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# Purification and Properties of Acylase from Ehrlich Ascites Carcinoma Cells

Yukio Kameda,[1a] Toshikatsu Nakabayashi,[1b] Shōichi Kanatomo,[1a] and Seimei Kitade[1b]

*School of Pharmacy, Hokuriku University[1a] and Faculty of Pharmaceutical Sciences, Kanazawa University[1b]*

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The acylase of Ehrlich ascites carcinoma cells was purified by fractionation with ammonium sulfate, diethylaminoethyl cellulose chromatography, Sephadex G-200 gel filtration, and hydroxylapatite chromatography. The acylase activity of the purified enzyme toward N-dichloroacetyl-L-aspartic acid was 1561 U/mg and it was represented about 410 fold purification over the cell free extract. The enzyme has a pH optimum around 8.5 toward N-dichloroacetyl-L-aspartic acid. It is inhibited by $Sn^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Zn^{2+}$, *p*-chloromercuribenzoate, $ICH_2COOH$, $H_2O_2$, and diisopropyl fluorophosphate. As a result of the investigation of substrate specificity, it was found that the enzyme hydrolyzed only N-dichloroacetyl-L-aspartic acid among eleven kinds of N-dichloroacetyl amino acids. But it could not hydrolyze D from of N-dichloroacetyl-aspartic acid. In order to test the effect of acyl groups toward the enzyme activity, eleven acyl aspartic acids were tested. N-Chloroacetyl, N-dichloroacetyl, N-trifluoroacetyl derivatives of L-aspartic acid were hydrolyzed.

**Keywords**——Ehrlich ascites carcinoma acylase; acylase purification; N-dichloroacetyl-L-aspartic acid; acylase; N-acyl-L-amino acid amidohydrolase; substrate specificity

In a previous paper,[2] the authors reported the acylase activity of lyophilized powder of ascites tumor cells such as Ehrlich ascites carcinoma, sarcoma 180, leukemia SN36, ascites hepatoma MH134 and MH129P in C3H mice, and AH7974, AH130, and Yoshida sarcoma in rats on N-dichloroacetyl derivatives of amino acids.

The purpose of this report is to describe in detail the purification and properties especially the substrate specificity of Ehrlich ascites carcinoma acylase.

## Experimental

**Materials**——The following compounds were prepared as described in the literatures: N-dichloroacetyl-glycine, N-dichloroacetyl-L-leucine,[3] N-dichloroacetyl-L-alanine,[4] N-dichloroacetyl-D- and L-aspartic acid, N-dichloroacetyl-L-glutamic acid,[5] N-dichloroacetyl-DL-serine, N-dichloroacetyl-DL-methionine,[6] N-dichloroacetyl-DL-phenylalanine,[7] α-dichloroacetyl-ε-benzoyl-DL-lysine,[8] N-chloroacetyl-L-aspartic acid,[9] N-benzoyl-L-aspartic acid,[10] N-phenylacetyl-L-aspartic acid,[11] trifluoroacetyl-DL-aspartic acid,[12] butyryl-L-aspartic

1) Location: *a) 3 Ho, Kanagawa-machi, Kanazawa 920-11, Japan; b) 13-1, Takara-machi, Kanazawa 920, Japan.*
2) Y. Kameda, K. Matsui, Y. Kimura, and E. Toyoura, *Nature* (London), **192**, 468 (1961).
3) E. Ronwin, *J. Org. Chem.*, **18**, 1546 (1953).
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10) Y. Kameda, E. Toyoura, Y. Kimura, and K. Yoshimura, *Yakugaku Zasshi*, **78**, 765 (1958).
11) T. Suyama, T. Toyoda, and S. Kanao, *Yakugaku Zasshi*, **85**, 279 (1965).
12) W.S. Fones and M. Lee, *J. Biol. Chem.*, **210**, 227 (1954).

acid, octanoyl-L-aspartic acid, and lauroyl-L-aspartic acid.[13] Dichloroacetyl derivatives of L-valine and DL-tryptophan were prepared by the application of the method of Kameda, *et al.*[7] Dichloroacetyl-L-valine, mp 169—170°, $[\alpha]_D^{21}$ +8.9° ($c$=1, EtOH), *Anal.* Calcd. for $C_7H_{11}Cl_2NO_3$: C, 36.86; H, 4.86; N, 6.14. Found: C, 36.76; H, 4.81; N, 6.05. Dichloroacetyl-DL-tryptophan, mp 136—138°, *Anal.* Calcd. for $C_{13}H_{12}Cl_2N_2O_3$: C, 49.54; H, 3.84; N, 8.89. Found: C, 49.46; H, 3.74; N, 8.80. N-Formyl-L-aspartic acid, N-acetyl-L-aspartic acid, N-glycyl-L-aspartic acid were purchased from Sigma Chemical Co. Diethylaminoethyl (DEAE) cellulose was a product of Brown Company and Sephadex G-200 was a product of Pharmacia Fine Chemicals. Hydroxylapatite was prepared by the method of Tiselius, *et al.*[14]

