POLAROGRAPHIC STUDY OF HYDROLYSIS OF [8-LYSINE]VASOPRESSIN AND ITS DERIVATIVES WITH BLOOD SERUM OF PREGNANT WOMEN

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A polarographic study of the hydrolysis of [8-lysine]vasopressin and some hormonogens of the vasopressin series with the blood serum of women in the last week of pregnancy was studied. The dependence of hydrolysis on pH (pH optimum: 7·4—7·5), substrate concentration (K_m 1·2 . . 10^{-5}M), pH stability and thermal stability were determined. The rate of hydrolysis of individual vasopressin analogues decreases in the order: [8-lysine]vasopressin > N^{α}-glycyl-prolyl[8-lysine]-vasopressin > N^{α}-leucyl-[8-lysine]vasopressin > N^{α}-alanyl-[8-lysine]vasopressin > N^{α}-phenyl-alanyl-[8-lysine]vasopressin > N^{α}-diglycyl-[8-lysine]vasopressin > N^{α}-prolyl-[8-lysine]vasopressin > N^{α}-triglycyl-[8-lysine]vasopressin > N^{α}-sarcosyl-glycyl-[8-lysine]vasopressin. The degree of hydrolysis gradually increases to a multiple with the length of the pregnancy in consequence of the presence of oxytocinase. However, vasopressin is also hydrolysed to a small extent with the enzymes from the blood sera of non-pregnant women. Under similar analytical conditions oxytocin was not hydrolysed with the sera of non-pregnant women and therefore oxytocin is a more suitable substrate than vasopressin for polarographic determination of serum oxytocinase.

Recently a polarographic method of following hydrolysis of oxytocin with serum oxytocinase[1-3] was elaborated. The enzyme activity was determined from the decrease of the catalytic wave of oxytocin as substrate, after incubation in the presence of serum. This paper summarizes experimental results concerning polarographic study of hydrolysis of vasopressin* with the serum of pregnant women and extends the data concerning the problems of the polarographic determination of serum oxytocinase activity, when vasopressin is the substrate instead of oxytocin. The utilisability of the polarographic method in the study of the inactivation of analogues of [8-lysine]vasopressin with a prolonged chain is demonstrated.

* Under vasopressin and its analogues, [8-lysine]vasopressin and the analogues derived from it are understood. With the exception of glycine and sarcosine all amino acids were of L-configuration. NAp means the *p*-nitroanilide residue.

EXPERIMENTAL

Material

Synthetic [8-lysine]vasopressin (LVP) and its structural analogues, N^{α}-alanyl (Ala-LVP), N^{α}-glycyl (Gly-LVP), N^{α}-diglycyl(Gly-Gly-LVP), N^{α}-triglycyl(Gly-Gly-Gly-LVP), N^{α}-glycyl-prolyl (Gly-Pro-LVP), N^{α}-leucyl (Leu-LVP), N^{α}-phenylalanyl (Phe-LVP), N^{α}-sarcosyl-glycyl (Sar-Gly--LVP), N^{α}-prolyl (Pro-LVP) (ref.[4]), were employed for the experiments. Store solutions of LVP and the mentioned analogues contained 0·5 mg of substance per millilitre. The following chromogenic substrates were also used in the form of their $2 \cdot 10^{-2}$M solutions in 50% acetone: S-methyl--cysteine-*p*-nitroanilide (Cys(Me)-NAp (ref.[5]), S-ethyl-cysteine-*p*-nitroanilide (Cys(Et)-NAp) (ref.[5]), alanine-*p*-nitroanilide (Ala-NAp), glycine-*p*-nitroanilide (Gly-NAp), diglycyl-*p*-nitroanilide (Gly-Gly-NAp), triglycyl-*p*-nitroanilide(Gly-Gly-Gly-NAp), leucine-*p*-nitroanilide(Leu-NAp) and lysine-*p*-nitroanilide (Lys-NAp). The hormonogens of vasopressin[4] and the *p*-nitroanilides of amino acids or peptides used were prepared in our laboratory. The following cobalt(III) solution was used for polarography: 10^{-3}M-$Co(NH_3)_6Cl_6$, 0·7M-NH_4OH and 0·1M-NH_4Cl. Blood serum of pregnant women in the ninth month of pregnancy and the serum of healthy non-pregnant women were used as a source of enzymes. The recording of polarographic curves was carried out on polarographs Radelkis OH-102 (Budapest) or LP-60 (Laborinstruments, Prague).

