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Studies on the Enzyme Immunoassay of Bio-Active Constituents Contained in Oriental Medicinal Drugs. III.[1] Enzyme Immunoassay of Paeoniflorin, a Constituent of Chinese Paeony Root

MATAO KANAOKA,*,[a] SABURO YANO,[b] HIROMI KATO,[b] KYOKO NAKANISHI,[b] and MASAO YOSHIZAKI[c]

Research Institute for WAKAN-YAKU (Oriental Medicines),[a] The First Department of Internal Medicine,[b] Herbal Garden,[c] Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan

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An enzyme immunoassay (EIA) for the determination of paeoniflorin (PA), which is a principal constituent of Chinese paeony root ("Shakuyaku" in Japanese), was established by the use of 6′-hemisuccinyl (III) and 6′-hemiglutaryl PA (IV) as haptens. Compounds III and IV were coupled with β-galactosidase (β-Gal) and bovine serum albumin (BSA) by the *N*-hydroxysuccinimide ester method to give hemisuccinyl PA-β-Gal (IX) (labeled antigen) and hemiglutaryl PA-BSA (X) (immunogen), respectively. A 30000-fold diluted solution of anti-PA antisera elicited in rabbits by immunization with the immunogen was used for the EIA, and the separation of bound and free fractions was performed by the double antibody method using a goat antiserum to rabbit IgG. 7-β-D-Galactopyranosyloxy-4-methylcoumarin was used as the substrate for the fluorometric assay of β-Gal activity. A satisfactory standard curve for EIA of PA was obtained in the range of 1—400 ng/ml and was only slightly interfered with the addition of serum and urine to the assay mixture of EIA. The PA antiserum reacted with oxypaeoniflorin (0.31%) and albiflorin (0.22%) which are also constituents of Chinese Paeony root.

Keywords——enzyme immunoassay; paeoniflorin; Chinese Paeony root; 6′-hemisuccinyl paeoniflorin; 6′-hemiglutaryl paeoniflorin; *N*-hydroxysuccinimidyl ester method; double antibody method; β-galactosidase; 7-β-D-galactopyranosyloxy-4-methylcoumarin; fluorometric assay

In the previous paper,[1,2] we described an enzyme immunoassay (EIA) of 18β-glycyrrhizin (GL) and 18β-glycyrrhetic acid (GA), the aglycone of GL, which is a principal constituent of Glycyrrhizae Radix. These EIA methods were applied to determine GL and GA concentrations in human blood after intravenous and oral administration of GL,[3] and those in the blood of patients with GL-induced pseudoaldosteronism.[4] On the other hand, Glycyrrhizae Radix has often been prescribed together with Chinese Paeony root ("Shakuyaku" in Japanese) as a traditional remedy. The available methods for the determination of the latter's major constituent, paeoniflorin (PA), such as thin-layer chromatography (TLC),[5] and high performance liquid chromatography (HPLC),[6] do not have sufficient sensitivity and specificity for quantitative analysis of the drug in biological fluids, tissues and organs. This led us to develop a more sensitive and specific EIA of PA for studies of the metabolism of PA in humans. This paper deals with the preparation of haptens, the production and specificity of anti-PA antibody, and the EIA of PA.

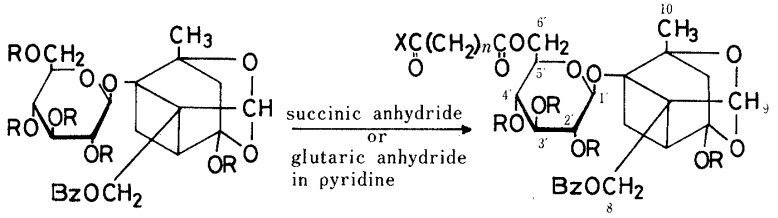
Hosoda *et al.*[7] claimed that the use of enzyme-labeled antigen prepared from a hapten having a bridge shorter than that used for antibody production is advantageous for obtaining increased sensitivity in enzyme immunoassay, and they used the hemisuccinyl group for enzyme-labeled antigen and the hemiglutaryl group for antibody production. Thus, hemisuccinyl and hemiglutaryl groups were chosen as bridges between PA and carrier protein, and were introduced at the 6′-hydroxyl group, which is most readily benzoylated with benzoyl chloride[8] among the hydroxyl groups in the PA molecule, by the reaction of PA with succinic

anhydride and glutaric anhydride in pyridine to give 6′-hemisuccinyl (III) and 6′-hemiglutaryl PA (IV), respectively. The PA used was obtained from commercial Chinese Paeony root according to the procedure of Shibata *et al.*[8,9]

