Inhibition of rat liver glutamine synthetase by phosphonic analogues of glutamic acid[1]

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Summary. Analogues of glutamic acid, α-methylglutamic acid and glutamine in which the α- or γ-COOH groups are replaced by PO_3H_2 or $P(O)(OH_3)OH$ functions competitively inhibit rat liver glutamine synthetase. The K_I values are comparable to or lower than K_M for L-glutamate.

Aminophosphonates related structurally to glutamic acid appear to be of some interest as biologically active substances. Thus, phosphinothricin (**2**, table) is a natural compound with antibiotic properties[2,3], while 2-amino-4-phosphonobutyric acid (**1**) competes with glutamate for receptors in nerve cells[4,5] and has been reported to have antiviral activity[6].

The mode of antibacterial action of phosphinothricin involves inhibition of glutamine synthesis in the bacterial cell by competition with glutamic acid[2]. Considering this fact, and the much earlier observation that **1** and its P-ethyl analogue are strong inhibitors of pigeon liver glutamine synthetase[7] it appeared worthwhile to extend the number of phosphonic analogues of glutamic acid examined for inhibitory properties in glutamine synthesizing enzyme systems. In this communication we report the inhibition of rat liver glutamine synthetase by phosphonic and P-methylphosphinic acids structurally related to glutamic acid, α-methylglutamic acid, glutamine and isoglutamine (table).

All analogues listed in the table were prepared in our laboratory in the form of racemic mixtures[8-11] and were used as such in enzyme tests.

The inhibition was studied using glutamine synthetase (EC.6.3.1.2) prepared from rat liver[12]. Enzyme activity with and without inhibitors was assayed by the hydroxamate procedure[13] in which K_M for L-glutamate was 2.4×10^{-3} M. Assays were conducted at 37 °C in 1 ml mixture which contained 50 μM imidazole, 20 μM $MgCl_2$, 100 μM NH_2OH:HCl, 10 μM ATP, 25 μM 2-mercaptoethanol, 2.5–20 μM L-glutamate and 0–60 μM of inhibitor. Final pH 7.2. Reaction time was 15 min. K_I values were determined from double reciprocal plots of velocity against glutamate concentration.

All analogues listed in the table, although structurally quite diverse, are effective inhibitors of glutamine synthetase, acting competitively against L-glutamate.

The inhibition constants K_I shown in the table indicate that all the analogues examined have substantial affinities to rat liver glutamine synthetase. In fact, the values of K_I obtained for most of our analogues are of the same order of magnitude as K_M for L-glutamate and in four cases (compounds **1**, **2**, **4** and **6**) the inhibitors are bound more tightly than the substrate. This demonstrates that substituting the phosphonic or P-methylphosphinic acid function for the COOH group, regardless of its position in the molecule, is an effective means of producing structural antagonists of glutamic acid. Comparison of inhibition by compounds **1**, **3**, **5**, **7** and **2**, **4**, **6**, **8** shows that the P-methylphosphinic analogues have higher affinities for glutamine synthetase than the compounds with a PO_3H_2 group. Another general trend is that analogues with PO_3H_2 or $P(O)(CH_3)OH$ group in γ position (compounds **1**–**4**) are better inhibitors that corresponding α analogues (compounds **5**–**8**).

The effect of an α methyl group is irregular: the K_I values for compounds **1** and **5** are higher than those found for corresponding α-methyl derivatives **3** and **7** (note that α-methylglutamate is nor as good substrate for glutamine synthetase as glutamate[14]) while the K_I for phosphinothricin (**2**) is lower than that observed for the α-methyl derivative (**4**). It is worth noting that compound **4** is a strong inhibitor of rat liver glutamine synthetase, second only to the transition state analogue methionine sulphoximine phosphate[15,16]. Conversion of a COOH group in α or γ phosphonic analogues to an amide or ester function (compounds **9**–**12**) produces inhibitors with K_I values of the same order of magnitude as K_M for L-glutamate. It thus appears that the COOH group is not essential for effective binding by glutamine synthetase.

