The Effect of 4-Substituent of Benzoic Acid on Glycine Conjugation

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The relationship between the glycine conjugation of 4-substituted derivatives of benzoic acid and physico-chemical property was studied using rats. A linear relationship was found between the lipophilicity and the glycine conjugation after the intravenous injection of the derivative into rats. But there was no relationship between the lipophilicity and permeability into the liver. The formation of the glycine conjugate using mitochondria fraction of the liver was responsible for the superdelocalizability indicating the electrophilicity of carboxyl carbon.

Keywords benzoic acid; 4-substitution; glycine conjugation; partition coefficient; permeability; superdelocalizability

Glycine conjugation is well known as a metabolic reaction of the detoxification of the derivatives of benzoic acid.[1] Therefore, the study on the relationship between chemical structure and glycine conjugation gives useful information about the detoxification mechanism of the derivatives of benzoic acid. The study on the relationship between chemical structure and enzymatic reactivity needs a complex combination of steric and physico-chemical features of chemicals under consideration. To clarify the basic relationship between the substituent of benzoic acid and glycine conjugation, the present study avoided a steric factor due to the difference of the substituent position and focused on the effect of 4-substituent on the conjugation.

Experimental

Animals Male Wistar rats weighing 220—240 g were used.

Materials The following labelled compounds were used: 4-nitrobenzoic acid (7-^{14}C), 4-hydroxybenzoic acid (7-^{14}C), 4-bromobenzoic acid (7-^{14}C) and benzoic acid (7-^{14}C) were synthesized by Daiichi Pure Chemicals Co. (Japan); 4-chlorobenzoic acid (^{14}C-u) and 4-aminobenzoic acid (^{14}C-u) were purchased from Amersham. Benzoic acid and its derivatives were purchased from Kanto Chemical Co. (Japan).

Analysis of Urinary Excretion Urine was collected 12 h after an intravenous injection of labelled compound (specific activity 50 μCi/mmol) at a dose of 0.4 mmol/kg. Urine was freeze-dried and residues were redissolved in 0.1 M sodium phosphate (2 ml). A 50 μl portion was subjected to thin layer chromatography (TLC).

Permeability into Liver The labelled compound was injected intravenously at a dose of 0.4 mmol/kg into rats. Blood and liver were obtained 10 min after the injection. A part of the liver was dissolved in tissue solubilizer (Soluene-350) and mixed with scintillator (Econofluor). The plasma was freeze-dried and residues were redissolved in 0.1 M sodium phosphate. Permeability into the liver was calculated from the ratio of the concentration of radioactive substances in the liver to the concentration of unchanged substance in the plasma (T/P value).

***In Vitro* Glycine Conjugation** The glycine conjugation of labelled compounds was carried out by using the mitochondria fraction of the rat livers according to the method of Dixon *et al.*[2] After the reaction was stopped by the addition of a half volume of methanol, proteins were removed by centrifugation. Supernatant was subjected to TLC.

Partition Coefficient The *n*-octanol–H_2O partition coefficient was determined according to the method of Hansch and Fujita.[3] π is defined as: $\pi = \log P_X - \log P_H \cdot P_X$ and P_H are the partition coefficients of the substituted and unsubstituted compounds, respectively.

TLC and Radioactivity Assay Plates (Merck Silica gel F_{254}) were developed in *n*-butanol saturated with 0.1 N HCl and contacted with X-ray film. The radioactive area on the plate was scraped off and mixed with toluene liquid scintillation medium. The radioactivity was assayed in the manner previously reported.[4]

Calculation of Superdelocalizability ($S_r^{(N)}$) $S_r^{(N)}$[5] for a nucleophilic reaction was calculated by using a single linear combination of the atomic orbital-molecular orbital (LCAO-MO) method. Parameters suggested by Streitwieser[6] were used.

Results and Discussion

To examine a structure–activity relationship in glycine conjugation, we attempted to correlate degrees of production of the conjugate with the physico-chemical property of the derivatives of benzoic acid. Figure 1 shows a plot of the amount of the conjugate in the urine against the partition coefficient. The Eq. 1 was calculated from the data in Fig. 1,

$$A_{in\ vivo} = 15.23\pi + 75.76 \quad (r = 0.9313,\ s = 4.604) \tag{1}$$

where $A_{in\ vivo}$ is the amount of the conjugate in the urine, r is the correlation coefficient and s is the standard deviation. A lipophilicity has been related to a biological activity of chemicals since a lipophilicity reflects permeability into the cell membrane.[7] Thus we examined the relationship between π value and T/P value of the liver, forming the conjugate. The relationship between π and T/P value is shown in Fig. 2. The increase of π did not correspond to the change of T/P value ($r = 0.5414$). This result suggests that the lipophilicity of derivatives was responsible for permeability into the intracellular membrane.[8] A glycine conjugate is formed by an enzymatic reaction of a carboxyl group to adenosine triphosphate (ATP), coenzyme A (CoA) and glycine.[1] Therefore, it is estimated that the influence of the 4-substituent of the derivative on the electronic property of the carboxyl group relates to the formation of the conjugate. We applied Hammett's rule to the influence of the 4-substituent on the reactivity in the glycine

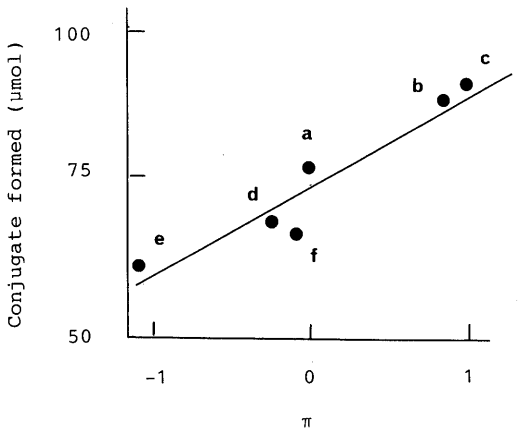

Fig. 1. Relationship between *in Vivo* Glycine Conjugation and Partition Coefficient

Key: a, benzoic acid; b, 4-chlorobenzoic acid; c, 4-bromobenzoic acid; d, 4-hydroxybenzoic acid; e, 4-aminobenzoic acid; f, 4-nitrobenzoic acid.

