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Preparation of Specific Antibody to 2,3-Trimethylene-4-quinazolone for the Immunoassay of Δ^1-Pyrroline

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Anti-2,3-trimethylene-4-quinazolone antibody was prepared from the sera of two rabbits immunized with an antigen which was a conjugate of 6-(β-carboxypropionyl)-amino-2,3-trimethylene-4-quinazolone and bovine serum albumin in a molar ratio of approximately 20 to 1. Specific affinity of the antibody for 2,3-trimethylene-4-quinazolone was confirmed by comparison of the affinities for thirteen structurally related compounds.

Keywords——Δ^1-pyrroline; diamine oxidase; putrescine; anti-2,3-trimethylene-4-quinazolone antiserum; immunoassay

In the previous paper,[2)] we reported that Δ^1-pyrroline, a catabolite of putrescine[3)] or spermidine,[4)] was stoichiometrically converted to 2,3-trimethylene-4-quinazolone (I) by a specific reaction with *o*-aminobenzaldehyde followed by chromic acid oxidation, and that the reaction was successfully applied to the determination of Δ^1-pyrroline added to a deproteinized supernatant of rat liver by gas chromatography. The gas chromatographic method, which is a hundred times more sensitive than the conventional colorimetry, can be easily modified to make it even more sensitive by using gas chromatography-mass spectrometry. Immunoassay is a convenient alternative method for the analysis of many samples with high sensitivity. The immunoassay for I is immediately applicable to the determination of Δ^1-pyrroline. The method is, thus, an example of how to determine immunologically a compound such as Δ^1-pyrroline which has no immunogenicity by itself because of its low molecular weight. This report is concerned with the preparation of a specific antibody to I.

Experimental

Materials——I was synthesized from isatoic anhydride and 2-pyrrolidone according to the method of Späth *et al.*[5)] 2,3-Tetramethylene-4-quinazolone was similarly synthesized. 2-Amino-N-alkylbenzamides, 3-alkyl-4-quinazolones, and 2-methyl-3-propyl-4-quinazolone for use in specificity experiments were synthesized by the reported methods.[6,7)]

N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Fluka AG, Buchs, Switzerland. Bovine serum albumin (BSA), rabbit serum albumin (RSA), anti-BSA antiserum (rabbit), and Freund's complete adjuvant were purchased from Miles Laboratories, Inc., IN, U.S.A. [1,4-^{14}C]-Succinic anhydride (9.32 mCi/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Fluorescamine was kindly donated by Nippon Roche. All other chemicals and solvents were commercial products of reagent grade.

Synthesis of Hapten——6-Nitro-2,3-trimethylene-4-quinazolone (II): I (2.7 g, 14.5 mmol) was dissolved in a mixed solution of 10 ml of nitric acid (60%) and 20 ml of concentrated sulfuric acid, and the mixture was kept at room temperature for 15 hr. The reaction mixture was then neutralized with 5 N NaOH in the presence of ice, and extracted twice with 100 ml of ethyl acetate. The extract was evaporated to dryness

1) Location: *Takada 3-41-8, Toshima-ku, Tokyo 171, Japan.*

2) Previous paper: S. Sakamoto and K. Samejima, *Chem. Pharm. Bull.*, **27**, 2220 (1979).
3) B. Holmstedt, L. Larsson, and R. Tham, *Biochim. Biophys. Acta*, **48**, 182 (1961).
4) U. Bachrach and I.S. Oser, *J. Biol. Chem.*, **238**, 2098 (1963).
5) E. Späth and N. Platzer, *Chem. Ber.*, **68**, 2221 (1935).
6) R.H. Clark and E.C. Wagner, *J. Org. Chem.*, **9**, 55 (1944).
7) B.R. Baker, M.V. Querry, A.F. Kadish, and J.H. Williams, *J. Org. Chem.*, **17**, 35 (1952).

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and the residue was recrystallized from 95% EtOH to give II (2.4 g, 71.6%) as pale yellow needles, mp 196—198°. IR ν_{max}^{KBr} cm^{-1}: 1350, 1560 (NO_2). NMR (5% solution in $CDCl_3$) δ: 9.10 (1H, d, $J_{5,7}=3$ Hz, H-5), 8.50 (1H, dd, $J_{7,8}=8$ Hz, $J_{5,7}=3$ Hz, H-7), 7.20 (1H, d, $J_{7,8}=8$ Hz, H-8). *Anal.* Calcd for $C_{11}H_9N_3O_3$: C, 57.14; H, 3.92; N, 18.17. Found: C, 57.54; H, 3.89; N, 17.65.