In view of the efficient inhibition of rat liver glutamine synthetase by phosphonic and P-methylphosphinic substrate analogues reported here it was of interest to examine their effect on other enzymes involved in the metabolism of glutamic acid. Studies performed so far have included *E. coli* glutamate decarboxylase (EC.4.1.1.15), porcine heart alanine and aspartate transaminases (EC.2.6.1.2, EC.2.6.1.1) and bovine liver glutamate dehydrogenase (EC.1.4.1.3). No effect was observed in the case of gluta-

Inhibition of rat liver glutamine synthetase ($K_m = 2.43 \cdot 10^{-3}$ M) by phosphonic analogues of glutamic acid and glutamine

Compound	Structure	K_i [M]
1	$H_2O_3P-CH_2-CH_2-CH(NH_2)(COOH)$	$8.8 \cdot 10^{-4}$
2	$HO(CH_3)(O)P-CH_2-CH_2-CH(NH_2)(COOH)$	$3.2 \cdot 10^{-4}$
3	$H_2O_3P-CH_2-CH_2-C(NH_2)(CH_3)(COOH)$	$6.3 \cdot 10^{-3}$
4	$HO(CH_3)(O)P-CH_2-CH_2-C(NH_2)(CH_3)(COOH)$	$5.6 \cdot 10^{-5}$
5	$HOOC-CH_2-CH_2-CH(NH_2)(PO_3H_2)$	$1.3 \cdot 10^{-3}$
6	$HOOC-CH_2-CH_2-CH(NH_2)(P(O)(CH_3)OH)$	$7.9 \cdot 10^{-4}$
7	$HOOC-CH_2-CH_2-C(NH_2)(CH_3)(PO_3H_2)$	$1.6 \cdot 10^{-2}$
8	$HOOC-CH_2-CH_2-C(NH_2)(CH_3)(P(O)(CH_3)OH)$	$9.5 \cdot 10^{-3}$
9	$H_2NOC-CH_2-CH_2-CH(NH_2)(PO_3H_2)$	$5.5 \cdot 10^{-3}$
10	$H_2O_3P-CH_2-CH_2-CH(NH_2)(CONH_2)$	$6.2 \cdot 10^{-3}$
11	$H_2O_3P-CH_2-CH_2-CH(NH_2)(COOCH_3)$	$8.7 \cdot 10^{-3}$
12	$H_3COOC-CH_2-CH_2-CH(NH_2)(PO_3H_2)$	$2.1 \cdot 10^{-3}$

mate dehydrogenase, while inhibition of decarboxylase and transaminases was insignificant. Thus, recognition of phosphonic and phosphinic analogues as being similar to glutamic acid might be a peculiar property of glutamine synthesizing enzymes. This might be due to the similarity of inhibitors to the intermediate γ-glutamyl phosphate.

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2 E. Bayer, K.H. Gugel, H. Hägele, M. Hagenmaier, S. Jessipow, W.A. Konig and H. Zähner, Helv. chim. Acta *55*, 224 (1972).
3 Y. Kondo, T. Shomura, Y. Ogawa, T. Tsuruorka, H. Watanabe, K. Tutsukawa, T. Suzuki, C. Moriyama, S. Inouye and T. Niida, Sci. Rep. Meji Seika Kaisha *13*, 34 (1973).
4 S.G. Cull-Candy, J.F. Donnellan, R.W. James and G.G. Lunt, Nature 408 (1976).
5 T.J. Mc Donald and R.D. O'Brien, J. Neurobiol. *3*, 277 (1972).
6 H. Fukujaasu, Z. Oya, K. Kawakami, T. Kikuchi, T. Shomura, T. Tsumoka, T. Watanabe, Y. Kazuno, S. Inaue and Y. Schizawa, Jap. Kokai Tokkyo Koho 7902, 336, CA *90*, 198338r (1979).
7 P. Mastalerz, Archwiss. Immun. Terap. doswiad. *7*, 201 (1959).
8 E. Gruszecka, P. Mastalerz and M. Soroka, Roczn. Chem. *49*, 2127 (1975).
9 E. Gruszecka, M. Soroka and P. Mastalerz, Pol. J. Chem. *53*, 937 (1979).
10 E. Gruszecka, M. Soroka and P. Mastalerz, Pol. J. Chem. *53*, 2327 (1979).
11 E. Gruszecka and P. Mastalerz, unpublished results.
12 Ch. Wu, B.B.A. *89*, 137 (1964).
13 S.P. Colowick and N.O. Kaplan, eds, Methods Enzymology, vol. XVII, A, p. 900. Academic Press, New York 1970.
14 H.M. Kagan, L.R. Mannig and A. Meister, Biochemistry *4*, 1063 (1965).
15 R.A. Ronzio and A. Meister, Proc. natl Acad. Sci. USA *59*, 164 (1968).
16 W.B. Rowe and A. Meister, Biochemistry *15*, 1578 (1973).