In the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra, the 6′-methylene protons of PA pentaacetate (II) exhibited a doublet signal at δ 4.14 (2H, J=7 Hz), while those of the acetyl derivatives of PA-hemiesters (V) and (VI) exhibited a pair of double doublet signals of ABX type at δ 4.13 (1H, J=12, 7 Hz), 4.24 (1H, J=12, 3 Hz) and 4.12 (1H, J=12, 7 Hz), 4.23 (1H, J=12, 3 Hz), and showed the signals of two methylene protons adjacent to carbonyl of the hemisuccinyl and hemiglutaryl groups as a singlet at δ 2.45 (4H) and a multiplet at 2.44 (4H), respectively (Table I).

These hemi-esters, III and IV, were then condensed with *N*-hydroxysuccinimide by the use of ethyl dimethylaminopropylcarbodiimide hydrochloride (EDC) to give succinimidyl esters, VII and VIII. The succinimidyl esters VIII was coupled with bovine serum albumin (BSA) in phosphate buffer (pH 7.3) to afford PA-hemiglutarate-BSA conjugate (X) (immunogen), which was used for immunization after purification by dialysis. Twenty-one molecules of PA were incorporated per BSA molecule in the BSA conjugate (X) as judged by ultraviolet (UV) spectral analysis. The succinimidyl ester VII was coupled with β-galactosidase (β-Gal) to give PA-hemisuccinate-β-Gal conjugate (IX), which was used as a labeled antigen for EIA after purification on a Sepharose 6B column.

Antisera for PA were obtained from rabbits immunized with PA-hemiglutarate-BSA conjugate (X) in a manner similar to that described previously.[2] EIA for PA was performed by a competitive binding procedure with goat antiserum to rabbit IgG as the second antibody and the bound enzyme activity in the immune precipitate was determined fluorometrically with 7-β-D-galactopyranosyloxy-4-methylcoumarin as a substrate. A typical anti-PA antiserum dilution curve is shown in Fig. 1. The intensities of fluorescence caused by PA-β-Gal conjugate bound to antibody in the absence (open circle) and presence of 50 ng of PA (closed circle) were plotted against the dilution of anti-PA antiserum on a semilogarithmic scale. A dilution of 1 : 30000 of the anti-PA antiserum was chosen for EIA of PA because the ratio of enzyme activity in the absence and presence of PA at this dilution was maximum. A typical standard curve is shown in Fig. 2 and the insert shows the linearized logit-log plot using the same experimental data. The measurable range was 1—400 ng/ml. Cross-reactivity of constituents of Chinese Paeony root and related compounds with anti-PA antiserum was determined at 50% inhibition of the binding of enzyme-labeled PA. The anti-PA antiserum reacted with oxypaeoniflorin (0.31%) and albiflorin (0.22%), as shown in Table II. Standard curves, which were prepared by the addition of water, serum and urine to the assay mixture of



I: R=H
II: R=Ac

III: R=H, X=OH, n=2
IV: R=H, X=OH, n=3
V: R=Ac, X=OH, n=2
VI: R=Ac, X=OH, n=3
VII: R=H, X=succinimidyl, n=2
VIII: R=H, X=succinimidyl, n=3
IX: R=H, X=β-Gal---NH, n=2
X: R=H, X=BSA---NH, n=3

Chart 1

TABLE I. ^{1}H-NMR Data for PA Acetate (II), Hemisuccinyl (V) and Hemiglutaryl PA Acetate (VI) ($CDCl_3$)