6-Amino-2,3-trimethylene-4-quinazolone (III): II (300 mg, 1.30 mmol) dissolved in 120 ml of 99% EtOH was added to previously activated palladium charcoal (30 mg) in 30 ml of EtOH under an H_2 atmosphere. The suspension was stirred at room temperature until the theoretical amount of H_2 was absorbed. The catalyst was then filtered off, and the filtrate was evaporated to dryness. The residue was recrystallized from 99% EtOH to give III (194 mg, 74.3%) as yellow crystals, mp 260—265°. IR ν_{max}^{KBr} cm^{-1}: 3350 (NH_2). *Anal.* Calcd for $C_{11}H_{11}N_3O$: C, 65.66; H, 5.51; N, 20.88. Found: C, 65.10; H, 5.42; N, 20.32.

6-(β-Carboxypropionyl)amino-2,3-trimethylene-4-quinazolone (IV): III (200 mg, 1.0 mmol) and succinic anhydride (200 mg, 2.0 mmol) were dissolved in 18 ml of pyridine, and heated at 80° for 5 hr. The pyridine was removed *in vacuo*, and the residue was recrystallized from 99% EtOH to give IV (187 mg, 62.4%) as pale yellow needles, mp 272—274°. IR ν_{max}^{KBr} cm^{-1}: 1710 (COOH). *Anal.* Calcd for $C_{15}H_{15}N_3O_4$: C, 59.80; H, 5.02; N, 13.95. Found: C, 59.72; H, 4.76; N, 14.00.

[^{14}C]-Labelled IV was similarly synthesized: III (100 mg, 0.5 mmol) and a mixture of [1,4-^{14}C]-succinic anhydride (0.5 mg) and the unlabelled anhydride (99.5 mg) were dissolved in 9 ml of pyridine. The mixture was sealed in an ampoule and heated at 70° for 5 hr. The crystals obtained were recrystallized three times up to constant specific activity.

6-Acetylamino-2,3-trimethylene-4-quinazolone was similarly synthesized, using acetic anhydride in place of succinic anhydride, and was obtained as colorless needles, mp 274—275°. *Anal.* Calcd for $C_{13}H_{13}N_3O_2$: C, 64.19; H, 5.39; N, 17.27. Found: C, 64.13; H, 5.20; N, 17.00.

Preparation of Antigen——EDC (200 mg) dissolved in 2.5 ml of H_2O was added dropwise to IV (100 mg) dissolved in 2.5 ml of 0.1 N NaOH with stirring at 9°. Stirring was continued for 1 hr, then 2.5 ml of an aqueous solution of BSA (122 mg) was added. The mixture was allowed to stand overnight at room temperature. This reaction mixture was then applied to a column of Sephadex G-25 (310×19 mm I.D.) equilibrated with phosphate buffer-saline (PBS). Fractions containing protein were collected, and lyophilized after intensive dialysis against H_2O. The powder obtained was used as the antigen.

[^{14}C]-Labelled conjugate for the determination of the binding ratio of IV to BSA was prepared from [^{14}C]-labelled IV and BSA by the procedures described above. Protein was fluorometrically determined by the method of Böhlen *et al.*[8]

The conjugate of IV with RSA was similarly prepared to confirm the production of anti-IV antibody by the double immuno diffusion method.[9]

Preparation of Antibody and Its Specificity——The foot pad, axillar site, and intraperitoneum of each of two female rabbits (domestic albino) were injected with 1 ml of an emulsion containing 5 mg of the antigen dissolved in 0.5 ml of PBS and 0.5 ml of Freund's complete adjuvant. Every week after the immunization, sera were collected and stored at −20°. Booster injections (5 mg of antigen/ml of PBS/rabbit) were repeated several times. Titers of the antisera were tested by a tube precipitin reaction.

