EXPERIMENTAL

Alkyl Benzimidazolylcarbamates. To 0.018 mole of 2-cyanamidobenzimidazole and 0.1 mole of the appropriate alcohol was added 0.036 mole of concentrated hydrochloric acid. The reaction was carried out at the boiling point of the reaction mixture for 3 h. During the boiling of the mixture, crystals of the alkyl benzimidazolylcarbamate gradually separated out. The yields, melting points, and other characteristics of the products are given in Table 1. The analyses of the compounds obtained for nitrogen agreed with the calculated figures.

SUMMARY

A method has been developed for obtaining alkyl esters of benzimidazolylcarbamic acid using alcohols of various structures. An advantage of the method is that it has been possible to avoid the employment of toxic lachrymatory reagents and to use readily available substances.

LITERATURE CITED

1. US Patent No. 2,933,502 (1961); Chem. Abstr., 55, 3617 (1961).
2. US Patent No. 2,933,504 (1961); Chem. Abstr., 55, 9431 (1961).
3. N. N. Mel'nikov, The Chemistry and Technology of Pesticides [in Russian], Moscow (1974), p. 635.
4. J. A. Murrow and F. B. Dains, J. Am. Chem. Soc., 56, 114 (1934).
5. Chem. Zentralbl., C, III, 527 (1921).
6. E. N. Zil'berman and A. E. Kulikova, Zh. Obshch. Khim., 29, 3039 (1959).
7. E. N. Zil'berman and A. M. Sladkov, Zh. Obshch. Khim., 31, 245 (1961).
8. Ch. Sh. Kadyrov, S. A. Khasanov, M. Akbarova, and S. Yu. Yunusov, USSR Inventor's Certificate No. 520,361; Byul. Izobret., No. 25 (1977).

SOME KINETIC FEATURES OF THE HYDROLYSIS OF L-ASPARAGINE WITH *E. coli* ASPARAGINASE

I. A. Milman, I. I. Geiman,
V. A. Slavinskaya, I. A. Vina,
and R. A. Zhagat

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The influence of various effectors (methanol, neutral salts) on the kinetic parameters K_M and k_{cat} has been studied. The hypothesis has been expressed that chloride ions are responsible for the worsening of the binding of the substrate to the enzyme. The temperature dependence of the kinetic parameters K_M and k_{cat} for the enzymatic hydrolysis of L-asparagine has been obtained. It has been shown that the graph of log k_{cat} versus 1/T has a break at 30°C. The effective activation energies below and above the critical point are 6.5 and 3.6 kcal/mole, respectively.

The kinetics of the enzymatic hydrolysis of L-asparagine have been little studied. In an investigation of the influence of hydroxylamine on the hydrolysis of L-asparagine, Ehrman et al. [1] put forward a hypothesis of a three-stage mechanism of the reaction. O'Leary [2] also assumed that L-asparaginase operates by a mechanism similar to that for chymotrypsin and other serine proteases:

$$E + S \rightleftarrows ES \rightarrow EA \rightarrow E + P.$$

However, the possibility of the formation of an intermediate acylenzyme (EA) has not hitherto been definitively demonstrated.

We have determined the kinetic parameters of the hydrolysis of L-asparagine in the presence of various effectors capable of selectively acting on one of the possible stages of the

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 707-709, September-October, 1979. Original article submitted April 12, 1979.

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TABLE 1. Influence of the Concentration of Neutral Salts on the Kinetic Parameters of the Hydrolysis of L-Asparagine

Salt	Concentration of the salt, M	Ionic strength	K_M, mM	V_{max}, mM/min
—	—	0,20	0,008	0,15
Na_2SO_4	0,04—0,15	0,33—0,64	0,008	0,18
NaCl	0,18—0,74	0,38—0,94	0,034	0,17
KCl	0,17-0,38	0,37-0,58	0,034	0,18

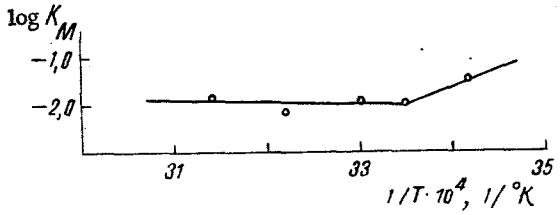

Fig. 1. Temperature dependence of the Michaelis constant of the hydrolysis of L-asparagine.

reaction mechanism put forward above. We have studied the influence of a nucleophilic agent — methanol — which is capable of competing with water at the acylenzyme deacylation stage. The addition of methanol to the reaction mixture over a wide range of concentrations (0.04-0.92 M) did not affect the kinetic parameters K_M and k_{cat}.

We have also considered the influence of the neutral salts NaCl, KCl, and Na_2SO_4 on the hydrolysis of L-asparagine. The addition of neutral salts to the reaction mixture and, consequently, the change in ionic strength caused by them had no effect on the maximum rate of the reaction (Table 1). At the same time, the Michaelis constant increased fivefold on the addition of NaCl and KCl, but did not change on the addition of sodium sulfate. The change in the value of K_M due to the addition of chlorides of sodium and potassium enables us to exclude the influence of the ionic strength and to assume that Cl^- ions are responsible for the weakening of tne binding of the substrate with the enzyme. In other words, apparently, an interaction of the Cl^- ion with the enzyme or the enzyme-substrate complex takes place.