Methods

Enzymatic hydrolysis of LVP took place at 37°C in 2·0 ml of a reaction mixture containing 1·7 ml of Sörensen's phosphate buffer of pH 7·4, 0·2 ml of substrate solution and 0·1 ml of serum. After 1 h incubation the reaction was stopped by addition of 0·5 ml of 20% sulfosalicylic acid. The precipitate was mixed and centrifuged after 10 min standing (3000*g*, 20 min), or filtered off. The supernatant (0·5 ml) was added to 2·5 ml of the cobaltic salt solution and polarographed. The polarographic curves were recorded from —1·00 V. The measurements were carried out in Kalousek's vessel[6,7] with a reference saturated calomel electrode. For comparison with a hydrolysed sample a sample was chosen the hydrolysis of which was blocked by instantaneous addition of sulfosalicylic acid. The activity of the enzymes cleaving LVP was expressed as the difference between the heights of the polarographic waves of the incubated and the non-incubated sample in mm, or as the degree of hydrolysis in %. The method of making shortened polarographic records is presented in ref.[3]. Enzymatic hydrolysis of Cys(Me)-NAp was carried out using a 2·0 ml volume containing 1·6 ml of buffer, 0·2 ml of substrate solution and 0·2 ml of serum. The reaction took place at 37°C for 10 min. The hydrolysis was stopped on addition of 1·0 ml of 0·2M-HCl. The amount of the liberated *p*-nitroaniline was measured at 405 nm with a Spectromom 202 (Budapest) spectrophotometer. Occasional differences from the procedures mentioned here are given in the text.

RESULTS

Dependence of the Height of the Polarographic Wave on Concentration of LVP *and Hormonogens*

In Brdička's cobaltic salt solution LVP gives a double polarographic wave at $-1{\cdot}4$ V and $-1{\cdot}6$ V. The double-wave is well developed mainly at lower concentrations of LVP. With increasing concentration of LVP the first part of the wave becomes

less distinct and finally, at the highest concentrations only the second part of the polarographic double-wave with the peak at $-1{\cdot}6$ V is visible, opposite to the saturated calomel electrode.

The dependence between the height of the polarographic double-wave and the concentration of LVP has a linear course within the investigated range of concentrations, *i.e.* $1{\cdot}05-6{\cdot}30 \,.\, 10^{-5}$M (Fig. 1). However, the beginning of this dependence does not start from zero, owing to the presence of the proteins of the sulfosalicylic acid filtrate of the serum in the investigated solutions. The height of the LVP wave is relatively strongly decreased in consequence of adsorption of LVP on the surface of the protein precipitate within the time interval of 0 to 7 min from the formation of the precipitate. Since further adsorption becomes negligible after 10 min we chose this time of standing for further experiments. All hormonogens of LVP investigated also give a polarographic double-wave in Brdička's cobaltic salt solution. The course and the properties of this wave are similar to those of LVP itself. However, they

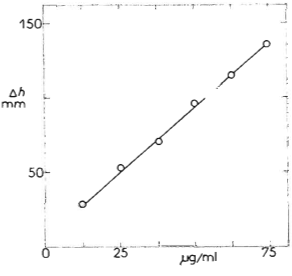

FIG. 1

Relationship between the Height of the Polarographic Wave, h, and the Concentration of [8-Lysine]vasopressin (LVP)

Concentration of LVP 12·5, 25·0, 37·5, 50·0, 62·5 and 75·0 μg/ml in 2 ml of phosphate buffer of pH 7·4, containing 0·1 ml of serum. Polarographed after precipitation with sulfosalicylic acid.