Specificity experiments were carried out by means of the tube precipitin test. Solutions prepared by mixing 0.1 ml of the antiserum treated with BSA and 0.1 ml of various concentrations of each test compound dissolved in PBS were kept overnight at room temperature. The equilibrated solutions were then diluted with PBS in serial two-fold dilutions. Each diluted solution (100 µl) was pipetted into a small glass tube (3 mm I.D.×5 cm), and an equal volume of antigen solution (1 µM as BSA) was carefully added to it, avoiding disturbance of the interphase where the precipitation line appears. The maximum dilution capable of forming a precipitation line was decided at various concentrations of each test compound. The maximum dilution for the control containing no test compound was sixteen-fold. The maximum concentration in which a precipitation line was observable at eight-fold dilution was compared for each test compound.

Results and Discussion

Of several derivatives proposed as a hapten for the preparation of anti-I antibody, we chose IV because of the availability of a simple procedure for introduction of a nitro group at the 6 position of I. The synthetic route from I to the antigen is shown in Chart 1. II derived from I was subjected to conventional reduction with a catalyst to obtain III. IV was obtained by the succinylation of III. All the reactions proceeded in high yields. The hapten, IV, was conjugated with BSA in the presence of water-soluble carbodiimide to obtain the antigen. The binding ratio of the hapten to BSA was calculated by means of ultraviolet spectrometry and by

8) P. Böhlen, S. Stein, W. Dairman, and S. Udenfriend, *Arch. Biochem. Biophys.*, **155**, 213 (1973).
9) O. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **32**, 231 (1953).

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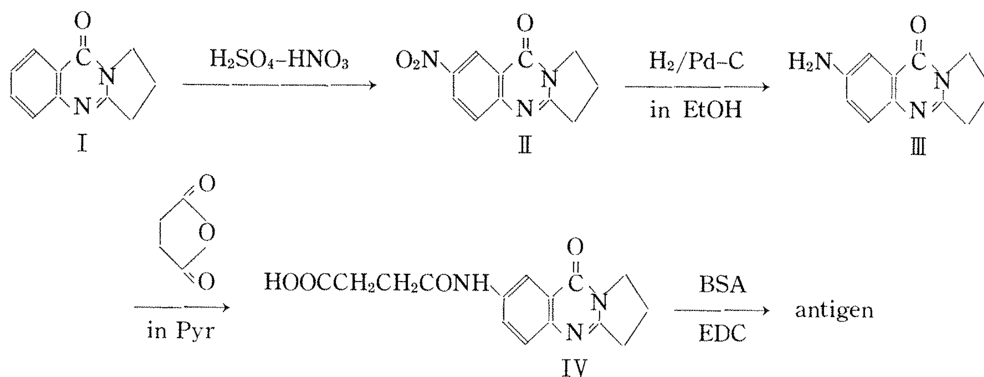

Chart 1

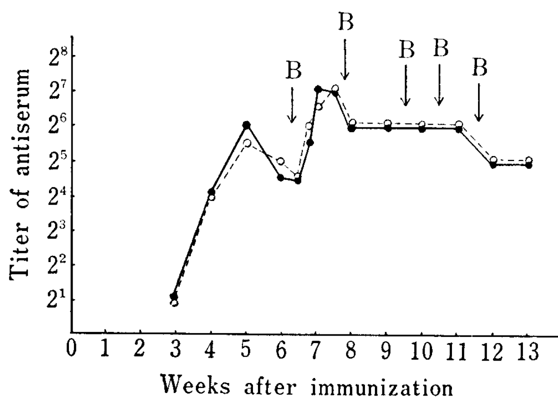

Fig. 1. Time Course of Titers of Antisera

Conditions are described in "Experimental."
—●—: rabbit A, ---○---: rabbit B.
B: booster injection.

using [^{14}C]-labelled IV. Both results showed that about twenty mol of hapten bound to one mol of BSA under the conditions described in "Experimental". This ratio was not altered by denaturation of the conjugate with 50% acetic acid.

Two rabbits were then immunized with the antigen emulsified with Freund's complete adjuvant. The time course of titers of antisera is shown in Fig. 1. After 3 weeks, the titers began to rise and attained the first maxima at 5 weeks. A booster injection after 6 weeks increased the production of antibody, inducing the second maxima a week or two later. Additional booster injections resulted in no further increase of antibody production. The maximum titers corresponded to a few mg of IgG per ml of antiserum.