**Assay of the Enzyme Activity**——(1) Standard Enzyme Reaction: The reaction mixture, containing 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.2), 0.25 ml of 0.1 M N-dichloroacetyl-L-aspartic acid and 0.25 ml of appropriately diluted enzyme solution was incubated at 37°. The liberated amino acid was measured by Moore and Stein's colorimetric ninhydrin method.[15] One unit (U) of acylase is defined as that amount of enzyme which caused the liberation of one micromole of amino acid per hr under the above condition.

(2) Estimation of Hydrolytic Activity toward α-N-Acyl Amino Acid: The method of estimation of hydrolytic activity toward α-N-acyl amino acids was entirely the same with the above described standard enzyme reaction.

**Protein Determination**——Protein concentration was determined by the method of Warburg, *et al.*[16] with absorbance at 260 and 280 nm.

**Preparation of Carcinoma Cell Suspension**——Ehrlich ascites carcinoma cells was maintained by weekly intraperitoneal transplantation in ddY mice. The ascites fluid, aspirated from mice, bearing 8 days old Ehrlich ascites carcinoma was suspended in chilled Dulbecco buffer A (DBA)[17] and centrifuged at 160 ***g*** for 5 min. The sedimented carcinoma cells were washed twice with chilled DBA and suspended in chilled 0.01 M Tris-HCl buffer (pH 7.2) containing 0.01% β-mercaptoethanol.

**Extraction of Acylase from Ehrlich Ascites Carcinoma Cells**——The cell suspension described above was subjected to sonic oscillation at 200 W for 10 min. The resultant sonicate was centrifuged at 20000 ***g*** for 20 min to remove cell fragments and unbroken cells. The supernatant was used as cell free extract.

**Purification of Acylase**——All purification steps reported in the following paragraphs were carried out at 4°.

(1) Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to the cell free extract with stirring, the solution was kept at pH 7.2 by occasional addition of 28% ammonium hydroxide. The resulting precipitate at 0.3—0.6 saturation was collected by centrifugation and dissolved in 0.01 M Tris-HCl buffer (pH 7.2) containing 0.01% β-mercaptoethanol and the solution was dialyzed against the same buffer overnight.

(2) Chromatography on DEAE Cellulose: The dialyzed enzyme solution (395 ml, 1748 mg protein) was applied to a column of DEAE cellulose (3.6×50 cm) and washed with the same buffer and then linear gradient elution was started with the same buffer containing 0.5 M NaCl. The active fractions were collected and concentrated by ultrafiltration.

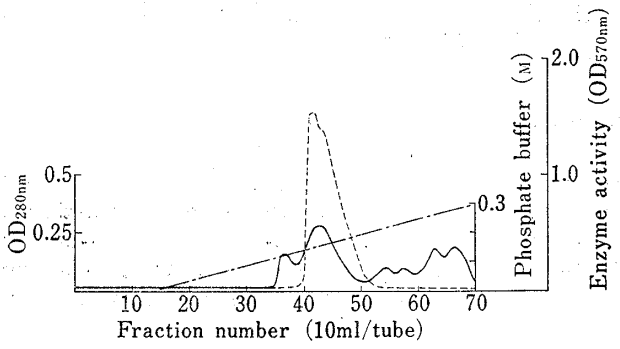


Fig. 1. Chromatography on Hydroxylapatite

Enzyme solution (92 ml, 25 mg protein) was loaded on Hydroxylapatite column (2.4×23 cm). After washing with buffer, linear gradient elution was carried out. Ten ml fractions were collected. Enzyme activity is indicated by (----) and protein concentration measured at $OD_{280\ nm}$ is indicated by (——) and phosphate buffer concentration is indicated by (-·-·-).

(3) Chromatography on Sephadex G-200: The concentrated enzyme solution (10 ml, 283 mg protein) was applied to gel filtration on Sephadex G-200 (2.6×90 cm). The active fractions were collected and concentrated. This preparation (10 ml, 58 mg protein) was purified by regelfiltration on Sephadex G-200. The active fractions were collected and dialyzed against 0.001 M phosphate buffer (pH 6.8) containing 0.01% β-mercaptoethanol.

