH 129- and ETH 1001-based PVC and PU membrane electrodes toward Ca^{2+} ion (A=ETH 129/PVC, B=ETH 1001/PVC, C=ETH 129/PU; see Table 1 for formulations).

Results and Discussion

The equilibrium potentiometric response of the ETH 129-based PVC and PU membrane electrodes toward calcium in the concentration range of 0 mM-10 mM is shown in Figure 3. Although the baseline potentials were somewhat different, response patterns (e.g. response slope, Table 3) were very similar. The detection limits were 1.2×10^{-5} M in both cases. For a comparison purpose, an equilibrium potentiometric response of ETH 1001-based PVC membrane electrode is also presented in Figure 3. Table 4 provides the potentiometric selectivity coefficient data of the ETH 129-based PVC and PU membranes for the interfering cations relative to calcium. The data for PVC-based electrodes show a similar tendency in selectivity pattern to those reported earlier. The slight difference in numbers between the reference and the present study are due to the difference in obtaining the coefficients. Note that both ETH 129-based PVC and PU membranes showed same coefficient numbers, indicating a substantial advantage of using ETH 129 over ETH 1001, as far as the selectivity coefficients are concerned. This is because concentrations for urine potassium and se-

Table 3. Calcium membrane response slopes in mV/dec at between 0.1 and 10 mM (at 18 ℃; theoretical 28.9 mV/dec)[a]

	A	B	C
fresh membranes[b]	28.5	28.6	28.5
after two months[c]	27.8	27.9	27.9

[a] A, B, and C denote membrane formulation described in Table 1. [b] average of three membranes. [c] average of two membranes.

Table 4. Comparison of the static mode potentiometric selectivity coefficients, $\log k^{pot}_{Ca,x}$, for electrodes prepared with PVC and PU as membrane supporting material[a]

Cation	Reference[b]		Present study[c]		
	A	B	A	B	C
$x=Na^+$	−3.7	−3.4	−3.9	−3.6	−3.9
$x=K^+$	−4.0	−3.8	−4.0	−3.8	−4.0

[a] A, B, and C denote membrane formulation described in Table 1. [b] From Schefer *et al.* [13], separate solution method using 0.1 M metal chloride solutions. [c] Fixed interference method ($[Na^+]$=100 mM, $[K^+]$=100 mM).

rum sodium can be quite high (reference range: 25-125 mM/d and 136-146 mM, respectively.).[18] In terms of lifetime of the PVC and PU membrane electrodes, more than two months of response stability was observed (see Table 3).

Despite of the superior selectivity and lower price, ETH 129-based membranes are not being used in most commercial analyzers.[11] This is presumably due to their poorer biocompatibility (*i.e.* larger asymmetry potential) than that of ETH 1001-based membranes. In fact, the asymmetry potential is found when the membrane electrode is in contact with protein-containing sample solutions (e.g. serum samples). As the membrane is in contact with serum sample proteins that constitute 6-8 wt.% of serum, the proteins are adsorbed on top of outer membrane surface. Because the adsorption is somewhat irreversible and the second and later readings tend to be stabilized,[6,11] the non-specific adsorption can result in a systematic error especially for the first measurement. This makes the reproducibility (%CV) of a replicate measurement poor. This effect is shown clearly in Figure 4 that compares the FIA data obtained with the system drawn in Figure 2. Membranes used in this study were the same membranes that used in the static mode experiments. When the protein solution was flowed in between the aqueous calcium standards (at the point of 150 sec for 180 sec), there were clear differences in response behavior for the three kinds of electrodes, in terms of total response and baseline potential for the aqueous calcium standards. That is, baseline potential for ETH 129-based PVC membrane electrode (see Figure 4 II-A) shifted in the positive direction. The amount of the shift was about 3.6 mV, while PU membranes doped with the same ionophore showed less than 0.5 mV. This small amount of baseline shift was quite comparable to that of ETH 1001-based PVC membrane electrodes (0.5 mV) (average of 3 determinations each). These baseline shifts resulted in span changes (ΔmV of two aqueous