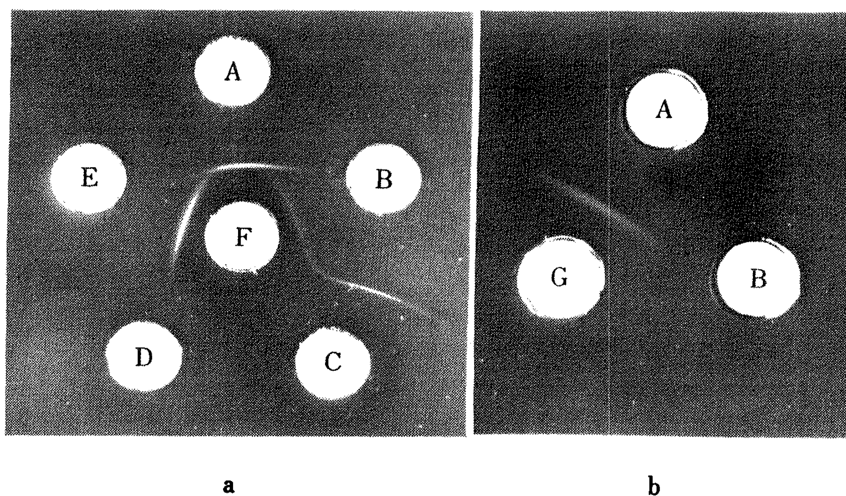

Fig. 2. Double Diffusion Gel Precipitation of Antiserum (F) with Antigen (A), BSA (B), Anti-BSA Antiserum (C), RSA (D), and Conjugate of RSA and Hapten (E)

G: antiserum treated with BSA.

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Production of antibody against the hapten was then examined by the double immunodiffusion method on agar gel plates (Fig. 2). The antiserum produced precipitation lines against the antigen, BSA, and the conjugate of RSR and IV. All three lines and the line between BSA and anti-BSA antiserum were connected, and both ends of the line between the antigen and the antiserum formed spurs (Fig. 2a). These results indicated that the antiserum contained antibidies against the hapten and BSA. Antiserum which had been treated with BSA to remove the anti-BSA antibody showed a precipitation line against the antigen alone (Fig. 2b).

Specific binding of the anti-hapten antibody to I was examined by means of a tube precipitin test using the BSA-treated antiserum and compounds related to I. The results are summarized in Table I. III, IV, and 6-acetylamino-2,3-trimethylene-4-quinazolone bound more tightly to the antibody than I, possibly because these compounds are the hapten itself and derivatives at the 6 position of I. Other compounds tested, however, showed negligible affinity for the antibody except in the case of 2,3-tetramethylene-4-quinazolone, which is closely related to I.

Table I. Relative Cross-Reactivity of the Antiserum[a] with Compounds Related to 2,3-Trimethylene-4-quinazolone

Compound	Relative cross-reactivity[b] (%)
2,3-Trimethylene-4-quinazolone (I)	100
6-Amino-2,3-trimethylene-4-quinazolone (III)	150
6-(β-Carboxypropionyl)amino-2,3-trimethylene-4-quinazolone (IV)	400
2,3-Tetramethylene-4-quinazolone	15
6-Acetylamino-2,3-trimethylene-4-quinazolone	150
2-Amino-N-methylbenzamide	<0.1
2-Amino-N-ethylbenzamide	<0.1
2-Amino-N-propylbenzamide	<0.1
2-Amino-N-butylbenzamide	<0.1
3-Methyl-4-quinazolone	<0.1
3-Ethyl-4-quinazolone	<0.1
3-Propyl-4-quinazolone	<0.1
3-Butyl-4-quinazolone	<0.1
2-Methyl-3-propyl-4-quinazolone	<0.1

a) Anti-BSA antibody was removed.
b) Molar ratio of I to each compound, calculated from the results of specificity experiments described in "Experimental."

It is concluded from these results that the anti-hapten antibody has a satisfactory specificity for I, and hence can be applied for an immunoassay of I. An enzyme immunoassay of I for the determination of Δ^1-pyrroline is being developed in this laboratory.

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