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High-performance liquid chromatographic study of the reduction of protected oxytocein by sodium in liquid ammonia

A. PÉTER*, F. LUKÁCS and K. BURGER
Department of Inorganic and Analytical Chemistry, A. József University, P.O. Box 440, 6701 Szeged (Hungary)
and
I. SCHŐN, M. LŐW and L. KISFALUDY
Gedeon Richter Chemical Works, P.O. Box 27, 1475 Budapest (Hungary)

SUMMARY

A combined high-performance liquid chromatographic (HPLC)–electrochemical method was developed to investigate the mechanism of reduction of protected oxytocein by metallic sodium in liquid ammonia. The changes in the redox potential and conductivity of protected oxytocein solution provided information on the stoichiometry of the reduction. HPLC methods elaborated for the identification and selective determination of the reaction intermediates and products were used to analyse the compounds formed. This combined procedure revealed the reaction path of the reduction processes and permitted optimization of the experimental conditions to increase the yield of oxytocin.

INTRODUCTION

Reductions by alkali metals in liquid ammonia have long been used in peptide chemistry to cleave tosyl and benzyl protecting groups[1]. The protected oxytocein investigated in this work contained one benzyloxycarbonyl (Z) and two benzyl (Bzl) groups: Z–Cys(Bzl)–Tyr–Ile–Gln–Asn–Cys(Bzl)–Pro–Leu–Gly–NH_2.

Different stoichiometries have been reported for the reduction of the benzyl group[2–5], the N-benzyloxycarbonyl group[5–7] and organic sulphides (*e.g.*, in cysteine)[8]. The application of this reduction for oxytocin production is based on empirical prescriptions, but the oxytocin yield does not exceed 55–60%.

In previous work[9], we applied a method that had been developed to measure the redox potentials and conductivities of model substances in liquid ammonia in the course of "titration" with a solution of metallic sodium. Data relating to redox potential and conductivity changes during "titration" provided information on the stoichiometry of the reduction of protected oxytocein, but not on the products, mechanism or degree of reaction.

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The degree of reduction has been observed to depend on, among other factors, the presence of proton donor molecules in organic substance–metallic sodium systems[8,10]. This phenomenon was explained in terms of the acidic behaviour and buffer action of proton donors. The proton donor molecules most often used are alcohols[10]. In this work we chose urea as proton donor; it acts as a dibasic acid in liquid ammonia[11] and has a pK_a of 12.9[12].

The direct product of reduction of protected oxytocein in liquid ammonia is not oxytocin, but the sodium salt of oxytocein. Oxytocin is formed in a further oxidation step in water.

High-performance liquid chromatography (HPLC) was found to be the most useful method for characterizing the reaction intermediates and products and for determining the mechanism of oxytocin synthesis. Several HPLC systems have been reported for the determination of oxytocin[13–30], but none of them was suitable for the separation of oxytocein and oxytocin.

We set out to investigate the steps of the oxytocin synthesis and to optimize the reaction conditions with the aim of increasing the oxytocin yield. HPLC methods were elaborated for these investigations.

EXPERIMENTAL

Apparatus

A glass apparatus was constructed for the quantitative characterization of reactions with metallic sodium in liquid ammonia[9]. In the "titration" process with sodium, the changes in redox potential and conductivity were recorded.

HPLC separations

Experiments were carried out with Model 2150 (LKB, Bromma, Sweden) and Liquochrom OE 330 (Labor-MIM, Budapest, Hungary) liquid chromatographs connected with LKB 2138 Uvicord S and UV 308 detectors. Rheodyne loop injectors (10 μl) were used for sample introduction. Prepacked Hypersyl ODS (10 μm) columns (250 $\times$ 4.6 mm I.D.) (Bio Separation Technology, Budapest, Hungary) were also used.

Materials

Protected oxytocein and oxytocin were obtained from Gedeon Richter (Budapest, Hungary) and urea and toluene from Reanal (Budapest, Hungary). Diphenylethane was prepared by the usual method.