Effect of cholinesterase on the chemiluminescence of luminol

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Summary. The chemiluminescent oxidation of luminol was activated by cholinesterase. Physostigmine and dimethoate inhibited the luminescence, TEA had no effect. These results indicate that the esteratic site is responsible for the activating action of the enzyme.

The chemiluminescent oxidation of luminol with hydrogen peroxide in aqueous alkaline solution occurs to a significant extent only in the presence of an 'activating agent'[1]. The activating action of organophosphorous cholinesterase (ChE) inhibitors on the oxidation of luminol was first described by Goldenson[2]. Weber and co-workers have extensively studied the activating action of DFP, tabun, sarin[3] and various organophosphorous insecticides[4,5]. In our previous papers, we reported that cholinomimetic agents (acetylcholine and pilocarpine) also activate the oxidation of luminol[6–8].

This study was undertaken to determine whether ChE exerts any action on the luminescence of luminol, and if so, to examine the interaction between the enzyme and its inhibitors.

Material and methods. Luminescence intensity was recorded as a function of time with a luminophotometer described by Weber[9]. Maximum luminescence intensity (Φ_m) was plotted against the concentration of the reagent investigated. All experiments were performed with a reaction mixture containing 0.4 mM luminol, 45 mM NaOH and various concentrations of H_2O_2. The total volume of reaction mixture was 50 ml, the pH 12.2 and the temperature 20 °C. Pseudocholinesterase (4 units/mg) was obtained from Calbiochem, and luminol from Koch-Light. All other reagents were of analytical grade.

Results and discussion. Upon addition of ChE to the reaction mixture an intense luminescence was immediately evident, indicating that the enzyme activates the oxidation of luminol. A linear relationship exists between maximum luminescence intensity and enzyme concentration. The intensity of light produced is markedly affected by the amount of H_2O_2. Figure 1 shows the effect of H_2O_2 concentration on maximum luminescence intensity in the

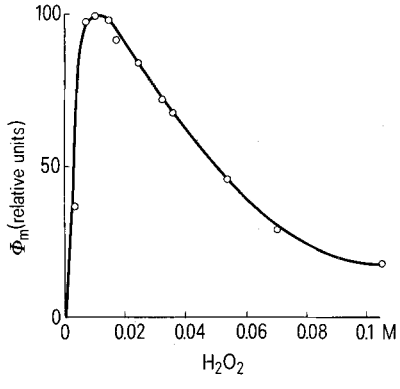

Fig. 1. Maximum luminescence intensity vs H_2O_2 concentration. Activator: ChE 0.04 mg%. Conditions: $4 \cdot 10^{-4}$ M luminol, reaction pH 12.2.

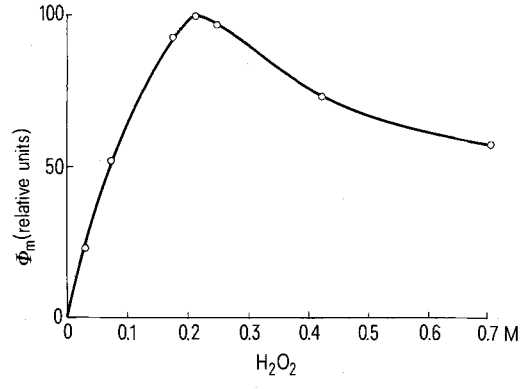

Fig. 2. Maximum luminescence intensity vs H_2O_2 concentration. Activator: dimethoate 0.1 mM. Conditions: $4 \cdot 10^{-4}$ M luminol, reaction pH 12.2.