(4) Chromatography on Hydroxylapatite: The dialyzed enzyme solution was applied to a column of hydroxylapatite. After washing with the same buffer as dialyzing solution, the column was eluted with a linear gradient of phosphate buffer increasing from

13) S. Nagai and J. Matsumoto, *J. Biochem.* (Tokyo), **56**, 465 (1964).
14) A. Tiselius, S. Hjerten, and Ö. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).
15) S. Moore and W.H. Stein, *J. Biol. Chem.*, **176**, 376 (1948).
16) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).
17) R. Dulbecco and M. Vogt, *J. Exp. Med.*, **99**, 167 (1954).

zero to 0.3 M (Fig. 1). The active fractions were dialyzed against 0.01 M Tris-HCl buffer (pH 7.2) containing 0.01% β-mercaptoethanol.

**Disc Electrophoresis**——Polyacryl amide disc gel electrophoresis was carried out according to the method of Nagai[18] using pH 8.3 gel.

## Results and Discussion

### Relation between the Days after Inoculation of Ehrlich Ascites Carcinoma Cells and Acylase Production

Female dd-Y mouse was transplanted intraperitoneally with approximately $1\times10^7$ Ehrlich ascites carcinoma cells. After 7—10 days, Ehrlich ascites carcinoma cells from ascites fluid of three animals each day were collected and subjected to sonic oscillation. The sonicate was centrifuged to obtain clear supernatant solution, and the acylase activity was measured. As shown in Fig. 2, the production of acylase was maximum on 8 days after inoculation.

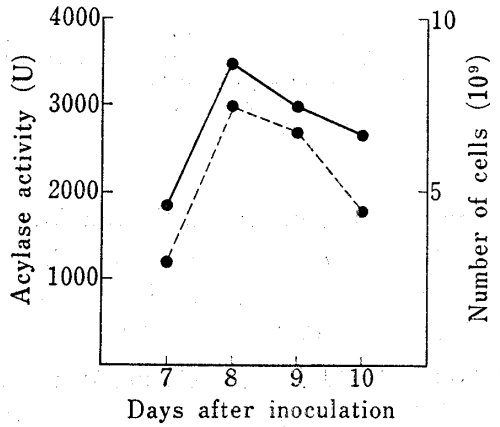


Fig. 2. Relation between the Days after Inoculation of Ehrlich Ascites Carcinoma Cells and Acylase Production

Ehrlich ascites carcinoma cells were collected as described in the text. Number of cells is indicated by (---●---) and enzyme activity is indicated by (—●—).

### Purification of Acylase of Ehrlich Ascites Carcinoma Cells

The results of the purification procedure are summarized in Table I. The specific activity was 1561 U/mg and represented about 410 fold purification over the original cell free extract. The purity of the enzyme preparation was tested by disc electrophoresis. In addition to a main band, some minor protein bands were observed. But the preparation was used as the purified enzyme for further experiments.

### Effect of pH on Activity and Stability

The effect of pH on the enzyme activity was determined over a pH range of 6.0 to 10.6. As shown in Fig. 3, optimal pH is found at the neighborhood of 8.5 and the activity is considerably low below pH 8.0 or above pH 9.2. Then, the effect of pH on the stability in the presence or absence of β-mercaptoethanol was examined. Mixture of the enzyme solution and various pH of buffers were allowed to stand at 4° for 15 days. As shown in Fig. 4, the enzyme was unstable, but in the presence of 0.01% β-mercaptoethanol it was stable at pH 7.0 to 8.0.

Table I. Purification of Ehrlich Ascites Carcinoma Acylase

| Step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Recovery (%) | Purification |
|---|---|---|---|---|---|---|
| Cell free extract | 380 | 8746.9 | 33440 | 3.8 | 100 | 1 |
| $(NH_4)_2SO_4$ fractionation 0.3—0.6 saturation | 395 | 1748.2 | 28440 | 16.3 | 85 | 4.3 |
| DEAE-cellulose | 158 | 282.7 | 19339 | 68.4 | 58 | 18.0 |
| 1st Sephadex G-200 | 100 | 58.4 | 19200 | 328.8 | 57 | 86.5 |
| 2nd Sephadex G-200 | 92 | 24.9 | 15336 | 615.8 | 46 | 162.0 |
| Hydroxylapatite | 61 | 7.5 | 11712 | 1561.6 | 35 | 411.0 |

18) Y. Nagai, *Tanpakushitsu Kakusan Koso*, **11**, 744, 818 (1966).