The organic solvents used were of HPLC grade (E. Merck, Darmstadt, F.R.G.). Buffers and salt solutions were prepared from Merck, BDH (Poole, U.K.) or Fluka (Buchs, Switzerland) reagents, using water doubly distilled from a glass apparatus and further purified by pumping through 10-μm filters.

RESULTS AND DISCUSSION

Fig. 1 shows the potentiometric and conductimetric titration curves of protected oxytocein with standard sodium solution in the presence and absence of urea. As can be seen from the potentiometric curves, the reduction requires about 7 equiv. of sodium in both instances. The presence of urea does not change the stoichiometry of reduction.

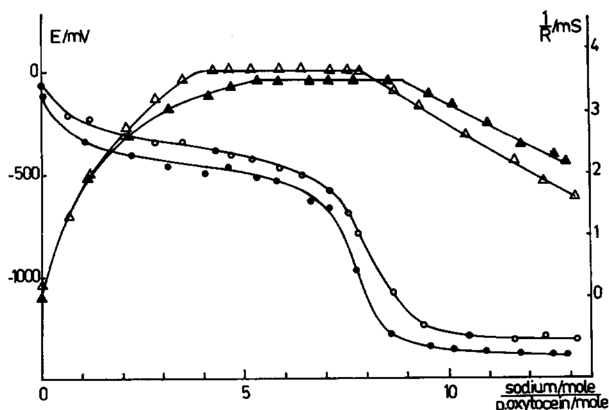

Fig. 1. Potentiometric (○, ●) and conductometric (△, ▲) titration curves of protected oxytocein in the absence (○, △) and presence (●, ▲) of urea. Concentration of protected oxytocein, $6 \cdot 10^{-3}$ mol dm^{-3}; concentration of urea, $3.6 \cdot 10^{-2}$ mol dm^{-3}.

To determine the reduction products, the reaction was stopped at different molar ratios of sodium to peptide, the ammonia was removed by evaporation and the product was dissolved in water (or in methanol for analysis of aromatic compounds), suitably oxidized and analysed by HPLC.

In the reduction of protected oxytocein with sodium, toluene and the sodium salt of oxytocein are first formed. The formation of toluene may reflect the splitting off of benzyl and N-benzyloxycarbonyl groups via a two-electron step mechanism. The formation of diphenylethane, indicating reduction via a one-electron step, is negligible.

The sodium salt of oxytocein was oxidized to oxytocin in a careful oxidation step. The degree of oxidation was followed by HPLC on the basis of the difference in polarity between oxytocein and oxytocin (the latter is less polar).

The eluent system applied to resolve oxytocin from oxytocein was acetonitrile–0.01 *M* phosphate buffer (pH = 2.2) (25:75) containing 0.1 *M* sodium perchlorate. The capacity factors (k') were 3.0 for oxytocein and 3.4 for oxytocin (Fig. 2).

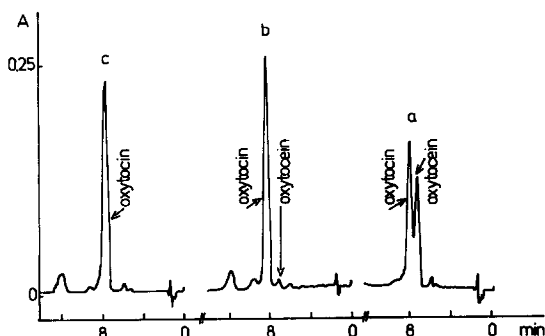

Fig. 2. Chromatograms of reduction products of protected oxytocein in various stages of oxidation. Eluent, acetonitrile–0.01 *M* KH_2PO_4 (pH 2.2) (25:75); detection, UV, 220 nm; concentration of protected oxytocein in liquid ammonia, $6 \cdot 10^{-3}$ mol dm^{-3}; molar ratio of sodium to protected oxytocein, 7.06; molar ratio of urea to protected oxytocein, 6.0; stirring time in air, (a) 20, (b) 70 and (c) 120 min.

