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Effect of Carbobenzoxy, Phenylacetyl and Phenylpropionyl Derivatives of Amino Acids on Mammalian and Bacterial Asparaginase

L-Asparaginase from various, although not all, sources was found to be effective against a variety of murine tumors, certain neoplasms of the cat and canine lymphosarcoma. In a greater number of human patients with acute lymphatic leukemia, treatment with asparaginase leads to a complete, although temporary, remission of symptoms[1,2].

It was found recently[3–5] that a number of carbobenzoxy derivatives of amino acids, especially derivatives with more than one aromatic group, markedly inhibit, in vivo, at non-toxic levels, several transplantable mouse tumors. Considerable inhibitory effects were also exerted by phenylacetyl and 3-phenylpropionyl amino acids[6]. It appeared therefore of interest to investigate whether a combined application of such derivatives and asparaginase would augment the antilymphoma activity of this enzyme. Obviously, any attempt at such a combined treatment would be justified only if the animo acid derivatives do not inhibit the asparaginase used. Previous experiments revealed that various carbobenzoxy derivatives of amino acids inhibit a number of isolated enzyme systems including rat liver asparaginase[7]. However, the latter finding does not imply that such derivatives also inhibit asparaginases from other sources.

The present paper deals with the effect of carbobenzoxy, phenylacetyl and phenylpropionyl derivatives of amino acids on rat liver asparaginase as well as on asparaginase from *E. coli* which is employed in most clinical studies. The effect of such compounds on a similar bacterial enzyme, asparaginase from *Erwinia carotovora*, is also dealt with.

Materials and methods. The preparation of phenylacetyl and 3-phenylpropionyl derivatives of amino acids is described elsewhere[8]. Carbobenzoxy L-glutamic acid γ-benzyl ester was prepared according to HANBY et al.[9]. N-Carbobenzoxy-*O*-benzyl-L-tyrosine was from Merck. All the other carbobenzoxy derivatives were from Fluka.

Rat liver enzyme was prepared and assayed as described previously[7]. *E. coli* asparaginase (Kyowa Hakko Kogyo Co. Ltd., Tokyo) and lyophylized preparations of asparaginase from *Erwinia cartovora*[10] were kindly donated by Prof. N. GROSSOWICZ, Hebrew University-Hadassah Medical School, Jerusalem. In the experiments with the bacterial enzymes, the reaction mixtures contained, in 1 ml 0.1 *M tris* buffer, pH 8: L-asparagine 2 µmoles; enzyme, an amount that hydrolyzed approximately 1.5 µmoles of the substrate in 5 min; amino acid derivatives, none or 20 µmoles. The incubation time was 5 min at 37 °C. At the end of the incubation 1 ml of 5% CCl_3COOH, 3 ml borate buffer, pH 10.1[11] and 0.5 ml tributyl phosphate were added. Ammonia was transferred by aeration for 20 min at 50 °C into 0.2 *M* citrate buffer, pH 5, and estimated colorimetrically by the method of MOORE and STEIN[12]. In several cases, the hydrolysis of asparagine was estimated as follows. To the reaction mixture 1 ml of 0.6 *M* CCl_3COOH and 2 ml of 0.3 *M*

Effect of phenylacetyl (PA) derivatives of amino acids on rat liver asparaginase

Derivative	Molar ratio derivative: L-asparagine	Inhibition (%)
PA-L-phenylalanine	10:1	89–94
PA-D-phenylalanine	6:1	67–73
N-PA-S-benzyl-L-cysteine	10:1	97–100
Di-PA-L-lysine	10:1	39–41[a]
Di-PA-L-cystine	10:1	59–63
PA-DL-phenylglycine	10:1	83–89
N-PA-*O*-benzyl-L-tyrosine	1:1	100
PA-L-asparagine	10:1	0
PA-L-glutamine	10:1	0

[a] At the same molar ratio, di-3-phenylpropionyl-L-lysine gave about 60% inhibition.

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CCl_3COOH were added. After centrifugation, to 0.5 ml of the supernatant 0.5 ml of 0.3 *M* trisodium citrate was added. This mixture was heated with 2 ml of ninhydrin reagent according to the modified method of MOORE and STEIN[13] in which, on molar basis, ammonia, aspartic acid and asparagine give practically the same color intensity.