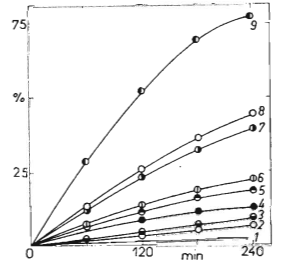

FIG. 2

Course of Hydrolysis of [8-Lysine]vasopressin and Synthetic Hormonogens

The reaction mixture contained 0·2 ml of serum, phosphate buffer of pH 7·4 and substrate in $4 \,.\, 10^{-5}$M concentration in a total volume of 2 ml. Incubation temperature 37°C. *1* Sar-Gly-LVP; *2* Gly-Gly-Gly-LVP; *3* Pro-LVP; *4* Gly-Gly-LVP; *5* Phe-LVP; *6* Ala-LVP; *7* Leu-LVP; *8* Gly-Pro-LVP; *9* LVP. Ordinate: Degree of hydrolysis in %.

differ from each other by the height of the double-wave at the same concentration of hormonogens in solutions and at the same conditions of polarography.

Dependence of Enzymatic Hydrolysis of [8-*Lysine*]*vasopressin and the Derived Hormonogens on Time*

The course of hydrolysis of LVP and hormonogens in the presence of the serum of pregnant women is shown in Fig. 2. It is evident that with the exception of Sar-LVP all substrates used were cleaved. The hydrolysis of substrates that are most strongly cleaved, *i.e.* LVP, Gly-Pro-LVP and Leu-LVP, takes place following the first order kinetics, while the hydrolysis of other substrates follows the zero order kinetics. Table I shows the comparison of the degree of hydrolysis of hormonogens with an index of persistence I_p (ratio of elimination constants of the analogue and of the standard) with the cleavage of corresponding *p*-nitroanilides of amino acids.

Parallelly with the polarographic following of the hydrolysis of hormonogens we also determined the amino acids set free by enzymatic hydrolysis of substrates. The results of the analyses are presented in Table II.

TABLE I

Comparison of the Degree of Hydrolysis of Hormonogens with Persistency Index and with the Hydrolysis of *p*-Nitroanilides of Amino Acids

Compound	Degree of hydrolysis (% of hydrolysis/4 h)	Persistency index I_p	AK-NAp[a]	μM pNA[b]/h
LVP	79·0	—		
Gly-Pro-LVP	41·5	1·90		
Leu-LVP	38·0	1·04	Leu-NAp	2 400
Ala-LVP	21·3	2·20	Ala-NAp	1 645
Phe-LVP	18·0	2·60		
Gly-LVP	15·2	2·80	Gly-NAp	112
Gly-Gly-LVP	10·8	3·20	Gly-Gly-NAp	3
Pro-LVP	7·1	4·68		
Gly-Gly-Gly-LVP	6·2	5·10	Gly-Gly-Gly-NAp	1·5
Sar-Gly-LVP	5·4	3·21		
Sar-LVP	0·0	(inhibitor)		
Lys-LVP			Lys-NAp	850

[a] AK-NAp *p*-nitroanilide of amino acid or peptide. [b] pNA *p*-nitroaniline.