Compd. No.	II	V	VI
Hydrogen			
8	4.54 (2H, ABq, J=12 Hz)	4.55 (2H, ABq, J=12 Hz)	4.55 (2H, ABq, J=12 Hz)
9	5.52 (1H, s)	5.56 (1H, s)	5.55 (1H, s)
10	1.34 (3H, s)	1.35 (3H, s)	1.36 (3H, s)
1′	4.76 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)
2′, 3′, 4′	4.95—5.20 (3H, m)	4.94—5.20 (3H, m)	4.94—5.20 (3H, m)
5′	3.63 (1H, br, m)	3.70 (1H, br, m)	3.66 (1H, br, m)
6′	4.14 (2H, d, J=7 Hz)	4.13 (1H, dd, J=12, 7 Hz) 4.24 (1H, dd, J=12, 3 Hz)	4.12 (1H, dd, J=12, 7 Hz) 4.23 (1H, dd, J=12, 3 Hz)
CH_3CO	1.98, 2.02, 2.03, 2.10, 2.10 (each s)	1.98, 2.04, 2.05, 2.09 (each s)	1.97, 2.03, 2.03, 2.09 (each s)
$-COCH_2-$		2.45 (4H, s)*	2.44 (4H, m)*

* These signals exhibited along with other signals.

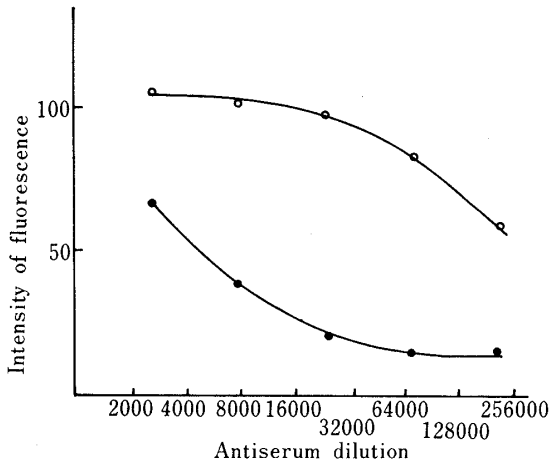


Fig. 1. Binding of the Enzyme-Labeled PA with Various Dilutions of Anti-PA Hemiglutarate-BSA Antiserum

In the presence of 50 ng of PA (—●—). In the absence of PA (—○—).

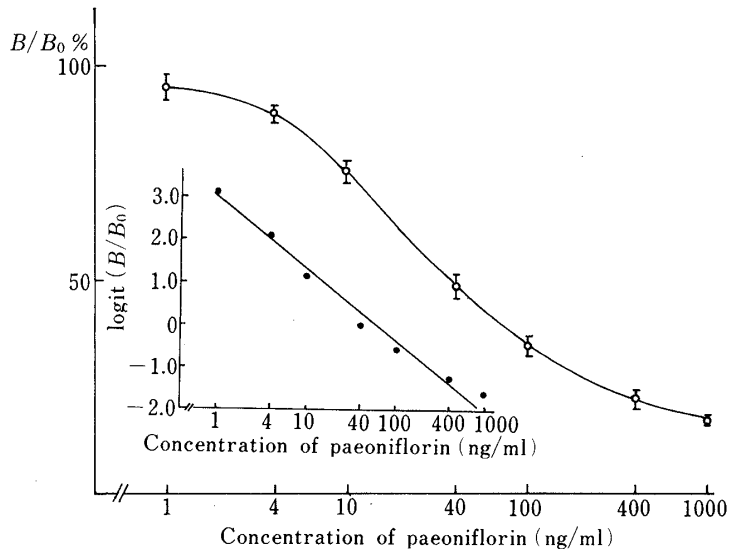


Fig. 2. Standard Curve for EIA of PA

Each point represents the mean ± S.D. of 8 replicate determinations and the insert shows the linearized logit-log plot using the same experimental data. B_0 = % binding in the absence of PA, B = % binding in the presence of PA. logit $(B/B_0) = \ln[B/B_0/(100 - B/B_0)]$.