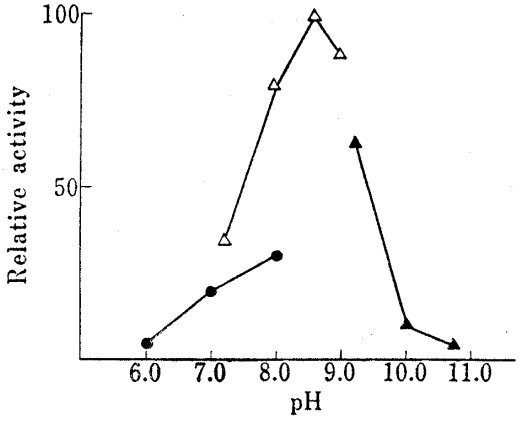


Fig. 3. Effect of pH on Activity of the Enzyme

The enzyme activity was measured under the standard assay condition using the following buffer: phosphate buffer (—●—) (pH 6.0 to 8.0), Tris–HCl buffer (—△—) (pH 7.2 to 9.0), and carbonate buffer (—▲—) (pH 9.2 to 10.7).

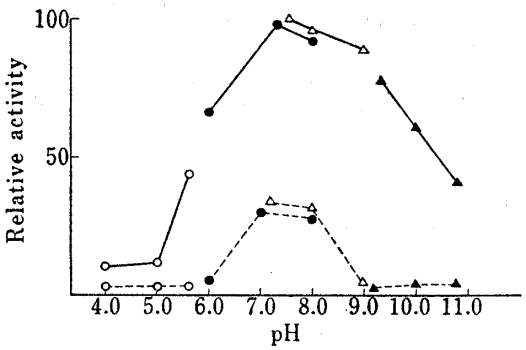


Fig. 4. Effect of pH on the Stability of the Enzyme Activity

Enzyme solution was adjusted to fixed pH values (pH 4.0 to 10.7) with respective buffer solution. After standing at 4° for 15 days, the residual enzyme activity was measured under the standard assay condition. Buffer used were acetate buffer (○) (pH 4.0 to 5.6), phosphate buffer (●) (pH 6.0 to 8.0), Tris–HCl buffer (△) (pH 7.2 to 9.0) and carbonate buffer (▲) (pH 9.2 to 10.7).
——: with β-mercaptoethanol,
– – –: without β-mercaptoethanol.

## Effect of Temperature on the Stability

To study the thermal stability, the enzyme solution was incubated at the indicated temperature for 10 min, the remaining activity was measured. As shown in Fig. 5, the activity was completely lost at 70°.

## Effect of Metal Ions, Chelating Reagents, and Other Compounds

It has been well known that various amino acylases are specifically activated by $Co^{2+}$ or $Zn^{2+}$.[19–22] In order to study the influence of metal ions on the acylase activity, various metal

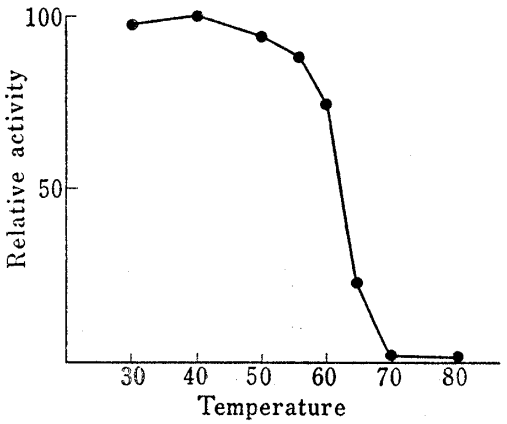


Fig. 5. Heat Stability of the Enzyme

The enzyme solution was incubated at indicated temperature for 10 min and the remaining activity was measured under the standard assay condition.

