POLAROGRAPHIC PROPERTIES OF AMINO ACID *p*-NITROANILIDES AND THEIR USE FOR ENZYME ACTIVITY ASSAY

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Polarographic reduction of *p*-nitroanilides of L-leucine, L-lysine, γ-L-glutamic acid, ε-aminocaproic acid and N^{α}-tosyl-L-arginine is described. Determination of enzyme activity with the aid of these substrates using the polarographic method is demonstrated on the cleavage of N^{α}-tosyl-L-arginine-*p*-nitroanilide with crystalline trypsin and duodenal content. Suitable conditions for hydrolysis are determined.

Papers dealing with the polarographic estimation of the activity of peptidolytic enzymes are most often based on the determination of cleavage products of enzyme hydrolysis of proteins and peptides using the Brdička filtrate reaction[1-4]. In this manner, the activity of trypsin[4], serum proteases[5] and oxytocinase[6] have been determined. The enzyme activity of trypsin was determined also on the basis of changes of the polarographic maximum in the system cobalt–oxyquinoline[7]. Other authors used the oscillopolarographic method for the determination of chymotrypsin activity during cleavage of *p*-nitrophenylacetate[8].

The present paper describes a polarographic method which, using amino acid *p*-nitroanilides as substrates, makes it possible to estimate the enzyme activity also in optically dense media.

EXPERIMENTAL

The following synthetic substrates were used for the experiments: L-leucine *p*-nitroanilide (L-Leu-NAp), L-lysine *p*-nitroanilide dihydrobromide (L-Lys-NAp), γ-*p*-nitroanilide of L-glutamic acid (L-Glu-NAp), ε-aminocaproic acid *p*-nitroanilide hydrobromide (Cap-NAp)[9] and N^{α}-tosyl-L-arginine *p*-nitroanilide hydrochloride (L-TAPA). The synthesis and properties of L-TAPA as a chromogenic substrate for trypsin were described before[10]. Reagent-grade *p*-nitroaniline was also used (Lachema).

Since amino acid *p*-nitroanilides as well as *p*-nitroaniline itself are not readily soluble in water the stock solutions of these compounds (10^{-3}M) were prepared in 30% acetone. The Britton-Robinson universal buffer and 0·5M-Tris-HCl buffer with 0·02M-$CaCl_2$ were used. For investigating the enzymic hydrolysis of L-TAPA we employed twice recrystallized trypsin (2850 NFU/mg) and duodenal content without stimulation, diluted 1 : 9 with a buffer. The hydrolysis itself took place in 5·0 ml of a system containing (unless stated otherwise) 4·65 ml Tris buffer, 0·25 ml substrate solution and 0·1 ml enzyme solution. The incubation took place at 25°C and, after flushing

with nitrogen, polarographic curves were recorded after 10 min on a Hungarian Radelkis OH-120 polarograph. Capillary constants: h 92 cm, t 3·64 s, m 2·06 mg s^{-1}. All the measurements were carried out against a saturated calomel electrode. The amount of liberated *p*-nitroaniline was determined from a calibration curve obtained under the same conditions. Study of polarographic properties of amino acid *p*-nitroanilides was performed in 5·0 ml volumes containing 4·65 ml Britton–Robinson buffer, 0·25 ml substrate solution and 0·1 ml 0·5% gelatin solution. The polarographic curves were obtained after 10 min of flushing with nitrogen under the same conditions as with enzyme hydrolysis.

RESULTS

p-Nitroanilides are reduced at the dropping mercury electrode in two waves. The half-wave potentials of both waves are shifted with increasing pH toward more negative values. With *p*-nitroaniline (in contrast with the *p*-nitroanilides) only a single wave was noted in the entire range examined (pH 3 – 10). Similarly to *p*-nitroanilides, the half-wave potential of *p*-nitroaniline is shifted toward negative values with increasing pH (Fig. 1). When examining the influence of trypsin concentration on the rate of hydrolysis of L-TAPA, a linear relationship was found between the enzyme concentration and the amount of *p*-nitroaniline released. A similar relationship exists between the amount of duodenal content and the rate of L-TAPA hydrolysis. The dependence of the rate of tryptic hydrolysis of L-TAPA on pH is represented by a typical curve with a maximum between pH 8·0 and 8·4 which is in fine agreement with the published data[10] based on the spectrophotometric method. The kinetics of hydrolysis of L-TAPA by trypsin was also studied polarographically and a Michaelis constant[11] of $2 \cdot 10^{-3}$M was found.

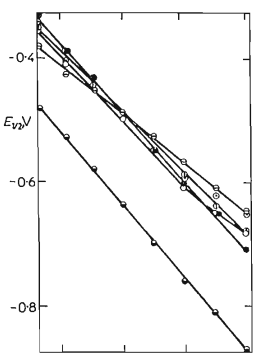

FIG. 1

Dependence of Half-Wave Potentials of *p*-Nitroaniline (0·5 mM) and of the First Wave of *p*-Nitroanilides of Amino Acids (0·5 mM) on pH

Britton–Robinson buffer with 1·5% acetone and 0·01% gelatin. ⊖ L-Lys-NAp, ⊕ L-Leu-NAp, ● L-Glu-NAp, ⊙ Cap-NAp, ○ L-TAPA, ◒ *p*-nitroaniline.