Dependence of Hydrolysis of [8-*Lysine*]*vasopressin and Some Hormonogens on* pH *and Temperature. Thermal and* pH *Stability of* LVP *Hydrolysis in the Presence of Serum of Pregnant Women*

The dependence of enzymatic hydrolysis of LVP on pH represents a curve with a maximum of hydrolysis at pH 7·3–7·5 (Fig. 3). For comparison the similar dependence of hydrolysis of Cys(Me)-NAp and Cys(Et)-NAp, *i.e.* of substrates cleavable with oxytocinase, is also presented. The pH optimum of the hydrolysis of these substrates is approximately equal to that for LVP. The dependence of the rate of hydrolysis on pH was also determined for Ala-LVP, Leu-LVP, Gly-LVP, Gly-Gly-LVP and Gly-Gly-Gly-LVP. All hormonogens investigated have their pH-optimum of hydrolysis at 7·5 (Fig. 4).

The rate of hydrolysis of LVP with serum enzymes of pregnant women increases with temperature up to 56°C and above this temperature a considerable decrease in enzymatic activity takes place. Thermal stability of these enzymes is relatively high

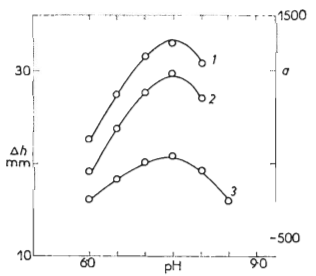

FIG. 3

Dependence of Hydrolysis of *1* Cys(Me)--NAp, *2* Cys(Et)-NAp and *3* LVP on pH

Δh decrease of the wave height during hydrolysis of LVP, *a* μM of *p*-nitroaniline set free within 1 h during hydrolysis of Cys(Me)-NAp and Cys(Et)-NAp.

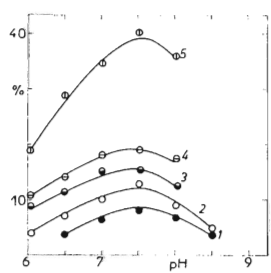

FIG. 4

Dependence of Hydrolysis of Some Hormonogens on pH

The reaction mixture contained 0·2 ml of serum, phosphate buffer of pH 7·4 and substrate at $4 \cdot 10^{-5}$ M concentration in total volume 0·2 ml. Incubated at 37°C for 4 h. *1* Gly-Gly-Gly-LVP. *2* Gly-Gly-LVP, *3* Gly-LVP, *4* Ala-LVP, *5* Leu-LVP. Ordinate: Degree of hydrolysis in %.

TABLE II

Free Amino Acids, Set Free by Enzymatic Hydrolysis of [8-Lysine]vasopressin and Its Analogues

Compound	Free amino acids[a]					
	Gly	Ala	Glu	Leu	Tyr	Phe
LVP					2·83	3·33
Gly-Pro-LVP	1·88				1·35	1·55
Leu-LVP				1·80	1·00	1·25
Ala-LVP		1·34			0·56	0·70
Phe-LVP					0·35	0·90
Gly-LVP	1·70				0·35	0·50
Gly-Gly-LVP	2·30				0·20	0·35
Gly-Gly-Gly-LVP	1·55				0·15	0·25
Pro-LVP					0·15	0·30
Sar-Gly-LVP					0·15	0·25
Sar-LVP					+	

[a] Amount of amino acids set free, expressed as the integral area under the peak.

TABLE III

Comparison of Polarographic Measurement of the Hydrolysis of Oxytocin, [8-Lysine]vasopressin and N^{α}-Leucyl-[8-lysine]vasopressin with the Serum of Non-Pregnant Women

Compound	Wave height, mm		Degree of hydrolysis[a]
	before incubation	after incubation	
	Pregnant women serum		
LVP	40	13	67·5
Oxytocin	12	8	33·3
Leu-LVP	28	20	28·6
	Non-pregnant women serum		
LVP	45	42	6·6
Oxytocin	13	13	0
Leu-LVP	26	22	10·4

[a] The degree of hydrolysis is expressed as % of hydrolysis/150 min at 37°C.

(Fig. 5). A 50% loss of enzymatic activity of the serum occurs after 90 minutes' incubation at 56°C. A full inactivation of the hydrolysing enzymes takes place after more than 180 min.