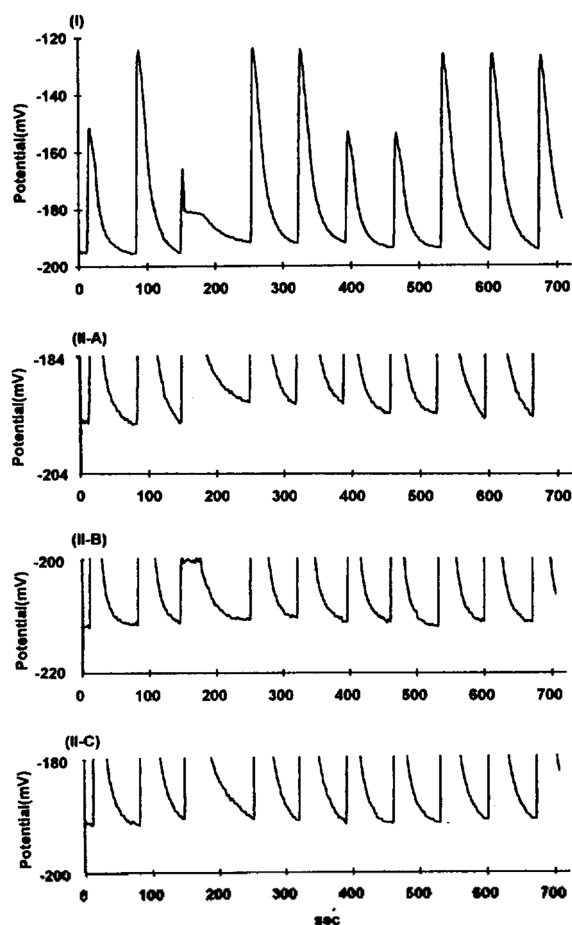

Figure 4. (a) Flow-injection response of a typical ETH 129-based PU membrane electrode; (b) Flow-injection responses of ETH 129-based PVC and PU membrane electrodes enlarged to compare the shifts in baseline potentials (A=ETH129/PVC; B=ETH 1001/PVC; C=ETH129/PU).

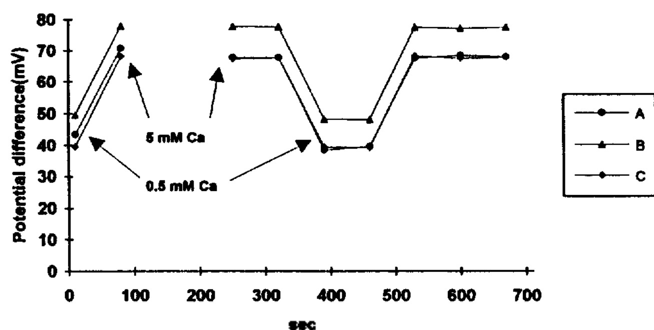

Figure 5. Tendency of span changes of the ETH 129- and ETH 1001-based PVC and PU membrane electrodes toward Ca standards before and after the protein exposure (see Table 1 for the formulation of A, B, and C).

standards, 0.5 and 5 mM Ca) in different ways for the three kinds of membranes. Calcium standards of 0.5 and 5 mM were used, as the reference range of total calcium in human serum(adult) is 2.10-2.55 mM.[18] Figure 5 shows the tendency of span changes before and after the membrane electrodes were in contact with the 7 wt.% dialyzed albumin solution. The significant span decrease of the ETH 129-based PVC membrane electrode immediately after the contact is mainly due to the shift in baseline. Although the span tends to be close, as time goes by, to the numbers obtained before the contact, this decreased span can give especially the first serum calcium reading a higher number than it should. The data obtained with ETH 129-based PU membranes were comparable to ETH 1001-based PVC membranes.