TABLE I

OXYTOCIN YIELD ON OXIDATION AT pH 6.8

Procedure	*Temperature (K)*	*Concentration of protected oxytocein in liquid ammonia (mol dm^{-3})*															
		3 · 10^{-3}						*6 · 10^{-3}*						*12 · 10^{-3}*			
		Concentration of urea in liquid ammonia (mol dm^{-3})															
		0		*18 · 10^{-3}*		*72 · 10^{-3}*		*0*		*36 · 10^{-3}*		*72 · 10^{-3}*		*0*		*72 · 10^{-3}*	
		r[a]	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*	*r*	*Yield (%)*
Addition of sodium																	
By "titration"	228	7.29	77	8.02	80	8.33	87	7.32	67	7.52	78			7.27	64	7.52	76
		8.45	79											7.75	62	7.89	74
In solid form	228	6.76	83	7.06	86	7.50	89	7.75	65			7.32	85	7.46	57	7.26	75
		6.98	83	8.47	76			7.76	67								
	240	7.12	82	7.57	79									6.80	64	7.34	71
		7.53	78	8.31	79									7.48	69	7.68	76
		7.65	78	8.50	76												

[a] r = Molar ratio of sodium to protected oxytocein in liquid ammonia.

Optimization of oxytocin production

The yield of oxytocin depends on the efficiency of the reduction and oxidation stages.

To optimize the reduction stage, first the optimum molar ratio of sodium to protected oxytocein was determined which was found to be about 7–8 (Fig. 3). At lower ratios unreacted protected oxytocein remained in the system, decreasing the yield. The protected oxytocein does not dissolve in water, only in hot dimethylformamide, and therefore it does not appear in HPLC analysis. Above the ratio of 7–8 the excess of sodium splits the peptide bonds. Peaks in the chromatograms characterizing smaller peptides increase with increasing sodium concentration.

To determine the dependence of the oxytocin yield on the concentration of protected oxytocein, three concentrations were studied, $12 \cdot 10^{-3}$, $6 \cdot 10^{-3}$ and $3 \cdot 10^{-3}$ mol dm^{-3}. Tables I and II show that a decrease in the protected oxytocein concentration leads to an increased oxytocin yield. This may be due either to suppression of the side-reactions or to the dissociation of the weakly acidic groups on protected oxytocein with dilution, leading to an increase in the proton concentration, favouring reduction.

The effect of the proton donor urea was investigated at various urea and protected oxytocein concentrations. At a constant protected oxytocein concentration of $12 \cdot 10^{-3}$ mol dm^{-3}, variation of the concentration ratio of urea to protected oxytocein between 3 and 12 revealed that the optimum ratio was about 6.

At a protected oxytocein concentration of $3 \cdot 10^{-3}$ mol dm^{-3}, no influence of urea was observed (Fig. 3b); at concentrations of $6 \cdot 10^{-3}$ and $12 \cdot 10^{-3}$ mol dm^{-3}, the oxytocin yield in the presence of urea was higher, the increase reaching 10–15% (Fig. 3a and Tables I and II).

For practical reasons, the influence of temperature was examined only at 228 and 240 K. The results did not reflect a temperature effect.

The results of the "titration" of the peptide with metallic sodium dissolved in liquid ammonia were compared with those for the reactions between solid sodium and