DISCUSSION

Substitution of the amino hydrogen in *p*-nitroaniline with the acyl residue of an amino acid results in a decrease of the electron density of the nitro group which is reflected in a shift of the half-wave potential toward more positive values as compared with *p*-nitroaniline. In agreement with the above assumption it may be predicted that *p*-nitroaniline will be reduced at the dropping mercury electrode at a more negative potential than amino acid *p*-nitroanilides (Fig. 1). With *p*-nitroanilides of basic amino acids (arginine, lysine) and of ε-aminocaproic acid, another shift of the half-wave potential toward positive values takes place due to protonation of the ω-amino groups. The effect of α-amino group is reflected best in the acid pH region by the induction effect (−I) of its positive charge, by a similar effect as with the ω-basic group while above pH 7 it plays no role (deprotonation). In the case of lysine, the protonation of the ε-amino group results in a weakening of the −I effect of the α-protonated amino group, *i.e.* in an increase of the electron concentration of the amide bond and hence in a shift of the half-wave potential to more negative values. This different behaviour is clearly apparent in comparison with the *p*-nitroanilide of ε-aminocaproic acid where no such shift of the half-wave potential is observed. With arginine (or L-TAPA), the α-amino group is acylated by tosyl group and hence shows practically no effect. On the contrary, with glutamic acid *p*-nitroanilide, even at pH 9, due to dissociation of the acidic α-carboxylic group, the half-wave potential is decreased toward rather negative values (the +I effect). Similarly, one can explain the shift of the second wave of γ-*p*-nitroanilide of glutamic acid beyond the exclusion potential of the buffer while the shift of the half-wave potential of the second wave of leucine *p*-nitroanilide (in the region above pH 7), can be attributed to a positive induction effect (+I) of the branched alkyl residue of the leucine side chain.

Of the double-wave polarographic reduction of *p*-nitroanilides, the first wave is more suitable for analytical purposes as it is better developed and about 2−3 times higher than the second wave. The differences betwen $E_{0.5}$ of the first wave of amino acid *p*-nitroanilides and that of *p*-nitroaniline lie between 100 and 200 mV. This difference, as is shown in the case of tryptic cleavage of L-TAPA, can be used for

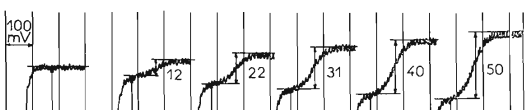

FIG. 2

Enzymic Hydrolysis of L-TAPA

Concentration of L-TAPA at the beginning of hydrolysis was 0·4mM in 0·5M Tris-HCl buffer at pH 8·1 with 0·02M-$CaCl_2$. Trypsin concentration 10 μg/ml. 25°C, flushed with nitrogen, start at −0·550 C. Hydrolysis (min): *1* 0, *2* 6, *3* 10, *4* 14, *5* 18, *6* 22.

a clear separation of the two waves. The height of the polarographic waves of *p*-nitroaniline and *p*-nitroanilides is proportional to the concentration of these compounds and does not depend on the solution pH between pH 3 and 9. This means that the degree of hydrolysis of amino acid *p*-nitroanilides may be followed simultaneously from the change of the height of substrate as well as of product, or from the total height of the two *p*-nitroanilide waves. Since the height of the *p*-nitroanilide wave is about 2–4 times greater than the height of the first wave of amino acid *p*-nitroanilides it is suitable during hydrolysis to follow only the changes of the polarographic wave of *p*-nitroaniline (Fig. 2). The increased buffer concentration results in a clearer distinction between the two waves. For this reason, a high concentration of Tris-HCl buffer was used in the present work. The polarographic method described here for assay of trypsin activity makes it possible to examine enzyme hydrolysis over a wide range of pH, it is sufficiently sensitive and does not require optimal transparency of the medium. In addition to the examination of L-TAPA hydrolysis by trypsin as described here, the method may be applied to a study of hydrolysis of other amino acid *p*-nitroanilides by different enzymes present in biological material.

REFERENCES

1. Březina M., Zuman P.: *Polarografie v lékařství, biochemii a farmacii*, p. 361. Published by Zdravotnické nakladatelství, Prague 1952.
2. Kalous V.: This Journal *21*, 1240 (1956).
3. Müller O. H.: Rev. Polarog. *11*, 79 (1963).
4. Štokrová S.: Chem. listy *48*, 1082 (1954).
5. Homolka J., Soušek O.: Čas. lékařů čes. *104*, 941 (1965).
6. Bartík M., Michnová E.: Veterinární medicína *11*, 645 (1966).
7. Ivanov I. D.: *Poljarografija Belkov, Enzimov i Aminokislet*, p. 180. Izd. Akad. Nauk SSSR, Moskva 1961.
8. Kozlov L. V., Smirnov P. N., Ginodman L. M., Antonov V. K.: Dokl. Akad. Nauk SSSR *180*, 1492 (1968).
9. Kasafírek E.: Unpublished results.
10. Kasafírek E., Chavko M., Bartík M.: This Journal *36*, 4070 (1971).
11. Lineweaver H., Burk D.: J. Am. Chem. Soc. *56*, 658 (1934).

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