CHAPTER III

METHODS

3.1 Preparation of Laboratory Reagents

3.1.1 Bacterial Culture Medium

The medium used for bacterial culture was LB broth (Luria, Adam and Teng, 1960). Tryptone (10 g), yeast extract (5 g) and sodium chloride (10 g) were dissolved in a portion of distilled water (about 800 ml), and the pH of the medium was adjusted to pH 7.0 with sodium hydroxide (6 mol/l). The medium was then adjused to 1 l with distilled water and autoclaved (120^{o} C, 15 psi, 15 minutes).

3.1.2 Reagents for Protein Determination by Lowry Method (Lowry, Rosenbrough, Farr and Randell, 1951)

3.1.2.1 Reagent A

Potassium sodium tartrate (2.000 g) was dissolved in water (100 ml).

3.1.2.2 Reagent B

$CuSO_4 \cdot 5H_2O$ (1.000 g) was dissolved in water (100 ml).

3.1.2.3 Reagent C

Sodium carbonate (2.000 g) was dissolved in sodium hydroxide (0.1 mol/l, 100 ml).

3.1.2.4 Reagent D

Reagent A (1 ml), reagent B (1 ml) and reagent C (100 ml) were mixed. The mixture was discarded after one day.

3.1.2.5 Reagent E

The Folin-Ciocalteu phenol reagent (2 mol/l) was diluted twofold with water.

3.1.3 Reagents for Protein Determination by Coomassie Brilliant Blue G-250 Binding Method (Bearden, 1978)

Commassie brilliant blue G-250 (100.0 mg) was dissolved in phosphoric acid (85% W/V, 100 ml). The resulting solution was diluted fivefold with distilled water. The solution was then filtered to remove undissolved solids and stored at room temperature.

3.1.4 Reagents for Disc Polyacrylamide Gel Electrophoresis (Davis, 1964; Weber and Osborn, 1969)

3.1.4.1 Reagent A

Tris (hydroxymethyl) aminomethane (36.6 g) and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (0.23 ml) were dissolved in a portion of distilled water (about 70 ml). The resulting solution was then adjust to pH 8.9 with concentrated hydrochloric acid, and the volume was adjusted to 100 ml with distilled water.

3.1.4.2 Reagent B

Tris (hydroxymethyl) aminomethane (5.98 g) and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (0.46 ml) were dissolved in a

portion of distilled water (about 70 ml). The resulting solution was then adjusted to pH 6.7 with concentrated hydrochloric acid, and the volume was adjusted to 100 ml with distilled water.

3.1.4.3 Reagent C

Acrylamide (28.0 g) and N,N'-methylenebisacrylamide (BIS) (0.7350 g) were dissolved in distilled water (100 ml).

3.1.4.4 Reagent D

Acrylamide (10.0 g) and N,N'-methylenebisacrylamide (BIS) (2.50 g) were dissolved in distilled water (100 ml).

3.1.4.5 Reagent E

Riboflavin (4.0 mg) was dissolved in water (100 ml).

3.1.4.6 Reagent F

Sucrose (40 g) was dissolved in distilled water (100 ml).

Reagents A-F were filtered and stored in brown glass bottles. The bottles were kept in a refrigerator, and the reagents were stable several months.

3.1.4.7 Reagent G

Ammonium persulfate (0.1400 g) was dissolved in water (100 ml). This reagent was stored in a refrigerator and used within seven days after preparation.

3.1.4.8 Stock Buffer Solution for the Reservoirs

Tris (hydroxymethyl) aminomethane (6.00 g) and glycine (28.8 g) were dissolved in distilled water (1 l). The pH of the solution was pH 8.3.

3.1.4.9 Fixative-Stain Solution

Coomassie brilliant blue (1.25 g) was dissolved in methanol (50%, 454 ml) and acetic acid (46 ml). The resulting solution was filtered and stored at room temperature.

3.1.4.10 Destaining Solution

Acetic acid (75 ml) and methanol (50 ml) were dissolved in distilled water. The final volume was adjusted to 1 l with distilled water.

3.1.5 Scintillation Fluid (Foye et al, 1982)

Naphthalene (150 g), PPO (8.00 g), dimethyl POPOP (0.600 g), ethylene glycol (20 ml) and ethoxyethanol (100 ml) were dissolved in toluene. The final volume was adjusted to 1 l with toluene.

3.1.6 Reagents for Colour Test of Amines (Cautrecasas, 1970)

3.1.6.1 Saturated Sodium Borate Solution

The solution of saturated boric acid in water was adjusted to pH 7.0 with sodium hydroxide solution (6 mol/l) and filtered.

3.1.6.2 2,4,6-Trinitrobenzenesulfonate (3%)

2,4,6-Trinitrobenzenesulfonate (3.00 g) was dissolved in water (100 ml).

3.1.7 Preparation of 2-Amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine pyrophosphate (DHPP)

The following method was modified from the method of Friedkin et al. (Friedkin et al. 1962).

2-Amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate (20 mg), sodium dithionite (20 mg) and 2-mercaptoethanol (1 mol/l, 1 ml) were mixed vigorously in a test tube. The tube was closed with paraffilm and left at room temperature for 30 minutes. The tube was then centrifuged at 2,000 xg for 15 minutes. The supernatant was collected and kept frozen at -20^{o} C. The amount of DHPP was measured by the ultraviolet absorption at 330 nm (pH 7.10). A molar extinction coefficient of 6,200 $(mol/l)^{-1}$ $(cm)^{-1}$ (Shiota, Baugh, Jackson and Dillard, 1969) was used for calculation of the concentration of DHPP.

3.1.8 Buffers for Sulfonamide-Sepharose Column Chromatography

3.1.8.1 Buffer I

The solution was made to contain the following components in a 1 l (pH 8.5) volume :(DHPP 9.4 µmol/l), dithiothreitol (0.1543 g, 1 mmol/l), $MgCl_2 \cdot 6\ H_2O$ (2.1686 g, equivalent to 5 mmol/l magnesium chloride), tris (hydroxymethyl) aminomethane (24.228 g, 0.2 mol/l) and distilled water. The pH of the solution was adjusted to pH 8.5 with concentrated hydrochloric acid.

3.1.8.2 Buffer II

Tris (hydroxymethyl) aminomethane (24.228 g, 0.2 mol/l) and sodium chloride (29.22 g, 0.5 mol/l) were dissolved in a portion of distilled water (about 800 ml). The pH of the solution was adjusted to pH 8.5 with concentrated hydrochloric acid, and the volume was adjusted to 1 l with distilled water.