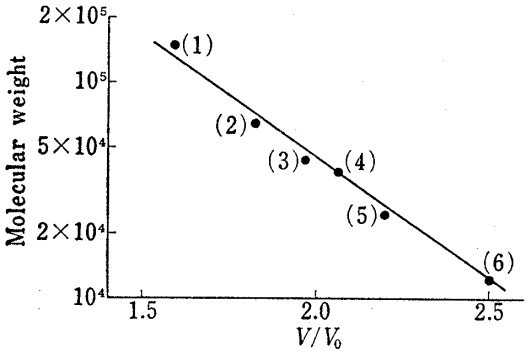


Fig. 6. Determination of Molecular Weight by Gel Filtration on Sephadex G 200

The enzyme was applied to a calibrated Sephadex G-200 column (2.6×90 cm, void volume 150 ml) as described in the text. The standard proteins used for calibration were following: (1) aldolase (rabbit, 158000), (2) albumin (bovine, 67000), (3) ovalbumin (hen egg, 45000), (4) Ehrlich ascites carcinoma acylase, (5) chymotrypsinogen (beef pancreas, 25000), (6) cytochrome c (horse heart, 12500). Fractions of 5 ml were collected and the standard proteins were estimated spectrometrically at $OD_{280\ nm}$.

19) R. Marshall, S.M. Brinbaum, and J.P. Greenstein, *J. Am. Chem. Soc.*, **78**, 4636 (1956).
20) R. Shirasaka, *Takamine Kenkyusho Nempo*, **10**, 74 (1958).
21) B.L. Vallee and H. Neurath, *J. Am. Chem. Soc.*, **76**, 5006 (1954).
22) S. Wada, *J. Biochem.*, **46**, 445, 1541 (1959).

Table II. Effect of Metal Ions, Chelating Reagents and Other Compounds

| Compounds | Relative activity[a)] | Compounds | Relative activity[a)] |
|---|---|---|---|
| $MgCl_2$ | 95 | $ZnCl_2$ | 10 |
| $SnCl_2$ | 22 | EDTA | 92 |
| $MnCl_2$ | 46 | *o*-Phenanthroline | 80 |
| $CaCl_2$ | 88 | PCMB | 27 |
| $BaCl_2$ | 95 | $ICH_2COOH$ | 33 |
| $NiCl_2$ | 92 | DFP | 0 |
| $CoCl_2$ | 99 | *β*-Mercaptoethanol | 99 |
| $FeCl_2$ | 96 | L-Cys | 98 |
| $CuCl_2$ | 55 | $H_2O_2$ | 26 |
| $HgCl_2$ | 5 | Ascorbic acid | 96 |

*a*) The activity under no addition is taken as control (100). Final concentration in reaction mixture is $1\times10^{-3}$ M.

ions were added to the enzyme assay. As shown in Table II, $Sn^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Hg^{2+}$, and $Zn^{2+}$ have considerably inhibitory effect. Then the effect of chelating reagents and other compounds on the activity was investigated. EDTA and *o*-phenanthroline have no inhibitory effect at $1\times10^{-3}$ M. The sulfhydryl reagents such as *p*-chloromercuribenzoate and $ICH_2COOH$ have inhibitory effect and diisopropyl fluorophosphate has completely inhibited at $1\times10^{-3}$ M. Hydrogen peroxide also has inhibitory effect. These facts suggest that cysteine and serine residues in the enzyme are involved in the active site.

**Estimation of Molecular Weight**

The determination of the molecular weight was performed by gel filtration through Sephadex G-200 column. Figure 6 shows the plots of the elution volume *versus* the logarithms of the known molecular weights of standard proteins. The approximate molecular weight of the enzyme was about 40000.

**Substrate Specificity**

α-Amino acylases are widely distributed in animals,[9)] plants,[23)] and microorganisms[24)] and they have different substrate specificities. For example, hog kidney amino acylase I

Table III. Substrate Specificity (Effect of Amino Acids)

| Substrate | Relative activity[a)] |
|---|---|
| Dichloroacetyl-glycine | 0 |
| Dichloroacetyl-L-alanine | 0 |
| Dichloroacetyl-L-valine | 0 |
| Dichloroacetyl-L-Leucine | 0 |
| Dichloroacetyl-DL-serine | 0 |
| Dichloroacetyl-L-aspartic acid | 100 |
| Dichloroacetyl-D-aspartic acid | 0 |
| Dichloroacetyl-L-glutamic acid | 0 |
| Dichloroacetyl-DL-methionine | 0 |
| Dichloroacetyl-DL-phenylalanine | 0 |
| Dichloroacetyl-DL-tryptophan | 0 |
| α-Dichloroacetyl-ε-benzoyl-DL-lysine | 0 |

*a*) The activity toward dichloroacetyl-L-aspartic acid is taken 100. Activities are measured by the procedure described in Experimental.