Thus, in spite of the variation in the nature and amount of the effectors, it proved to be impossible to determine the individual rate constants of the separate stages of the reaction.

We have studied the influence of the temperature on the kinetic parameters K_M and k_{cat} for the hydrolysis of L-asparagine. As follows from Fig. 1, the Michaelis constant scarcely changed in the range of temperatures from 25 to 45°C. At temperatures below 25°C a weakening of the binding of the substrate was observed. The graph of the temperature dependence of the maximum rate (k_{cat}) of the reaction has a break at 30°C (Fig. 2). The effective activation energies below and above the critical point are 6.5 and 3.6 kcal/mole, respectively. According to the literature, E_{eff} for the hydrolysis of L-asparagine by native asparaginase is 12.6 [3], 12.9 [4], and 11.2 [5] kcal/mole. The observed difference is connected with the fact that the values given above for the effective activation energy reflect the temperature dependences of different kinetic parameters: k_{cat} in our case and V_0 according to the literature. The treatment of the experimental results obtained in the form of plots of log V_0 versus 1/T at temperatures of 19-30°C gave a value of the effective activation energy of 13.1 kcal/mole, agreeing with the literature figures.

Among the factors responsible for the formation of a break may be the existence of two successive reactions with different temperature coefficients as, for example, if the reaction took place by a three-stage scheme, or there were a change in the ionization constants of ionogenic groups. In favor of the latter possibility is the work of Homer [6]. The temperature dependence of pK_a in his paper is anomalous (it has a minimum at 25°C) and he explains it as a change in the conformation around a histidine residue.

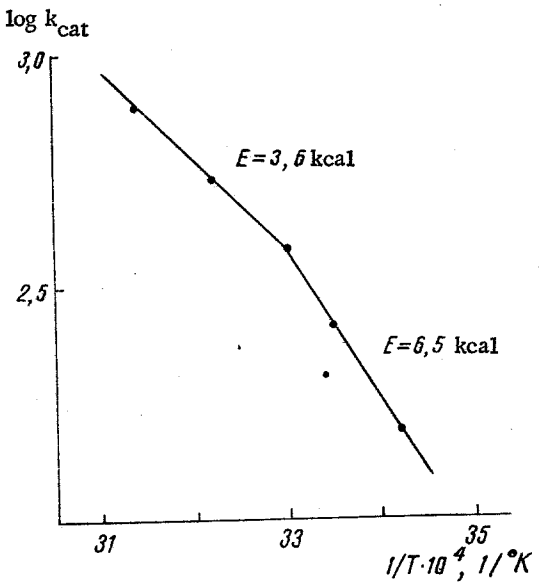

Fig. 2. Graph of the dependence of log k_{cat} on 1/T.

Since the values obtained for the activation energy are effective values having no definite physical sense, a more detailed thermodynamic analysis seems possible only after the determination of the individual kinetic constants.

EXPERIMENTAL

The work was carried out with *E. coli* L-asparaginase from the Riga medical preparations factory. The composition of the incubation mixture used in the experiments and the method of treating the experimental results obtained in the determination of K_M and k_{cat} have been described previously [7]. All the solutions were prepared in double-distilled water. Reagents of kh.ch. ["chemically pure"] and ch.d.a. ["pure for analysis"] grades were used.

The rate of the hydrolysis of asparagine under the conditions considered depends linearly on the concentration of enzyme in the range of $(0.2\text{-}1.4)\cdot10^{-6}$ M.

SUMMARY

The addition of methanol (0.04-0.92 M) to the reaction mixtures does not affect K_M and k_{cat}.

The influence of the temperature on the kinetic parameters, K_M and k_{cat}, of the hydrolysis of asparagine has been studied for the first time. Effective values of the activation energy have been determined.

On the basis of a study of the results of the influence of additions of NaCl and KCl it is suggested that Cl^- ions are responsible for a weakening of the binding of the substrate with the enzyme.

LITERATURE CITED

1. M. Ehrman, H. Cedal, and J. H. Schwartz, J. Biol. Chem., 246, 88 (1971).
2. M. H. O'Leary and S. L. Mattes, Biochim. Biophys. Acta, 522, 238 (1978).
3. L. S. Hersh, J. Polym. Sci., Polym. Symp., No. 47, 55 (1974).
4. N. Citri and N. Zyk, Biochem., 11, 2103 (1972).
5. A. J. Charlson, A. J. Coman, T. A. Karossi, F. S. Stephens, R. S. Vagg, and E. C. Watton, Inorg. Chem. Acta, 28, 217 (1978).
6. R. B. Homer and S. R. Allsopp, Biochim. Biophys. Acta, 434, 100 (1976).
7. I. A. Milman, I. I. Geiman, U. A. Pinka, I. P. Kirstukas, V. A. Slavinskaya, and R. A. Zhagat, Khim. Prir. Soedin., 364 (1978).