3.1.8.3 Buffer III

Tris (hydroxymethyl) aminomethane (24.228 g, 0.2 mol/l) was dissolved in a portion of distilled water (about 800 ml). The pH

of the solution was adjusted to pH 8.5 with concentrated hydrochloric acid, and the volume was adjusted to 1 l with distilled water.

3.1.8.4 Buffer IV

Tris (hydroxymethyl) aminomethane (24.228 g, 0.2 mol/l) and sodium chloride (58.44 g, 1 mol/l) were dissolved in a portion of distilled water (about 800 ml). The pH of the solution was adjusted to pH 8.5 with concentrated hydrochloric acid, and the volume was adjusted to 1 l with distilled water.

3.1.9 Enzyme Modifying Agents

3.1.9.1 Iodoacetamide (856.25 mmol/l)

Iodoacetamide (0.3168 g) was dissolved in Tris-hydrochloride buffer (1 mol/l, pH 8.55), and the final volume was adjusted to 2 ml with Tris buffer.

3.1.9.2 p-Chloromercuribenzoic Acid (PMB) (53.28 mmol/l

PMB (0.0380 g) was dissolved in Tris-hydrochloride buffer (1 mol/l, pH 8.55), and the final volume was adjusted to 2 ml with Tris buffer.

3.1.9.3 Phenylglyoxal (116.23 mmol/l)

Phenylglyoxal hydrate (0.0370 g) was dissolved in N-ethylmorpholine acetate buffer (1 mol/l, pH 8.0), and the final volume was adjusted to 2 ml with N-ethylmorpholine acetate buffer.

3.1.9.4 2,4-Pentanedione (971.3 mmol/l)

2,4-Pentanedione (1 ml) was dissolved in sodium phosphate buffer (0.2 mol/l, pH 7.0, 10 ml).

3.1.9.5 2,3-Butanedione (1.08 mol/l)

2,3-Butanedione (0.94 ml) was dissolved in Hepes buffer (1 mol/l, pH 8.2), and the final volume was adjusted to 10 ml with Hepes buffer.

3.1.9.6 Phenylmethylsulfonylfluoride (PMSF) (9 mmol/l)

The isopropanol solution was prepared by dissolving 3 ml of iso-propanol in about 4 ml of distilled water. PMSF (0.0155 g) was dissolved in this iso-propanol solution. The pH of the resulting solution was adjusted to pH 8.55, and the volume was adjusted to 10 ml with distilled water.

3.2 Protein Determination by Lowry Method (Lowry et al. 1951)

Sample (0.1 ml) was dissolved in reagent D (section 3.1.2.4, 3.0 ml), and the resulting solution was left at room temperature for 10 minutes. Reagent E (section 3.1.2.5, 0.3 ml) was added into the solution and mixed immediately. The solution was then left for a furthur 30 minutes at room temperature. The optical density of the solution at 650 nm was measured. The protein concentration was read from the standard curve in which bovine serum albumin was used as the standard protein.

3.3 Protein Determination by Coomassie Brilliant Blue G-250 Binding Method (Bearden, 1978)

Sample (1.5 ml) was dissolved in the coomassie reagent (section 3.1.3, 1.5 ml). The resulting solution was left for 2 minutes, and the optical density was measured at 595 nm. The protein concentration

was read from the standard curve in which bovine serum albumin was used as the standard protein.

3.4 Colour Test of Amines (Cautrecasas, 1970)

A small amount of sample was added into a test tube containing a saturated solution of sodium borate (section 3.1.6.1, 1 ml). The drops of 2,4,6-trinitrobenzenesulfonate reagent (3%) (section 3.1.6.2) was added to the mixture. The colour change was observed after the mixture was left for 30 minutes at room temperature. The colour changing from yellow to red indicates the presence of amines.

3.5 E. coli Culture

E. coli K12 No.3110 was cultured in LB broth (section 3.1.1) in the fermenter at 37^{o} C. The agitation rate was 400 rpm, and the aeration rate was 0.3 kg/cm^{2}. The cell was harvested at late log phase, centrifuged and kept frozen at -70^{o} C.

3.6 Preparation of Affinity Chromatography Gel (Sulfonamide-Sepharose Gel)

Agarose in the form of the beaded derivative (Sepharose 4B) was activated with 2,4-dichloro-6-methoxy-s-triazine and coupled to the spacer arm [bis-(3-aminopropyl) amine]. The sulfonamide, 4-(4-aminobenzenesulfonamido) benzenesulfonylglycine, was condensed with the coupled Sephasose 4B by 1-cyclohexyl-3-(2-morpholinoethyl) carbodi-imide methotoluene-p-sulphonate in aqueous dimethylformamide. The detail procedures were described in sections 3.6.1 - 3.6.4.

3.6.3 Preparation of 4-[3-(3-Aminopropyl) aminopropylamino]-6-methoxy-s-triazine-2-ylagarose (Lang et al. 1977)

Bis-(3-aminopropyl) amine (13.2 ml, 0.09 mol) was dissolved in a portion of distilled water (about 120 ml). The solution was adjusted to pH 8.5 with concentrated hydrochloric acid, and the volume was adjusted to 187 ml with distilled water. The 4-chlorotriazinylagarose (84 g) was then added into this solution. The resulting suspension was stirred for 24 hours at room temperature. The triazinylagarose was collected and washed on a buchner funnel with distilled water (2.33 l), a mixture solution of an equal volume of hydrochloric acid (0.01 mol/l) and sodium chloride (0.1 mol/l) (467 ml), distilled water (467 ml), a mixture solution of an equal volume of sodium hydroxide (0.01 mol/l) and sodium chloride (0.1 mol/l) (467 ml), and sodium chloride (0.1 mol/l, 3.73 l), respectively. The triazinylagarose was finally washed with distilled water (2.33 l). The filtrate was collected and examined for the presence of amines by the method described in section 3.5. The red colour was not occurred in the reaction mixture; therefore, the excess spacer arm (bis-(3-aminopropyl) amine) was washed out from the gel. The gel particles also examined for the presence of amines, and the red colour was observed at the gel particles, hence, the spacer arm was coupled to the activated gel to some extent.