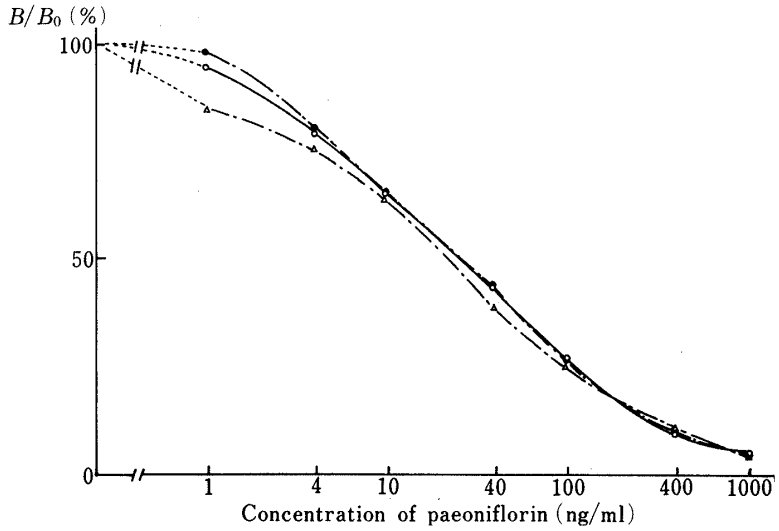
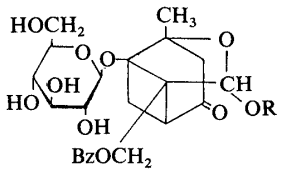


Fig. 3. Standard Curves for EIA of PA Measured with Samples Containing Water (—○—), Serum (---●---), and Urine (---△---)

TABLE II. Specificity of Anti-paeoniflorin Hemiglutarate-BSA Antiserum

Compound	Cross reaction (%)	Compound	Cross reaction (%)
Paeoniflorin	100	Paeoniflorin K_1 [a)]	0.003
Oxypaeoniflorin	0.31	Paeoniflorin K_2 [a)]	0.003
Albiflorin	0.22	Paeoniflorigenone	0.10
Desbenzoylpaeoniflorin	0.03	Glycyrrhizin	<0.001
Paeoniflorin tetraacetate	0.07	Glycyrrhetic acid	<0.001
Paeoniflorin pentaacetate	0.01		

a)



K_1: R=Me, K_2: R=Et

Cross-reaction ratio (%)=PA concentration required to induce 50% inhibition of anti-PA antiserum binding/sample concentration required to induce 50% inhibition of anti-PA antiserum binding.

EIA, are shown in Fig. 3. The addition of serum and urine interfered slightly with the EIA of PA.

Experimental

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and are uncorrected. Optical rotation was measured with a JASCO DIP-4 polarimeter. Spectra were obtained with the following machines: UV on a Beckman model 24, ^{1}H-NMR on Varian XL 200 and EM 390 spectrometers (solvent, $CDCl_3$; internal standard, tetramethylsilane; chemical shifts, δ (ppm); abbreviations are s, singlet; d, doublet; m, multiplet; q, quartet; br, broad). Fluorimetry was performed on a Shimadzu RF-503 recording spectrofluorophotometer. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel F_{254}, Merck) or 2 mm thick for preparative TLC, and detection was achieved by UV irradiation (254 nm) or by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 followed by heating. Buffer A: 0.02 M phosphate-buffered saline containing 0.1% BSA, 0.1% NaN_3, 0.001% $MgCl_2$.

Buffer B: 0.02 M phosphate-buffered saline containing 0.1% γ-globulin, 0.1% NaN_3, 0.001% $MgCl_2$.

Paeoniflorin (I) and Paeoniflorin Pentaacetate (II)——Crude PA (30 g) was isolated from methanol extract of commercial Chinese Paeony root (1 kg) according to Shibata's procedure[8,9] and was subjected to silica gel column chromatography with MeOH–$CHCl_3$ (1 : 9) to give PA (I) (*ca.* 12 g) as an amorphous powder. This product was identical with an authentic sample on the basis of TLC and IR (KBr) comparisons. I (200 mg) was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) overnight at room temperature. After usual work-up, the product was subjected to preparative TLC to give PA pentaacetate (II) (173 mg) as colorless needles, mp 161—163 °C. *Anal.* Calcd for $C_{32}H_{38}O_{16}$: C, 57.39; H, 5.51. Found: C, 57.40; H, 5.56.