Conclusion

We prepared ETH 129-based PVC and PU membranes to test their biocompatibility in protein-containing sample solutions. Minor changes in span mainly due to baseline shifts were observed using a flow-injection system. By continuously/alternately flowing aqueous Ca^{2+} standard and protein-containing solution, the span changes were able to be compared. ETH 129-based PU membranes showed a better biocompatibility than PVC membranes doped with the same ionophore, resulting in much reduced shifts in baseline potential. The biocompatibility was quite comparable to that of ETH 1001-based PVC membrane electrodes. Confirmed selectivity toward Ca^{2+} over Na^{+} and K^{+} as well as the comparable biocompatibility suggest that ETH 129-based PU membrane can replace ETH 1001-based PVC membrane electrodes currently used in most clinical autoanalyzers.

Acknowledgment. This work was supported by the Korea Science and Engineering Foundation (94-0800-07-01-3) and Grant from Inje University (1994).

References

1. Rabenstein, D. L. *Anal. Chem.* **1988**, *64*, 1R.
2. Arnold, M. A.; Meyerhoff, M. E. *Anal. Chem.* **1984**, *56*, 20R.
3. Czaban, J. D. *Anal. Chem.* **1985**, *57*, 345A.
4. Oesch, U.; Ammann, D.; Simon, W. *Clin. Chem.* **1986**, *32*, 1448.
5. Umezawa, Y. *Handbook of Ion-Selective Electrodes: Selectivity Coefficients*; CRC Press: Boca Raton, 1990.
6. Durselen, L. F. J.; Wegmann, D.; May, K.; Oesch, U.; Simon, W. *Anal. Chem.* **1988**, *60*, 1455.
7. Cha, G. S.; Liu, D.; Meyerhoff, M. E.; Cantor, H. C.; Midgley, A. R.; Goldberg, H. D.; Brown, R. B. *Anal. Chem.* **1991**, *63*, 1666.
8. Fogh-Andersen, N.; Christiansen, T. F.; Komarmy, L.; Siggaard-Andersen, O. *Clin. Chem.* **1978**, *24*, 1545.
9. Cha, G. S.; Meyerhoff, M. E. *Talanta* **1989**, *36*, 271.
10. Park, S. B.; Matuszewsky, W.; Meyerhoff, M. E.; Liu, Y. H.; Kadish, K. M. *Electroanalysis* **1991**, *3*, 909.
11. D'Orazio, P.; Laios, I.; Bowers, G. N. *Proceedings of Int'l symposium of Am. Assoc. for Clin. Chem.* **1992**, 21.
12. D'Orazio, P.; Bowers, G. N. *Clin. Chem.* **1992**, *38*, 1332.
13. Schefer, U.; Ammann, D.; Pretsch, E.; Oesch, U.; Simon, W. *Anal. Chem.* **1986**, *58*, 2282.
14. Schulthess, P.; Ammann, D.; Simon, W.; Caderas, C.; Stepanek, R.; Krautler, B. *Helv. Chim. Acta* **1984**, *67*, 1026.
15. Moody, G.; Thomas, J. *Selective Ion-Sensitive Electrodes*;

Merrow: Watford, England, 1971.
16. Trojanowicz, M.; Meyerhoff, M. E. *Anal. Chem.* **1989**, *61*, 787.
17. Binkley, D.; Dessy, R. *J. Chem. Educ.* **1979**, *56*, 148.
18. Tietz, N. *Textbook of Clin. Chem.*; Saunders: Philadelphia, 1986.

1,2,4-Triazole Fused Heterocycles; Part 3. Preparation of 1-(1-Phenylethenyl)-5-(*N*-substituted amino)-1,2,4-triazoles and 4*H*-1,2,4-Triazolo[1,5-c][1,3,5]oxadiazines

Kee-Jung Lee*, **You-Suk Lee**, and **Dong-Hyuk Song**

Department of Industrial Chemistry, Hanyang University, Seoul 133-791, Korea
Received June 12, 1995

The reaction of acetophenone 1-ureidoethylidenehydrazones **6** with a mixture of triphenylphosphine, carbon tetrachloride, and triethylamine in dichloromethane provides a general route to 1-(1-phenylethenyl)-5-(*N*-substituted amino)-1,2,4-triazoles **11** *via* the electrocyclization of the expected azino carbodiimide intermediates **9** to give the resonance stabilized azomethine imine **10a** followed by a proton abstraction from the methyl group by amide anion. However, the same reaction of benzaldehyde 1-ureidoethylidenehydrazones **5** was unsuccessful. Under the same conditions, the reactions of benzaldehyde 1-*N*-acylureidoethylidenehydrazones **7** or acetophenone 1-*N*-acylureidoethylidenehydrazones **8** afforded 4*H*-1,2,4-triazolo[1,5-*c*][1,3,5]oxadiazines **16** or **17** *via* the zwitterionic species **15**, or a [4+2] intramolecular cycloaddition from the carbodiimide intermediates **14**, respectively.