The range of pH stability of LVP-hydrolysing enzymes in the serum of pregnant women is relatively broad (pH 4 – 11).

Effect of Concentration of Serum Enzymes of Pregnant Women on the Rate of Hydrolysis of [8-Lysine]vasopressin and the Dependence on the Concentration of Substrate. Dependence of the Hydrolysis of LVP *on the Length of Pregnancy*

For the investigation of the concentration dependence we carried out the incubation of LVP with varying amounts of serum to which we added a serum inactivated at 56°C for 210 min, so that the resulting volume of the serum in the incubation mixture was always equal. Under these conditions the hydrolysis of LVP is a linear function of the amount of the enzyme (native serum). Finally we determined the dependence of the hydrolysis of LVP with serum enzymes on substrate concentra-

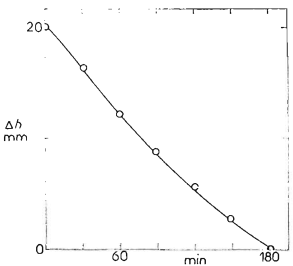

FIG. 5

Thermal Stability of Enzymes Hydrolysing [8-Lysine]vasopressin Present in the Serum of Pregnant Women

A mixture of 1·7 ml of a phosphate buffer of pH 7·4 and 0·2 ml of the basic solution of LVP was additioned with 0·1 ml of serum preincubated at the given intervals at 56°C. Hydrolysed at 37°C for 60 min.

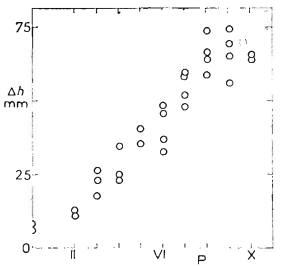

FIG. 6

Relationship between the Length of Pregnancy and the Hydrolysis of [8-Lysine]vasopressin

The reaction mixture contained 1·6 ml of phosphate buffer of pH 7·4, 0·2 ml of serum and $4{\cdot}2 \cdot 10^{-5}$M concentration of LVP. Incubated at 37°C for 120 min. The length of pregnancy is given in lunar months. Δh is the decrease of the wave height during the hydrolysis of LVP.

tion. The Michaelis constant K_m calculated from these data according to Lineweaver–Burk[8,9] is $1{\cdot}2 \cdot 10^{-5}$M.

In order to determine the dependence of hydrolysis of LVP on the length of pregnancy we carried out the hydrolysis of LVP with 28 various sera of pregnant women in various months of pregnancy and with the sera of non-pregnant women. As evident from Fig. 6, the hydrolysis of LVP increases with the length of pregnancy, even though the difference of the values obtained at the same time of pregnancy is in individual cases considerable. Table III indicates the fact that, in contrast to oxytocin, LVP and Leu-LVP are split to a small extent even with the sera of non-pregnant women.