6′-Hemisuccinyl Paeoniflorin (III)——A solution of I (400 mg, 0.84 mmol) and succinic anhydride (100 mg, 1 mmol) in pyridine (2 ml) was warmed on a water bath at 60—70 °C for 6 h. The reaction mixture was poured into cold 10% Na_2CO_3 (5 ml) and washed with AcOEt. The Na_2CO_3 solution was acidified with 1 N HCl and extracted with AcOEt. The AcOEt extract was washed with H_2O, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was subjected to preparative TLC with 20% MeOH–$CHCl_3$ as a developing solvent. The zone with *Rf* 0.3 gave 6′-hemisuccinyl PA (III) (256 mg, 52%) as an amorphous powder, $[\alpha]_D^{25}$ −16.0° ($c=1$, pyridine). III (60 mg) was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) overnight at room temperature. After usual work-up, the product was recrystallized from CH_2Cl_2–hexane to give hemisuccinyl PA tetraacetate (V) as colorless needles, mp 85 °C, $[\alpha]_D^{25}$ +4.3° ($c=1.8$, $CHCl_3$). *Anal.* Calcd for $C_{35}H_{40}O_{18}$: C, 56.15; H, 5.39. Found: C, 56.51; H, 5.59.

6′-Hemiglutaryl Paeoniflorin (IV)——A solution of I (400 mg, 0.84 mmol) and glutaric anhydride (114 mg, 1 mmol) in pyridine (2 ml) was worked up as described above to give 6′-hemiglutaryl PA (IV) as an amorphous powder (283 mg, 56%). IV (80 mg) was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) overnight at room temperature. After usual work-up, the product was recrystallized from CH_2Cl_2–hexane to give hemiglutaryl PA tetraacetate (IV) as colorless needles, mp 76—78 °C, $[\alpha]_D^{25}$ −4.6° ($c=1.2$, $CHCl_3$). *Anal.* Calcd for $C_{36}H_{42}O_{18}$: C, 56.69; H, 5.55. Found: C, 56.40; H, 5.77.

Succinimidyl Paeoniflorin Hemisuccinate (VII)——EDC (50 mg, 0.26 mmol) was added to a stirred mixture of III (100 mg, 0.17 mmol) and *N*-hydroxysuccinimide (30 mg, 0.26 mmol) in dioxane (2 ml) under ice cooling and the mixture was stirred for 20 h at room temperature. After addition of H_2O, the mixture was extracted with AcOEt (80 ml). The AcOEt extract was washed with H_2O, dried ($MgSO_4$) and concentrated *in vacuo* to give succinimidyl PA hemisuccinate (VII) (98 mg) as a semicrystalline material.

Succinimidyl Paeoniflorin Hemiglutarate (VIII)——EDC (87 mg, 0.46 mmol) was added to a stirred mixture of IV (180 mg, 0.3 mmol) and *N*-hydroxysuccinimide (53 mg, 0.46 mmol) in dioxane (3 ml) under ice cooling and the mixture was worked up as described above to give succinimidyl PA hemiglutarate (VIII) (194 mg) as a semicrystalline meterial.

Preparation of Paeoniflorin Hemiglutarate-BSA Conjugate (X)——A solution of BSA (33 mg, 0.5 μmol) in phosphate buffer (pH 7.3, 0.5 ml) was added to a solution of VIII (21 mg, 30 μmol) in pyridine (0.3 ml) under stirring at 5 °C. After being stirred for 24 h at the same temperature, the mixture was dialyzed successively for 5 d against 50, 25, 15 and 10% pyridine–H_2O and H_2O. The dialysate was further purified by gel filtration on a Sephadex G-25 column to exclude unbound hapten completely.

Determination of the Number of Paeoniflorin Molecules Linked to One BSA Molecule——UV spectrometric analysis was performed by comparing the absorbance at 275 nm of the PA hemiglutarate-BSA conjugate (X) with those of BSA and hemiglutaryl PA as controls in 0.05 M phosphate buffer (pH 7.3) and by using the following constants: molecular weight of BSA, 67000; ε value for BSA, 49900; that for hapten, 870. The protein content of the conjugate (X) was determined by the method of Lowry *et al.*[10] The number of hapten molecules coupled to one BSA molecule was determined to be 21.