3.6.4 Reaction of the Triazinylagarose with 4-(4-Aminobenzenesulfonamido) benzenesulfonylglycine (Lang et al. 1977)

The solution containing 4-(4-aminobenzenesulfonamido) benzenesulfonylglycine (1.23 g), triazinylagarose (80 ml settled

volume) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodi-imide methotoluene-p-sulfonate (2.91 g) was shaken gently for 48 hours at room temperature in aqueous dimethylformamide (50%, 291 ml). The resulting suspension was filtered, and the product gel was washed with distilled water (2.91 l), aqueous dimethylformamide (50%, 2.91 l) methanol (2.91 l), and distilled water (1.45 l) respectively.

A small portion of washed gel was suspened in aqueous dimethylformamide (50%, 1 ml). The suspension was stirred for 3 minutes and centrifuged. The supernatant was collected and examined for the presence of amines (section 3.5). The red colour was not formed in the reaction mixture; therefore, the excess sulfonamide was washed out from the gel. The washed gel was also examined for the presence of amines, and the red colour was observed at the gel particles.

A small portion of the coupled gel (15 ml settled volume) was washed with methanol and sucked dry. The gel was kept in the oven (60^{o} C) overnight and sent to the Department of Science Service, Ministry of Science, Technology and Energy for sulfur analysis by the general bomb method (Priemon, 1983). The analysis indicated the presence of 435.44 µg of sulfur (equivalent to 6.79 µmol of the sulfonamide)/ml of settled gel. This value was almost agreed with 4.5 ± 0.8 µmol of sulfonamide/ml of settled gel reported by the other investigator (Lang et al. 1977).

3.7 Partial Purification of Dihydropteroate Synthase

3.7.1 Preparation of the Crude Cell-Free Extract

E. coli cells (20 g) were suspended in Tris buffer (0.05 mol/l, pH 8.0, 40 ml) and centrifuged (11,400 xg, 4^{o} C, 10 minutes) to wash out the media. The washed cells were suspended in the same buffer (40 ml) and sonicated three times, (three minutes at a time, 40% duty cycle) to break cells. The suspension was then centrifuged (18,000 xg, 4^{o} C) for 50 minutes, and the supernatant (46 ml) was collected.

3.7.2 Preparation of 30-70% Ammonium Sulfate Saturation Fraction

The solid ammonium sulfate (7.38 g) was added to the cold cell-free extract (45 ml) slowly with gentle stirring. The ammonium sulfate in this solution was 30% ammonium sulfate saturation. The solution was centrifuged (18,000 xg, 4^{o} C) for 50 minutes. The supernatant (47 ml) was collected and ammonium sulfate (11.703 g) was added to bring the solution to 70% ammonium sulfate suturation. The solution was centrifuged (18,000 xg, 4^{o} C) for 60 minutes, and the precipitate was collected. The precipitate was suspended in Tris buffer (0.2 mol/l, pH 8.5, 15 ml), and the mixture was dialysed in the same buffer (3 l) for 8 hours. The dialysing process was repeated twice. The volume of the solution was found to be 31 ml after dialysis.

3.7.3 The Enzyme Purification by Sulfonamide-Sepharose Column (I)

The following method was modified from the method of Suckling et al. (Suckling et al. 1977).

The sulfonamide-Sepharose gel prepared by the method described in section 3.6 was added into a column (2.5 x 37 cm) until the gel height was 28 cm. The column was equilibrated with buffer I (section 3.1.8.1) with the flow rate of 21 ml/hour for 24 hours. The enzyme solution (21 ml) from 30-70% ammonium sulfate saturation fraction was diluted with buffer I (51.5 ml) until the protein concentration was 6.25 mg/ml. After the diluted enzyme solution was loaded, buffer I was applied into the column. The collected fractions (11 ml/fraction) were measured for the optical density at 280 nm. When the optical density were near zero, buffer II (section 3.1.8.2) was applied into the column and a further 132 fractions (7 ml/fraction) were collected. These fractions were assayed for the enzyme activity, and the protein concentrations were also measured by Lowry's method described in section 3.2. The fractions containing the enzyme activity (fractions No. 159-215) were pooled and concentrated by diaflo ultrafiltration (PM 10 membrane). The concentrated solution was dialysed in Tris buffer (0.2 mol/l, pH 8.5, 3 l) for 8 hours, and the dialysing process was repeated twice.

3.7.4 The Enzyme Purification by Sulfonamide-Sepharose Column (II)

The sulfonamide-Sepharose gel prepared by the method described in section 3.6 was added into a column (1.5 x 30 cm) until the gel height was 25.5 cm. The column was equilibrated with buffer III (section 3.1.8.3) with flow rate of 21.5 ml/hour for 12 hours. The enzyme solution (18 ml) obtained from sulfonamide-Sepharose column (I) was loaded and a linear gradient mixture of buffer III (section 3.1.8.3, 250 ml) and buffer IV (section 3.1.8.4, 250 ml) was applied into the

column. The fractions (2.5 ml/fraction) collected were assayed for the enzyme activity, and the protein concentrations were also measured by the coomassie method described in section 3.3. The fractions containing the enzyme activity (fractions No. 34-54) were pooled and concentrated by diaflo ultrafiltration (PM 10 membrane). The concentrated solution was dialysed in Tris buffer (0.2 mol/l, pH 8.5, 1 l) for 8 hours, and the dialysing process was repeated twice. The solution was then kept frozen at -70° C.

3.7.5 Methods for Regenerating Used Sulfonamide-Sepharose Gel

After the gel was used, the denatured protein on sulfonamide-Sepharose gel was removed by washing the gel on a buchner funnel with a mixture solution of urea (6 mol/l) and potassium chloride (2 mol/l) (Work and Work, 1979).

3.8 Disc Polyacrylamide Gel Electrophoresis for the Protein Solution from All Enzyme Purification Steps

3.8.1 Preparation of Disc Polyacrylamide Gels

Acrylamide gel (7%) was made to contain the total 8 parts of the following components : reagent A (section 3.1.4.1, 1 part), reagent C (section 3.1.4.3, 2 parts) distilled water (1 part), and reagent G (section 3.1.4.7, 4 parts). The solution was pipetted into the 0.5 x 11 cm tubes of which the bottom ends were sealed with paraffilm until the height of the solutions were 9 cm. A water layer were placed on top of the gel solutions. The tubes were then left at room temperature. After the gel was complete polymerization, water was removed.