23) I. Chibata and T. Tosa, *Bull. Agr. Chem. Soc. Jpn.*, **23**, 370 (1959).
24) Y. Kameda, E. Toyoura, Y. Kimura, K. Matsui, and R. Hotta, *Yakugaku Zasshi*, **78**, 754 (1958).

acts more readily on N-acylated aliphatic amino acids, whereas pancreatic carboxypeptidase acts more readily on N-acylated amino acids containing aromatic substituents on the $\beta$-carbon atom. The authors have reported that the acylase from *KT* 801 (*Pseudomonas* sp.) hydrolyzes $\alpha$-N-benzoyl-L-amino acids but not phenylacetyl-amino acids[25] and the acylase from *Escherichia coli K*-12 recombinant has a specificity toward phenylacetyl-L-amino acids.[26] Recently, two acylases, short acyl aminoacylase and long acyl aminoacylase, were isolated from *Mycobacterium smegmatis*.[27] The former exhibited hydrolytic activity toward N-short chain fatty acyl amino acids, and the latter hydrolyzed N-long chain fatty acyl amino acids. In order to clarify the substrate specificity of the acylase of Ehrlich ascites carcinoma cells, the susceptibility of the various acyl derivatives of amino acids toward the enzyme was investigated.

1) **Optical Specificity**——As shown in Table III, the acylase could hydrolyze dichloroacetyl-L-aspartic acid but could not hydrolyze dichloroacetyl-D-aspartic acid. The acylase has an optical specificity.

2) **Effect of Amino Acids**——Among the dichloroacetyl derivatives of amino acids, indicated in Table III, only dichloroacetyl-L-aspartic acid was hydrolyzed. Dichloroacetyl-D-aspartic acid, which is enantiomer, and dichloroacetyl-L-glutamic acid, which is acidic amino acid as aspartic acid, were not hydrolyzed.

3) **Effect of Substrate Concentration**——The effect of substrate concentration on the enzyme activity was investigated, and the results obtained have been plotted by the method of Lineweaver-Burk[28] for the estimation of *K*m. The *K*m value was calculated to be $5\times10^{-3}$ M, $V_{max}$=1500 U/mg for dichloroacetyl-L-aspartic acid. On the other hand, in the presence of $1\times10^{-2}$ M of dichloroacetyl-D-aspartic acid $K_i$ was calculated to be $8\times10^{-3}$ M, $V_{max}$=1500 U/mg. These facts suggest that dichloroacetyl-D-aspartic acid acts as competitive inhibitor toward dichloroacetyl-L-aspartic acid.

Table IV. Substrate Specificity (Effect of Acyl Groups)

| Substrate | Relative activity[a] |
|---|---|
| Formyl-L-aspartic acid | 0 |
| Acetyl-L-aspartic acid | 0 |
| Chloroacetyl-L-aspartic acid | 15 |
| Dichloroacetyl-L-aspartic aicd | 100 |
| Trifluoroacetyl-DL-aspartic acid | 140 |
| Butyroyl-L-aspartic acid | 0 |
| Octanoyl-L-aspartic acid | 0 |
| Lauroyl-L-aspartic acid | 0 |
| Benzoyl-L-aspartic acid | 0 |
| Phenylacetyl-L-aspartic acid | 0 |
| Glycyl-L-aspartic acid | 0 |

a) The activity toward dichloroacetyl-L-aspartic acid is taken 100. Activities are measured by the procedure described in Experimental.

4) **Effect of Acyl Groups**——In order to test the effect of acyl groups toward the enzyme activity, a number of N-acyl-aspartic acid, indicated in Table IV, were prepared and the susceptibilities to the enzyme were investigated. As a result, three acyl-aspartic acids were

25) Y. Kameda, T. Kuramoto, K. Matsui, and T. Ebara, *Chem. Pharm. Bull.* (Tokyo), **16**, 1023 (1968).
26) Y. Kameda and T. Iwahi, presented at the 24th Meeting of the Pharmaceutical Society of Japan, Kyoto, April 8, 1967.
27) J. Matsumoto and S. Nagai, *J. Biochem.*, **72**, 269 (1972).
28) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

hydrolyzed in the following order: trifluoroacetyl>dichloroacetyl>chloroacetyl. But acetyl-, lauroyl-, benzoyl-, and phenylacetyl derivatives of L-aspartic acid were not hydrolyzed.

As described above, the Ehrlich carcinoma acylase hydrolyzed N-dichloro- and N-trifluoroacetyl-L-aspartic acid specifically. Based on this result, we wish to find inhibitors of the acylase and test the antitumor effect of the acylase inhibitors against Ehrlich carcinoma.

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