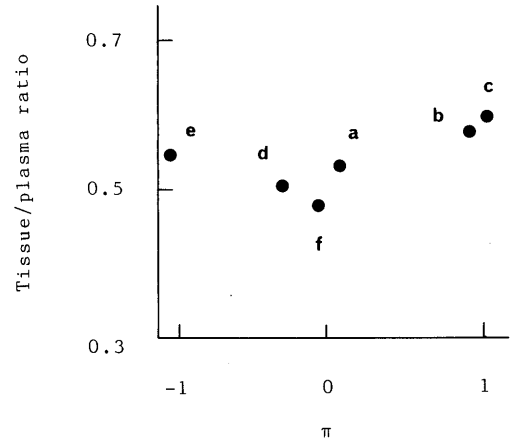

Fig. 2. Relationship between Permeability into Liver and Partition Coefficient

Key to derivatives corresponds to Fig. 1.

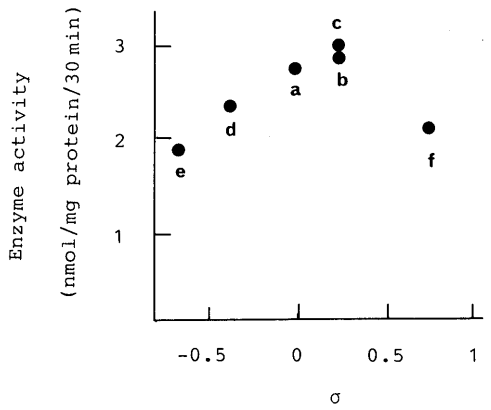

Fig. 3. Relationship between *in Vitro* Glycine Conjugation and Hammett

Key to derivatives corresponds to Fig. 1.

conjugation. Figure 3 shows a plot of Hammett's σ constant against the *in vitro* formation of the conjugate. A low correlation coefficient ($r=0.2316$) was observed due to the deviation of 4-nitrobenzoic acid. The deviation of 4-nitrobenzoic acid, a strong electron-withdrawing substituent, may be attributed to the increase in acid strength caused by the electron-withdrawing conjugative effect of the nitro group. A glycine conjugation proceeds as an electrophilic attack of the carboxyl carbon to ATP, CoA and glycine. Thus, the $S_r^{(N)}$ value of carboxyl carbon was calculated by the LCAO-MO method to determine the electrophilic reactivity of carboxyl carbon. The LCAO-MO, approximate calculation method, has been used to study the structure–activity relationship since electronic property obtained by the LCAO-MO method reflects its change due to substitution.[9,10] $S_r^{(N)}$ value by the LCAO-MO method in the present study, restricted to the 4-substituent, is estimated to reflect the change of electrophilicity of carboxyl

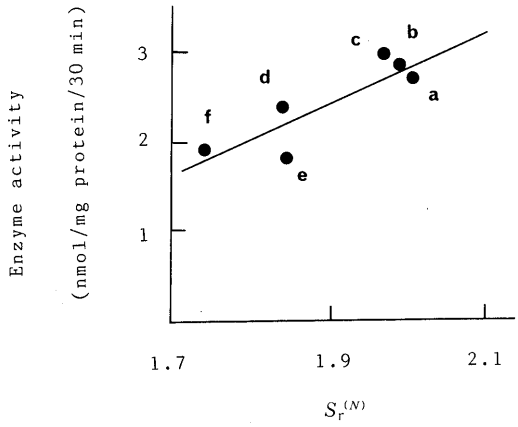

Fig. 4. Relationship between *in Vitro* Glycine Conjugation and Superdelocalizability

Key to derivatives corresponds to Fig. 1.

carbon by 4-substitution. Figure 4 shows the relationship between $S_r^{(N)}$ value and the *in vitro* formation of the conjugate. Using the data in Fig. 4, the following equation wad derived.

$$A_{in\ vitro}=4.106S_r^{(N)}-5.354 \quad (r=0.8807,\ s=0.2277) \tag{2}$$

where $A_{in\ vitro}$ is the *in vitro* formation rate of the conjugate. The correlation coefficient in Eq. 2 suggests that the influence of the 4-substituent on the electrophilic reactivity of the derivative is responsible for the formation of the conjugation. From the above results, the *in vitro* formation of the conjugate was expressed as a combination of Eqs. 1 and 2.

$$A_{in\ vivo}=11.85\pi+48.34S_r^{(N)}-16.01 \quad (r=0.9899,\ s=1.793) \tag{3}$$

This combination resulted in an increase in the correlation coefficient. Therefore Eq. 3 is estimated to be an adequate description of the influence of the 4-substituent on the *in vivo* conjugation.

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