The stacking gel was made to contain the total 8 parts of the following components : reagent B (section 3.1.4.2, 1 part), reagent D (section 3.1.4.4, 2 parts), reagent E (section 3.1.4.5, 1 part) and reagent F (section 3.1.4.6, 4 parts). The stacking gel solution was placed on top of previously prepared gels for a further 0.7 cm height, and the distilled water was then placed on top of the stacking gel solution. The resulting gels were left under the fluorescent light for polymerization. After the polymerization was completed, water was removed. The gels were ready to use.

3.8.2 Preparation of the Protein Solutions

The protein sample from crude cell-free extract (300 µg proteins, 10 µl) was used in the electrophoretic experiment.

The protein sample from 30-70% ammonium sulfate saturation fraction (216 µg proteins, 10 µl) was used in the electrophoretic experiment.

The protein sample from sulfonamide-Sepharose column I (1.3 ml) was concentrated in a dialysis bag by Aquacide II-A until the volume was reduced to 87 µl. The aliquot of 50 µl (105 µg proteins) was used in the electrophoretic experiment.

The protein sample from sulfonamide-Sepharose column II (0.9 ml) was concentrated in a dialysis bag by Aquacide II-A until the volume was reduced to 52 µl. This concentrated solution (54 µg proteins, 52 µl) was used in the electrophoretic experiment.

All protein samples were mixed with equal volumes of sucrose (80%) before use.

3.8.3 Electrophoresis

The gel tubes were placed into the upper reservior, and this reservior was filled with the stock buffer solution (section 3.1.4.8) diluted to 1/10 strength with distilled water. Bromphenol blue in distilled water (0.01%, about 0.6 ml) was stirred into the upper buffer. The upper buffer reservoir was then lowered so that the bottoms of the gel tubes were immersed in the same diluted stock buffer in the lower reservior. The protein samples were loaded in the gel tubes, and the current (3 mAmp/tube) was applied through the gels. The current was shut off when the dye had migrated to about 8.7 cm from the top of stacking gel.

3.8.4 Gel Staining

The gels were removed from the tubes and placed in the fixative-stain solution (section 3.1.4.9) for 1 hour. The gels were then destained with the destaining solution (section 3.1.4.10) until the background was clear, and the gels were kept in the destaining solution.

3.9 Measurement of Dihydropteroate Synthase Activity.

The assay mixtures was composed of the following components : Tris buffer (0.4 mol/l, pH 8.5), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.8 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The assay mixture was preincubated at 37^{o} C for 10 minutes. The enzyme solution was then added and the final volume of the mixture was 200 µl. The resulting mixture was incubated at 37^{o} C for 15 minutes. The reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). An aliquot of

100 µl (divided into two equal portions) was transferred by precalibrated micro-hematocrit capillary tube and spotted on a Whatman 3MM chromatographic paper. The chromatogram was developed in a descending fashion by immersing the lower margin of the paper in potassium phosphate buffer (0.1 mol/l, pH 7.0) inside a chamber at room temperature. After the buffer had moved 15 cm from the origin, the paper was removed from the chamber and dried. The product, DHP, was still located at the origin whereas unreacted ^{14}C-PABA migrated with an Rf value of 0.78 (Richey and Brown, 1969) or 0.76 (Iwai and Okinaka, 1980). The area around the origin (2 x 2 cm) was cut into small pieces and put into the vial containing scintillation fluid (section 3.2.4, 5 ml). The amount of radioactive substance was determined by liquid scintillation counter. The amount of DHP was calculated from the specific activity of [7-^{14}C]-PABA which was equal to 12,870 dpm/nmol. One enzyme unit was defined as the amount of the enzyme that catalyzes the formation of 1.0 nmol of DHP per minute under the experimental conditions. The efficiency of counting was about 86%. The counting time was 10 minutes. At a confidence level of 95%, the percentage of the proportional errer is about 2%.

3.10 The Methods of the Modification of Dihydropteroate Synthase by the Modifying Agents

3.10.1 The Enzyme Modification

The modifying agent and the enzyme were allowed to react for a period of time. The control experiment was that only the enzyme was incubated for the same period of time. If the enzyme activities obtained from these two conditions are not definitely different, it may be interpreted that the enzyme was not modified by the modifying agent.

3.10.1.1 Modification of Dihydropteroate Synthase by Iodoacetamide

The solutions in three tubes were made to contain iodoacetamide, Tris buffer (0.4 mol/l, pH 8.55) and the enzyme (0.05 units) in a total volume of 55 µl. Each tube contains different concentration of iodoacetamide. The concentration of iodoacetamide were 68.5, 6.85 and 0.137 mmol/l. The tubes were allowed to incubate at 25° C for 1 hour. Aliquots of 50 µl were then transferred into the assay mixtures (150 µl) preincubated at 37° C, and the final volume of 200 µl was obtained. The components in the three resulting mixtures (200 µl) were iodoacetamide (17.12, 1.71, and 0.034 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.8 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). The mixtures were then incubated for 15 minutes at 37° C, and the enzyme activity was measured. The enzyme reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol), and the enzyme activity was assayed by the method described in section 3.9.

The control experiment was done in the same manner as described above except that iodoacetamide was not present in the reaction mixture.

3.10.1.2 Modification of Dihydropteroate Synthase by PMB

The experiment was done in the same manner as the experiment of modification of enzyme by iodoacetamide (section 3.10.1.1) except that the concentrations of PMB in the incubation mixtures (55 µl) were 426.24, 42.62 and 0.852 µmol/l.

3.10.1.3 Modification of Dihydropteroate Synthase by 2,4-Pentanedione

The experiment was done in the same manner as the experiment of modification of enzyme by iodoacetamide (section 3.10.1.1) except that the concentrations of 2,4-pentanedione, the type of buffer, the incubation temperature and the incubation time were different. The concentrations of 2,4-pentanedione in the incubation mixtures (55 µl) were 77.70, 38.85 and 7.77 mmol/l. The buffer in the incubation mixture (55 µl) was sodium phosphate buffer (0.2 mol/l, pH 7.0), and the buffer in the assay mixtures (200 µl) was also sodium phosphate buffer (0.4 mol/l, pH 8.0). The incubation mixtures (55 µl) were incubated at 21°C for 24 hours.