Introduction

Recent interest in the electrocyclic reaction of conjugated heterocumulenes as a synthetic route to heterocycles,[1] prompted us to report on this subject. The previous papers in these series have shown that 1,2,4-triazole fused heterocyclic compounds with one of its nitrogen atom in a bridgehead position such as 5,10-dihydro-1,2,4-triazolo[5,1-*b*]quinazolines[2] and 4*H*-1,2,4-triazolo[1,5-*c*][1,3,5]oxadiazines[3] can readily be prepared from the reactions of benzophenone 1-ureidoethylidenehydrazones and benzophenone 1-*N*-acyl-ureidoethylidenehydrazones with a mixture of triphenylphosphine, carbon tetrachloride, and triethylamine in dichloromethane by dehydration[4] and subsequent electrocyclic ring closure of the azino carbodiimides. In the case of the reaction of benzophenone 1-ureidoethylidenehydrazones, either of two phenyl groups of benzophenone moiety was always participated in the ring closure process.[2]

With our continued interest in the reactions of azine substituted heterocumulenes to prepare fused triazolo ring systems, we chose to examine the reactions of benzaldehyde 1-ureidoethylidenehydrazones **5** or acetophenone 1-ureidoethylidenehydrazones **6** with triphenylphosphine, carbon tetrachloride, and triethylamine to see whether different triazole products such as **12** or **13** can be formed, because of the possibility of the participation of phenyl group in benzaldehyde or acetophenone, *N*-substituted aromatic group, or methyl group in the ring forming step (Scheme 2).

Results and Discussion

The starting compounds, benzaldehyde 1-aminoethylidenehydrazone (**3**) and acetophenone 1-aminoethylidenehydrazone (**4**), were obtained by the reaction of acetamidrazone hydrochloride (**1**) with benzaldehyde,[5] and by the reaction of acetophenone hydrazone with *S*-methylthioacetimidate hydroiodide (**2**), respectively. The ureas **5** and **6** were produced by the reactions of hydrazones **3** and **4** with isocyanates in dichloromethane at room temperature (Scheme 1). Thin layer chromatography showed one spot (silica gel, ethyl acetate-hexane, 1 : 1), however, ^{1}H NMR showed a mixture of two isomers, and the ratios found were 1.7-3.6/1 for the ureas **5** and 1.9-3.5/1 for the ureas **6** (Table 1). When the reaction of ureas **5** with triphenylphosphine, carbon tetrachloride, and triethylamine in dichloromethane was heated at reflux temperature for 4-5 h, the reaction mixture turned brown solution and thin layer chromatography showed the disappearance of **5** and the formation of a number of very small products along with triphenylphosphine oxide. All attempts to separate these complex mixture proved fruitless except triphenylphosphine oxide. We presume that although the azino carbodiimide intermediate 9 was formed, the electrocyclic reaction of **9** to give **12** or **13** did not occurr, but decomposed under the reaction conditions. These facts suggest that the steric interactions between phenyl and R groups push force the *N*-aromatic group into a transoid position relative to the triazole-*N*-substituents to give resonance form such as **10e**. Thus the resonance forms **10c** and **10d** are not favored to produce 1,2,4-triazoloquinazolines **12** or **13** (Scheme 2).

On the other hand, treatment of ureas **6** with triphenylphosphine, carbon tetrachloride, and triethylamine in dichloromethane at reflux temperature smoothly afforded the 1-(1-phenylethenyl)-5-(*N*-substituted amino)-1,2,4-triazoles **11** pre-