DISCUSSION

The observed kinetic characteristics of the hydrolysis of LVP and the fact that the cleavage of LVP was carried out with the serum of highly pregnant women, as well as the increase of hydrolytic activity with the length of pregnancy, lead to the conclusion that the inactivation of LVP is catalysed in the serum mainly by oxytocinase[8,9]. From the polarographic point of view the well explicable gradual decrease of the polarographic wave during the hydrolysis of LVP also leads to such a conclusion. That is, in LVP the Cys^1-Tyr^2 bond is hydrolysed primarily, analogously as in the case of oxytocin[10–13,15], under formation of tyrosyl-phenylalanyl-glutaminyl--asparaginyl-S-(S-cysteine)-cysteinyl-propyl-lysyl-glycinamide (1,2-acyclic vasopressin). We assume that this octapeptide will still possess an almost equal polarographic activity as the intact LVP. Only after gradual degradation of N-terminal amino acid residues of tyrosine, phenylalanine, glutamine and asparagine gradually shorter peptidic fragments will be formed which give gradually lower waves in ammoniacal cobaltic salt solution, or even do not appear in the given medium on the polarogram. In view of the presence of the imido group of proline the remaining peptide, cystinyl--prolyl-lysyl-glycinamide no longer undergoes degradation[8,13]. The experiments with the sera of pregnant women have shown (Table III) that oxytocinase is not the only serum enzyme which decreases the polarographic waves of LVP and Leu-LVP in consequence of hydrolysis. However, the polarographic wave of oxytocin did not decrease under equal experimental conditions in the presence of the serum enzymes of non-pregnant women. The decrease of the wave of LVP and Leu-LVP during the incubation with the sera of non-pregnant women can be explained on the basis of the experiments carried out by Čunderlíková[14] who found on the basis of chromatographic separation methods that serum enzymes of non-pregnant women weakly attack LVP and Leu-LVP from both ends of the peptide chain. We consider that this minor decrease of the catalytic wave of LVP is also caused by the hydrolysis of the Cys^1-Tyr^2 bond, since it can hardly be assumed that the mere small shortening of the polypeptide chain with a slow splitting off of glycinamide from the C-terminal end would cause any pronounced effect on the height of the polarographic wave. Hence,

a further enzyme must be present in the blood serum which hydrolyses LVP, Leu-LVP and probably even some other vasopressin hormonogens, but does not hydrolyse oxytocin to an appreciable extent. Whether this enzymatic factor is identical with the unspecific aminopeptidase hydrolysing synthetic substrates of the type Cys(Bzl)-NAp or Cys(Et)-NAp must be decided on the basis of a specifically aimed experiment. The non-hydrolysability – or more accurately the extremely slow hydrolysability – of oxytocin with the sera of non-pregnant women is rather dependent on its difference in the sequence at position 3, than at position 8. From the experiments carried out and from their analysis conclusions of a methodical character can be drawn. From Fig. 6 it is evident that the polarographic following of the hydrolysis of LVP with the sera of pregnant women can be exploited practically for the determination of serum oxytocinase. However, in view of the requirement of specificity, oxytocin is a more suitable substrate for such analyses than LVP.

From Table I it is evident that a good agreement exists between the rate of hydrolysis and the persistency index of the hormonogens investigated. This indicates the possibility of substituting the more elaborate biological or chemical tests by the polarographic method described. The results of quantitative determination of amino acids set free on the cleavage of hormonogens (Table II) also correspond to the results obtained polarographically. These analyses show that in the enzymatic hydrolysis of hormonogens the added amino acid (peptide) is really first split from the molecule, followed by subsequent degradation of the hormone proper, according to the scheme: hormonogen → hormone → degradation product[15,16]. Of all the substances investigated the hormone alone is most easily split, that means, the limiting factor for the rate of activation and inactivation of the hormone is the rate of splitting off of the additional amino acid (peptide) from the hormonogen molecule. In agreement with this assumption the rate of cleavage of *p*-nitroanilides of amino acids with the serum of pregnant women decreases in the following order: Leu-NAp, Ala-NAp, Gly-NAp, Gly-Gly-NAp, Gly-Gly-Gly-NAp (Table I).

In view of the methylated free amino group Sar-LVP is not hydrolysed and has an inhibitory effect[4]. In comparison with the cleavage of Sar-LVP, Gly-LVP and Pro-LVP the hormonogens Gly-Pro-LVP and Sar-Gly-LVP have an unexpectedly high degree of hydrolysis. The explanation which seems obvious in this connection assumes the participation of such enzymes as split off Gly-Pro and Sar-Gly directly from the molecule of the hormonogen. Glycylprolyldipeptidyl aminopeptidase was indeed already found as an enzymatic component of the blood serum[17]. The splitting off of the dipeptide Sar-Gly or even the tripeptide Sar-Gly-Cys from the molecule can be catalysed by some enzymes of the endopeptidase type.

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