3.10.1.4 Modification of Dihydropteroate Synthase by 2,3-Butanedione

The experiment was done in the same manner as the experiment of modification of enzyme by iodoacetamide (section 3.10.1.1) except that the concentration of 2,3-butanedione and the type of buffer were different. The concentration of 2,3-butanedione in the incubation mixture (55 µl) was 86.4 mmol/l. The buffer in the incubation mixtures (55 µl) was Hepes buffer (0.24 mol/l, pH 8.2), and the buffer in the assay mixtures (200 µl) was also Hepes buffer (0.4 mol/l, pH 8.2).

3.10.1.5 Modification of Dihydropteroate Synthase by Phenylglyoxal

The experiment was done in the same manner as the experiment of modification of enzyme by iodoacetamide (section 3.10.1.1) except that the concentration of phenylglyoxal and the type of buffer

were different. The concentration of phenylglyoxal in the incubation mixture (55 µl) was 29.59 mmol/l. The buffer in the incubation mixtures (55 µl) was N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), and the buffer in the assay mixtures (200 µl) was also N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0).

3.10.1.6 Modification of Dihydropteroate Synthase by PMSF

The experiment was done in the same manner as the experiment of modification of enzyme by iodoacetamide (section 3.10.1.1) except that the concentrations of PMSF in the incubation mixture (55µl) were 720, 72 and 1.44 µmol/l.

3.10.2 Investigation of the Possible Reaction Between the Modifying Agents (M) and the Substrates (S)

In the enzyme modification experiment, an aliquot of the incubation mixture was transferred directly to the assay mixture without prior separation of that modifying agent after the modification reaction was terminated; hence, it is possible that the reaction between the modifying agents and the substrates (DHPP and PABA) may occur.

The modifying agent was allowed to incubate with the substrate for 15 minutes. The enzyme (E) was then added and assayed for the activity by the method described in the section 3.9. The control experiment was that only substrates were incubated for 15 minutes. After incubation, the enzyme and the modifying agent were then added. The enzyme activity was assayed as described in the section 3.9. If the enzyme activities obtained from these two conditions are not

3.6.1 Preparation of 2,4-Dichloro-6-methoxy-s-triazine (Dudley, Thurston, Schaefer, Hansen, Hull and Adams, 1951)

Sodium bicarbonate (36.6 g, 0.4 mol) and trichloro-s-triazine (36.8 g, 0.2 mol) were added into the solution of methanol (200 ml) and water (25 ml). This mixture was stirred at 30° C for 30 minutes until the evolution of carbon dioxide had nearly ceased. Water was then added into the mixture. The crystalline solid separated was filtered, washed with water, and dried in a vacuum desiccator. The yield of crude 2,4-dichloro-6-methoxy-s-triazine was 17.5 g, mp 85-88° C. Literature yield : 10.5 g, mp : 87-89° C (Dudley et al. 1951). After the product was recrystallized from heptane, the melting point of the recrystallized compound was 87-88° C. Literature mp is 88-90° C (Dudley et al. 1951).

3.6.2 Preparation of 4-Chloro-6-methoxy-s-triazine-2-ylagarose (Lang et al. 1977)

Dioxane-washed agarose (100 g) was added into a solution of 2,4-dichloro-6-methoxy-s-triazine (4.50 g, 0.025 mol) in dioxane (200 ml), and the suspension was stirred for 15 minutes at room temperature. Distilled water (200 ml) and sodium carbonate (2 mol/l, 20 ml) was added into the resulting suspension respectively. The temperature of the suspension was then kept to 25-30° C, and sodium hydroxide (1 mol/l, 40 ml) was added dropwise slowly. After the mixture was stirred for a furthur 20 minutes, it was acidified to pH 6 with hydrochloric acid (1 mol/l). The triazinylagarose was obtained by filtration. The product was then washed with dioxane (1 l), sodium chloride (0.1 mol/l, 5 l) and distilled water (2.5 l) respectively.

definitely different, it may be interpreted that the reaction between the modifying agent and the substrates is not occured within 15 minutes. The simplified diagram was as following :

$$M + S \xrightarrow[15\ \text{minutes}]{\text{Incubation}} + E \Longrightarrow \text{Assay for the enzyme activity}$$

$$\text{Control tube ; } S \xrightarrow[15\ \text{minutes}]{\text{Incubation}} + E + M \Longrightarrow \text{Assay for the enzyme activity}$$

For the investigation of the possible reaction between the substrates and iodoacetamide, the solution was made to contain iodoacetamide (17.12 mmol/l), Tris-hydrochloride buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercapto-ethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l) in total volume of 191 µl. After the mixture was incubated at 37^{o}C for 15 minutes, the enzyme solution (9 µl, 0.045 enzyme units) was then added into the mixture. The concentration of each component was calculated from the final volume of 200 µl including the volume of the enzyme solution. The reaction mixture was further incubated at 37^{o} C for 15 minutes, and the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l) in a total volume of 187 µl. The concentration of each component was calculated from the final volume of 200 µl including the volume of iodoacetamide and the enzyme solution. After the tube was allowed to

incubate at 37° C for 15 minutes, iodoacetamide (17.12 mmol/l) and the enzyme (9 µl, 0.045 units) were then added, and a total volume of 200 µl was obtained. The reaction mixture was further incubated at 37° C for 15 minutes, and the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The modification procedures for the other four modifying agents (PMB, 2,4-pentanedione, 2,3-butanedione and phenylglyoxal) were the same as the procedure described for iodoacetamide except that the concentrations of the modifying agents and the types of buffers were different. The concentrations of PMB, 2,4-pentanedione, 2,3-butanedione and phenylglyoxal were 106.56 µmol/l, 19.43 mmol/l, 21.60 mmol/l and 7.40 mmol/l respectively. The buffer in the modification procedure for PMB, 2,4-pentanedione, 2,3-butanedione and phenylglyoxal were Tris buffer (0.4 mol/l, pH 8.55), sodium phosphate buffer (0.4 mol/l, pH 8.0), Hepes buffer (0.4 mol/l, pH 8.2) and N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0) respectively.

3.10.3 Investigation of the Possible Reaction Between the Modifying Agents and Sulfanilamide

In the protection experiment of the enzyme described in section 3.10.6, sulfanilamide was used as an inhibitor for the protection of the enzyme from the modifying agents; hence, it is possible that the reaction between the modifying agents and sulfanilamide may occur.

The simplified diagram for the procedure was the following

$$M + I \xrightarrow{\text{Incubation}} + S + E \Longrightarrow \text{Assay for the enzyme activity}$$

Control tube; $I \xrightarrow{\text{Incubation}} + S + E + M \Longrightarrow$ Assay for the enzyme activity

I = Sulfanilamide

If the enzyme activities obtained from these two conditions are not definitely different, it may be interpreted that the reaction between the modifying agent and the inhibitor (sulfanilamide) is not occurred within the incubation time.