Preparation of Antiserum to Paeoniflorin——The PA hemiglutarate-BSA conjugate (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with the same amount of complete Freund's adjuvant (Difco, Detroit, Mich., U.S.A.). The emulsion was injected into domestic albino female rabbits subcutaneously and intramuscularly at multiple sites on the back and legs. Half the initial dose of immunogen was used as a booster once two weeks for two months and monthly thereafter. The blood was collected from an ear vein 10 to 14 d after the last booster injection. The sera were separated by centrifugation at 3000 rpm for 15 min and were stored at −20 °C until use.

Preparation of Paeoniflorin-β-D-galactosidase Conjugate (IX)——VII (54 μg, 80×10^{-9} mol) [5.4 μl of a solution of VII (10 mg) in dioxane (1 ml) was pipetted off] was added to a solution of β-galactosidase (2 mg, 4×10^{-9} mol) in 0.05 M phosphate buffer (pH 7.3, 0.4 ml) and the mixture was stirred at 0 °C overnight. The reaction mixture was directly chromatographed on a Sepharose 6B column (1.5 × 30 cm) using buffer A as the eluent. The fractions of eluate containing the peak of enzyme activity were collected and pooled at 4 °C until use. The amount of enzyme conjugate was expressed as units of enzyme activity, one unit of enzyme activity being defined as the amount that hydrolyzes 1 μmol of 7-β-D-galactopyranosyloxy-4-methylcoumarin per min.

Antiserum Dilution Curve——Unless otherwise stated, dilution was carried out with buffer B. A mixture of 20 μ units of 2000-fold diluted PA-β-Gal conjugate (50 μl) and 100 μl of 2000-fold or more diluted anti-PA-antiserum was incubated at room temperature for 2 h. Ten-fold diluted goat antiserum to rabbit IgG (50 μl) and 100-fold diluted normal rabbit serum (20 μl) were added to the incubation mixture, and the mixture was allowed to stand at 4 °C

overnight. After addition of buffer A (1 ml), the resulting mixture was centrifuged at 2000 rpm, for 20 min. The supernatant was removed, and the immune precipitate was washed with buffer A (1 ml) and recentrifuged. The precipitate was used for measurement of enzyme activity. At the same time, the procedure with addition of PA (50 ng, 100 μl) was performed to provide competitive reaction PA and labeled PA. (Fig. 1.)

Assay Procedure——Sample or standard solution of PA (100 μl) was added to 30000-fold diluted anti-PA antiserum (100 μl) and PA-β-Gal conjugate (50 μl). The mixture was incubated at room temperature for 2 h and then 100-fold diluted normal rabbit serum (20 μl) and 10-fold diluted goat antiserum to rabbit IgG (50 μl) were added. After further incubation at 4 °C overnight, the resulting immune precipitate was treated according to the same procedure as described above.

Measurement of β-D-Galactosidase Activity——The immune precipitate was incubated with 1×10^{-4} M 7-β-D-galactopyranosyloxy-4-methylcoumarin (150 μl) at 30 °C for 30 min. After incubation, 2 ml of 0.1 M glycine–NaOH buffer (pH 10.3) was added to the reaction mixture, and the fluorescence intensity of 7-hydroxy-4-methylcoumarin formed was measured (364 and 448 nm for excitation and emission, respectively).

Influence of Serum and Urine on EIA of Paeoniflorin——An aliquot of 50% serum (100 μl) or 50% urine (100 μl) in H_2O was added to various amounts of PA (0.1—100 ng/100 μl) solution (100 μl) and each PA sample was assayed by the procedure described above (Fig. 3).

Specificity of the Anti-PA Antiserum——The cross-reactions of eleven kinds of PA-related compounds with anti-PA antiserum were examined by using PA hemisuccinate-β-Gal conjugate according to the assay procedure described above (Table II).

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References and Notes

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