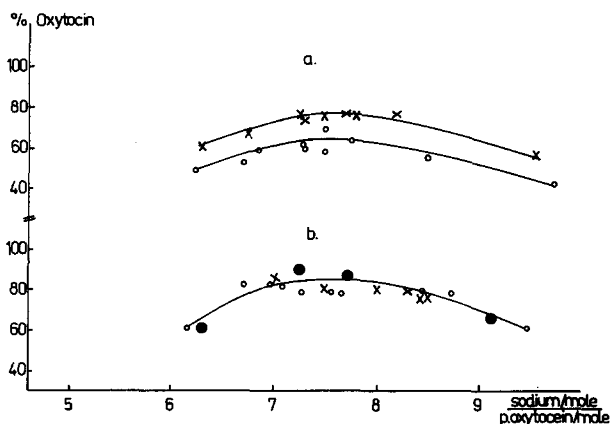

Fig. 3. Plots of oxytocin yield (%) *versus* molar ratio of sodium to protected oxytocein in the absence and presence of urea. pH = 6.8. Initial concentration of protected oxytocein in liquid ammonia: (a) $12 \cdot 10^{-3}$ and (b) $3 \cdot 10^{-3}$ mol dm^{-3}. Ratio of urea to protected oxytocein: ○ = 0; × = 6; ● = 24.

TABLE II

OXYTOCIN YIELD ON OXIDATION AT pH 11.9

Procedure	Temperature (K)	Concentration of protected oxytocein in liquid ammonia (mol dm^{-3})															
		$3 \cdot 10^{-3}$						$6 \cdot 10^{-3}$						$12 \cdot 10^{-3}$			
		Concentration of urea in liquid ammonia (mol dm^{-3})															
		0		$18 \cdot 10^{-3}$		$72 \cdot 10^{-3}$		0		$36 \cdot 10^{-3}$		$72 \cdot 10^{-3}$		0		$72 \cdot 10^{-3}$	
		r^a	Yield (%)	r	Yield (%)	r	Yield (%)	r	Yield (%)	r	Yield (%)	r	Yield (%)	r	Yield (%)	r	Yield (%)
Addition of sodium																	
By "titration"	228	7.29	86	8.02	99	8.33	99	7.32	80	7.52	78			7.27	74	7.52	83
		8.45	90					7.90	75	7.90	75			7.75	71	8.17	83
In solid form	228	6.76	90	7.06	97	7.50	96	7.75	70			7.32	91	7.46	70	7.26	86
		6.98	95	8.47	88			7.76	69								
	240	7.12	95	7.57	87									6.80	75	7.34	86
		7.53	87	8.31	90									7.48	75	7.68	78
		7.65	90	8.50	91												

[a] r = Molar ratio of sodium to protected oxytocein in liquid ammonia.

peptide solution (Tables I and II). No significant difference in oxytocin yield was observed under the conditions studied.

To optimize the oxidation stage in oxytocin synthesis, the kinetics of oxidation were investigated. The product of sodium reduction, the solid sodium salt of oxytocein, was dissolved in water to give different concentrations. The pH of these oxytocein solutions was adjusted to 11.9 or 6.8 and the solutions were allowed to stand in open beakers in contact with air. At fixed intervals, samples were taken from the solutions and the oxytocein and oxytocin contents were determined by HPLC.

The $3 \cdot 10^{-3}$ mol dm^{-3} oxytocein solution was oxidized more slowly than the $7.5 \cdot 10^{-4}$ mol dm^{-3} solution; it reached its maximum value 2–6 h later. This may be due to the high ratio of oxytocein to oxygen in solution, because the oxygen concentration was lower than the concentration of oxytocein and oxygen was replaced by diffusion only. When the replacement of oxygen was enhanced by stirring the solution, the rate of oxidation increased. On the other hand, the concentration of oxytocin started to decrease after reaching a maximum value. This phenomenon may be explained by oligomerization and degradation of the peptide being favoured in concentrated solution, as reflected in the chromatograms by some peaks after the oxytocin peak, these peaks increasing in time.

In some experiments, hydrogen peroxide was used instead of oxygen as the oxidant. The oxidation with hydrogen peroxide must be performed carefully, as too fast an addition or overaddition decreases the yield of oxytocin.

When oxidation was carried out in basic solution, the chromatograms contained higher peaks at the position of oxytocin, indicating a higher yield. Biological activity measurements confirmed the results of HPLC measurements.

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