3.10.3.1 Investigation of the Possible Reaction Between Iodoacetamide and Sulfanilamide

For the investigation of the possible reaction between iodoacetamide and sulfanilamide, the solution was made to contain iodoacetamide (68.5 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an alquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9

The control tube containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l), ^{14}C-PABA (328.12 µmol/l) and iodoacetamide (17.12 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.3.2 Investigation of the Possible Reaction Between PMB and Sulfanilamide

For the investigation of the possible reaction between PMB and sulfanilamide, the solution was made to contain PMB (426.24 µmol/I), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8,55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay

mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l), ^{14}C-PABA (328.12 µmol/l) and PMB (106.56 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzyme reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.3.3 Investigation of the Possible Reaction Between 2,4-Pentanedione and Sulfanilamide

For the investigation of the possible reaction between 2,4-pentanedione and sulfanilamide, the solution was made to contain 2,4-pentanedione (77.70 mmol/l), sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl. After the solution was incubated for 24 hours at 21° C, an aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing sodium phosphate

buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl was incubated for 24 hours at 21^{o} C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37^{o} C) containing sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l), ^{14}C-PABA (328.12 µmol/l) and 2,4-pentanedione (19.43 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.3.4 Investigation of the Possible Reaction Between 2,3-Butanedione and Sulfanilamide

For the investigation of the possible reaction between 2,3-butanedione and sulfanilamide, the solution was made to contain 2,3-butanedione (86.4 mmol/l), Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was then transferred into an assay mexture (141 µl, preincubated at 37° C) containing Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercapto-ethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l), ^{14}C-PABA (328.12 µmol/l) and 2,3-butanedione (21.60 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained.

The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.3.5 Investigation of the Possible Reaction Between Phenylglyoxal and Sulfanilamide

For the investigation of the possible reaction between phenylglyoxal and sulfanilamide, the solution was made to contain phenylglyoxal (29.59 mmol/l), N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and sulfanilamide (3.58 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), DHPP (1.1 mmol/l), 2-mercaptoethanol (0.1 mol/l), sodium dithionite (9.80 mmol/l), ^{14}C-PABA (328.12 µmol/l) and phenylglyoxal (7.40 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.4 Investigation of the Possible Reaction Between the Modifying Agents and DHPP

In the experiment of the protection of the enzyme (the method described in section 3.10.6), DHPP was used as a substrate for the protection of the enzyme from the modifying agents; hence, it is possible that the reaction between the modifying agents and DHPP may occur.

The procedure was similar to the experiment in the section 3.10.3. The simplified diagram was the following

$$M + DHPP \xrightarrow{\text{Incubation}} + PABA + E \Rightarrow \text{Assay for the enzyme activity}$$

$$\text{Control tube; } DHPP \xrightarrow{\text{Incubation}} + PABA + E + M \Rightarrow \text{Assay for the enzyme activity}$$

If the enzyme activity obtained from these two conditions is not definitely different, it may be interpreted that the reaction between the modifying agent and DHPP is not occurred within the incubation time.

3.10.4.1 Investigation of the Possible Reaction Between Iodoacetamide and DHPP

For the investigation of the possible reaction between iodoacetamide and DHPP, the solution was made to contain iodoacetamide (68.5 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride

(5 mmol/l), ^{14}C-PABA (328.12 µmol/l) and iodoacetamide (17.12 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic raction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.4.2 Investigation of the Possible Reaction Between PMB and DHPP

For the investigation of the possible reaction between PMB and DHPP, the solution was made to contain PMB (426.24 µmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercapto-ethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mexture (141 µl, preincubated at 37° C) containing Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), ^{14}C-PABA (328.12 µmol/l) and PMB (106.56 µmol/l). The enzyme (9 µl, 0.045 units)was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.4.3 Investigation of the Possible Reaction Between 2,4-Pentanedione and DHPP

For the investigation of the possible reaction between 2,4-pentanedione and DHPP, the solution was made to contain 2,4-pentanedione (77.70 mmol/l), sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl. After the solution was incubated for 24 hours at 21°C, an aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C) containing sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of

200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl was incubated for 24 hours at 21° C. An aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C) containing sodium phosphate buffer (0.4 mol/l,pH 8.0), magnesium chloride (5 mmol/l), ^{14}C-PABA (328.12 µmol/l) and 2,4-pentanedione (19.43 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercapto-ethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.4.4 Investigation of the Possible Reaction Between 2,3-Butanedione and DHPP

For the investigation of the possible reaction between 2,3-butanedione and DHPP, the solution was made to contain 2,3-butane-dione (86.4 mmol/l), Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C)

containing Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol/l). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25^{o} C. An aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37^{o} C) containing Hepes buffer (0.4 mol/l, pH 8.2), megnesium chloride (5 mmol/l), ^{14}C-PABA (328.12 µmol/l) and 2,3-butanedione (21.60 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.4.5 Investigation of the Possible Reaction Between Phenylglyoxal and DHPP

For the investigation of the possible reaction between phenylglyoxal and DHPP, the solution was made to contain phenylglyoxal (29.59 mmol/l), N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0),

magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl. After the solution was incubated for 1 hour at 25° C, an aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C) containing N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and ^{14}C-PABA (328.12 µmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37°C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The control tube containing N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l) and DHPP (4.2 mmol/l) in a total volume of 55 µl was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (141 µl, preincubated at 37° C) containing N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), ^{14}C-PABA (328.12 µmol/l) and phenylglyoxal (7.40 mmol/l). The enzyme (9 µl, 0.045 units) was then added into the mixture and the total volume of 200 µl was obtained. The concentration of each component in the assay mixture was calculated from the final volume of 200 µl. After the reaction mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.5 Investigation of the Possible Reaction Among the Modifying Agents, 2-Mercaptoethanol and Sodium Dithionite

2-Mercaptoethanol and sodium dithionite were contained in the solution of DHPP in the experiment of the protection of the enzyme (the method described in section 3.10.6), hence, it is possible that the reactions among the modifying agents, 2-mercaptoethanol and sodium dithionite may occur.