Results. A strong inhibition of rat liver asparaginase by carbobenzoxy derivatives of amino acid with more than one aromatic group was described previously[7]. As can be seen from the Table, this enzyme is also markedly inhibited by phenylacetyl derivatives of amino acids with more than one aromatic group. We found, however, that the following carbobenzoxy (CBZ), 3-phenylpropionyl (PP) and phenylacetyl (PA) derivatives of amino acids did not inhibit, at a molar ratio of 10:1 of derivative to substrate, the *E. coli* asparaginase (as tested by the above methods): CBZ-L-phenylalanine; N-CBZ-S-benzyl-L-cysteine; di-CBZ-L-lysine; N-CBZ-*O*-benzyl-L-tyrosine; CBZ-L-glutamic acid-γ-benzyl ester; PP-L-phenylalanine; N-PP-S-benzyl-L-cysteine; di-PP-L-lysine; PA-L-phenylalanine; PA-D-phenylalanine; N-PA-S-benzyl-L-cysteine; di-PA-L-lysine; N-PA-*O*-benzyl-L-tyrosine; di-PA-L-cystine; PA-L-phenylglycine; PA-L-asparagine; PA-L-glutamine.

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Since the experiments with mammalian and *E. coli* asparaginase were carried out in different buffers, some of the experiments with the latter enzyme were repeated using phosphate buffer instead of *tris*, with the same results. We also tested, at the same molar ratio, the effect of most of the above derivatives on asparaginase from *Erwinia carotovora*. With this enzyme, too, no inhibition was observed.

It follows from these findings that whenever one of the above, or similar, amino acid derivatives inhibits an asparaginase-sensitive tumor, a test for the combined action of this compounds and *E. coli* (or *Erwinia*) asparaginase appears desirable.

Zusammenfassung. Während Acylderivate von Aminosäuren mit mehr als einer aromatischen Gruppe (wie z.B. N-Phenylacetyl-S-benzyl-L-cystein) auf Rattenleberasparaginase eine stark hemmende Wirkung ausüben, werden Asparaginase aus *Escherichia coli* und *Erwinia cartovora* durch solche Verbindungen nicht gehemmt.

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In vitro Binding of ^3H-Acrolein to Regenerating Rat Liver DNA Polymerase

Our previous studies on DNA synthesis in vitro[1] suggested that the inhibitory action of acrolein on regenerating rat liver DNA polymerase was located in the sulfhydryl groups essential for the enzyme activity. The present investigation was designed in order to test this hypothesis. For this purpose (^3H) acrolein has been used. The binding of the labelled aldehyde to regenerating rat liver DNA polymerase and to *Escherichia coli* DNA polymerase I was studied. As previously pointed out[1], the enzymatic activity of the first is associated with functional thiol group(s), whereas the single cysteine residue of the second is not included in the active centre of the molecule. The competition between (^3H) acrolein and 2-mercaptoethanol for the enzymes as well as the action of iodoacetamide were investigated. The fixation of (^3H) acrolein to the templates, DNA or synthetic polymers, was also measured.

Table I. Labelling of DNA polymerase templates by (^3H) acrolein

(^3H) acrolein concentration (*M*)	6×10^{-6}	6×10^{-5}	6×10^{-4}
Native DNA	0	0	65
Denatured DNA	0	0	35
Poly [d(A–T) · (T–A)]	0	0	23
Poly (dC)	0	0	12

The various templates were incubated with increasing concentrations of (^3H) acrolein (10 mCi/m*M*) for 60 min at 38 °C. The reaction mixtures (0.5 ml) contained native or denatured DNA (100 μM), poly [d(A–T) · d(T–A)] or poly dC (23 μM). The results are expressed in pmoles of (^3H) acrolein incorporated per ml of reaction mixture in 1 h.

Material and methods. We used freshly prepared (^3H) acrolein (CEA, Saclay, France) and unlabelled acrolein (Prolabo) distilled just before use, 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y., USA), iodoacetamide (Sigma Chemical Co.). The templates were native or heat denatured calf-thymus DNA (Choay, France), double stranded alternating copolymer poly [d(A-T)·d(T-A)] and single stranded homopolymer poly (dC) (Biopolymers Inc.). DNA dependent DNA polymerase of regenerating rat liver was prepared according to the method published elsewhere[2]. Fraction IV obtained after hydroxylapatite chromatography was used. The specific activity of this fraction in the presence of poly [d(A-T) · d(T-A)] was equal to 100 U/ml. *E. coli* DNA polymerase I (Fraction VII) (Biopolymers Inc.) was diluted to obtain a specific activity of 300 U/ml in the presence of poly [d(A-T) · d(T-A)].

Two types of assays were performed: 1. (^3H) acrolein was incubated 1 h at 38 °C with templates, enzymes, reagents or alone as a control. 2. Regenerating rat liver DNA polymerase or *E. coli* enzyme was preincubated 30 min at 38 °C with templates or other products before (^3H) acrolein was added and incubated 30 min at 38 °C. The incubations were performed as previously described[1]. The results, obtained by determining the difference between assays and controls, were expressed in pmoles of (^3H) acrolein incorporated per ml or reaction mixture.

Results. Binding to DNA or synthetic templates. The data in Table I show that only with 6×10^{-4} *M* (^3H) acrolein can an incorporation be measured in native or denatured DNA, poly [d(A-T) · d(T-A)] or poly (dC). Calculations

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