In the experiment, the modifying agent, 2-mercaptoethanol, sodium dithionite and the enzyme were allowed to incubate together. The enzyme activity was then assayed. In the control tube, the modifying agent and the enzyme were allowed to incubate, and the enzyme activity was then assayed. If the enzyme activities obtained from these two conditions are not definitely different, it may be interpreted that the reaction among the modifying agent, 2-mercapto-ethanol and sodium dithionite is not occurred within the incubation time.

3.10.5.1 Investigation of the Possible Reaction Among Iodoacetamide, 2-Mercaptoethanol and Sodium Dithionite

Three tubes used in the experiment were tube No. 1 (E + R + M, R = mercaptoethanol and sodium dithionite), tube No. 2 (E + M), and tube No. 3 (E).

The solution in tube No. 1 (E + R + M) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), iodoacetamide (68.5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated at 25^{o} C for 1 hour. An

aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C), and the final volume of the mixture was 200 µl. The components in the mixture were Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.195 mol/l), sodium dithionite (19.12 mmol/l), DHPP (1.1 mmol/l), iodoacetamide (17.12 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol) and the enzyme activity was assayed by the method described in section 3.9.

The solution in tube No. 2 (E + M) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), iodoacetamide (68.5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No. 2 (E+M) was almost the same as that for tube No. 1 except that 2-mercapto-ethanol and sodium dithionite were not present in the incubation mixture (55 µl).

The solution in tube No. 3 (E) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No. 3 (E) was almost the same as that for tube No. 1 except that 2-mercaptoethanol, sodium dithionite, and iodoacetamide were not present in the incubation mixture (55 µl).

3.10.5.2 Investigation of the Possible Reaction Among PMB, 2-Mercaptoethanol and Sodium Dithionite

Three tubes used in the experiment were tube No. 1 (E + R + M), tube No. 2 (E + M), and tube No. 3 (E).

The solution in tube No. 1 (E + R + M) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l) sodium dithionite (37.26 mmol/l), PMB (426.24 µmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated at 25° C for 1 hour. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C), and the final volume of the mixture was 200 µl. The components in the mixture were Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.195 mol/l), sodium dithionite (19.12 mmol/l), DHPP (1.1 mmol/l), PMB (106.56 mol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercapto-ethanol (80 µl, 1.14 mmol) and the enzyme activity was assayed by the method described in section 3.9.

The solution in tube No. 2 (E + M) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), PMB (426.24 µmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No.1 (E + R + M).

It can be seen that the procedure for tube No. 2 (E + M) was almost the same as that for tube No. 1 except that 2-mercaptoethanol and sodium dithionite were not present in the incubation mixture (55 µl).

The solution in tube No. 3(E) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No. 3(E) was almost the same as that for tube No. 1 except that 2-mercaptoethanol, sodium dithionite, and PMB were not present in the incubation mixture (55 µl).

3.10.5.3 Investigation of the Possible Reaction Among 2,4-Pentanedione, 2-Mercaptoethanol and Sodium Dithionite

Three tubes used in the experiment were tube No. 1 (E + R + M), tube No. 2 (E + M), and tube No. 3 (E).

The solution in tube No. 1 (E + R + M) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), 2,4-pentanedione (77.70 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated at 21° C for 24 hours. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of the mixture was 200 µl. The components in the mixture were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.195 mol/l), sodium dithionite (19.12 mmol/l), DHPP (1.1 mmol/l), 2,4-pentanedione (19.43 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol) and the enzyme activity was assayed by the method described in section 3.9.

The solution in tube No. 2 (E + M) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2,4-pentanedione (77.70 mmol/l), and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1(E + R + M).

It can be seen that the procedure for tube No. 2 (E + M) was almost the same as that for tube No.1 except that 2-mercaptoethanol and sodium dithionite were not present in the incubation mixture (55 µl).

The solution in tube No. 3 (E) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in the total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No.3 (E) was almost the same as that for tube No. 1 except that 2-mercaptoethanol, sodium dithionite and 2,4-pentanedione were not present in the incubation mixture (55 µl).

3.10.5.4 Investigation of the Possible Reaction Among 2,3-Butanedione, 2-Mercaptoethanol and Sodium Dithionite

Three tubes used in the experiment were tube No. 1 (E + R + M), tube No. 2 (E + M) and tube No. 3 (E).

The solution in tube No. 1 (E+ R + M) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), 2,3-butanedione (86.4 mmol/l) and the enzyme (0.05 units) in

a total volume of 55 µl. The tube was incubated at 25° C for 1 hour. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C), and the final volume of the mixture was 200 µl. The components in the mixture were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.195 mol/l), sodium dithionite (19.12 mmol/l), DHPP (1.1 mmol/l), 2,3-butanedione (21.60 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol) and the enzyme activity was assayed by the method described in section 3.9.

The solution in tube No. 2(E + M) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2,3-butanedione (86.4 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No.2 (E + M) was almost the same as that for tube No.1 except that 2-mercaptoethanol and sodium dithionite were not present in the incubation mixture (55 µl).

The solution in tube No. 3 (E) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No. 3 (E) was almost the same as that for tube No. 1 except that 2-mercapto-ethanol, sodium dithionite, and 2,3-butanedione were not present in the incubation mixture (55 µl).

3.10.5.5 Investigation of the Possible Reaction Among Phenylglyoxal, 2,-Mercaptoethanol and Sodium Dithionite

Three tubes used in the experiment were tube No. 1 (E + R + M), tube No.2(E +M) and tube No. 3 (E).

The solution in tube No. 1 (E + R + M) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), phenylglyoxal (29.59 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated at 25°C for 1 hour. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of the mixture was 200 µl. The components in the mixture were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.195 mol/l), sodium dithionite (19.12 mmol/l), DHPP (1.1 mmol/l), phenylglyoxal (7.40 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol) and the enzyme activity was assayed by the method described in section 3.9.

The solution in tube No. 2(E + M) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), phenylglyoxal (29.59 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E + R + M).

It can be seen that the procedure for tube No.2 (E + M) was almost the same as that for tube No.1 except that 2-mercaptoethanol and sodium dithionite were not present in the incubation mixutre (55 µl).

The solution in tube No. 3(E) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The rest of the experiment was the same as that described for tube No. 1 (E+ R + M).

It can be seen that the procedure for tube No. 3 (E) was almost the same as that for tube No. 1 except that 2-mercapto-ethanol, sodium dithionite and phenylglyoxal were not present in the incubation mixture (55 µl).

3.10.6 Protection of the Enzyme from the Modifying Agents

The purpose of this experiment was to determine whether DHPP and sulfanilamide can protect the enzyme from the modifying agents.

3.10.6.1 Protection of the Enyme by DHPP and by Sulfanilamide in the Presence of DHPP

3.10.6.1.1 Protection of the Enzyme from Iodoacetamide

Seven tubes were used in the experiment.

Tube No. 1 (E +M) was used for studying the enzyme modification by iodoacetamide.

Tube No. 2 (E + M + R) was used for studying the enzyme modification by iodoacetamide in the presence of 2-mercaptoethanol and sodium dithionite. The purpose of measuring the enzyme activities in tube No. 1 and tube No. 2 was to determine the effect of 2-mercaptoethanol and sodium dithionite on iodoacetamide.

Tube No. 3 (E + R) was used as the control for tube No. 2. The materials in tube No. 3 were the same as that in tube No. 2 except that iodoacetamide in tube No. 3 was absent. The enzyme activities in the tube No. 2 and tube No. 3 were measured, and the degree of enzyme modification by iodoacetamide was expressed as percentage of control which was given below.

$$\text{\% Control}_{(E+R+M)} = \frac{\text{The enzyme activity in the tube No. 2}}{\text{The enzyme activity in the tube No. 3}} \times 100$$

Tube No. 4 (E + M + R + DHPP) was used for studying the protection of the enzyme from iodoacetamide by DHPP.

Tube No. 5 (E + R + DHPP) was used as the control for tube No. 4. The materials in tube No. 5 were the same as that in tube No. 4 except that iodoacetamide in tube No. 5 was absent. The enzyme activities in tube No. 4 and tube No. 5 were measured, and percentage of control was expressed as follows:

$$\text{\% Control}_{(E+M+R+DHPP)} = \frac{\text{The enzyme activity in the tube No. 4}}{\text{The enzyme activity in the tube No. 5}} \times 100$$

Tube No. 6 (E + M + R + DHPP + I) was used for studying the protection of the enzyme from iodoacetamide by sulfanilamide in the presence of DHPP.

Tube No. 7 (E + R + DHPP + I) was used as the control for tube No. 6. The materials in tube No. 7 were the same as that in tube No. 6 except that iodoacetamide in tube No. 7 was absent. The enzyme activities in tube No. 6 and tube No. 7 were measured, and percentage of control was expressed as follows:

$$\text{\% Control (E+M+R+DHPP+I)} = \frac{\text{The enzyme activity in the tube No. 6}}{\text{The enzyme activity in the tube No. 7}} \times 100$$

If the value of the % control (E + M + R + DHPP) is higher than that of the % control (E + M + R), it may be interpreted that DHPP can protect the enzyme from iodoacetamide. If the value of the % control (E + M + R + DHPP + I) is higher than that of the % control (E + M + R), it may be interpreted that sulfanilamide in the presence of DHPP can protect the enzyme from iodoacetamide.

The experimental details were as follows:

The solution in tube No. 1 (E + M) was made to contain iodoacetamide (137 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C), and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were iodoacetamide (34.25 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes.

the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 2 (E + M + R) was made to contain iodoacetamide (137 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (54.54 mmol/l), sodium dithionite (5.34 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were iodoacetamide (34.25 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 3 (E + R) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (54.54 mmol/l), sodium dithionite (5.34 mmol/l), and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), iodoacetamide

(34.25 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 4 (E+ M + R + DHPP) was made to contain iodoacetamide (137 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (54.54 mmol/l), sodium dithionite (5.34 mmol/l), DHPP (0.6 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The componentsin the resulting mixture (200 µl) were Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), iodoacetamide (34.25 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzymatic activity was then assayed by the method described in section 3.9.

The solution in tube No. 5 (E + R + DHPP) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (54.54 mmol/l), sodium dithionite (5.34 mmol/l), DHPP (0.6 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Tris buffer (0.4

mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), iodoacetamide (34.25 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 6 (E + M + R + DHPP + I) was made to contain iodoacetamide (137 mmol/l), Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (54.54 mmol/l), sodium dithionite (5.34 mmol/l), DHPP (0.6 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25^{o} C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37^{o} C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), iodoacetamide (34.25 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 7 (E + R + DHPP + I) was made to contain Tris buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol(54.54 mmol/l), sodium dithionite (5.34 mmol/l), DHPP (0.6 mmol/l) sulfanilamide (3.58 mmol/l) and the

enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Tris-hydrochloride buffer (0.4 mol/l, pH 8.55), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), iodoacetamide (34.25 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.6.1.2 Protection of the Enzyme from PMB

The experiment was done in the same way as that in the section 3.10.6.1.1 except that PMB (34.10 mmol/l) was used instead of iodoacetamide.

3.10.6.1.3 Protection of the Enzyme from 2,4-Pentanedione

The experiment was done similar to that in the section 3.10.6.1.1 except that tube No. 2 (E + R + M) was excluded. Thus, six tubes were included in the experiment.

The solution in tube No. 1 (E + M) was made to contain 2,4-pentanedione (77.70 mmol/l), sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was transferred into an assay mixutre

(150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were 2,4-pentanedione (19.43 mmol/l), sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 2 (E) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,4-pentanedione (19.43 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 3 (E + M + R + DHPP) was made to contain 2,4-pentanedione (77.70 mmol/l), sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The

tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,4-pentanedione (19.43 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 4 (E + R + DHPP) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,4-pentanedione (19.43 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 5 (E + M + R + DHPP + I) was made to contain 2,4-pentanedione (77.70 mmol/l), sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,4-pentanedione (19.43 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 6 (E + R + DHPP + I) was made to contain sodium phosphate buffer (0.2 mol/l, pH 7.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 24 hours at 21° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were sodium phosphate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12

µmol/l), 2,4-pentanedione (19.43 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.6.1.4 Protection of the Enzyme from 2,3-Butanedione

The experiment was done similar to that in the section 3.10.6.1.3. Six tubes were used in the experiment.

The solution in tube No. 1 (E + M) was made to contain 2,3-butanedione (86.4 mmol/l), Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were 2,3-butanedione (21.60 mmol/l), Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No.2(E) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was

incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,3-butanedione (21.60 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 3 (E + M + R + DHPP) was made to contain 2,3-butanedione (86.4 mmol/l), Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,3-butanedione (21.60 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 4 (E + R + DHPP) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,3-butanedione (21.60 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 5 (E + M + R + DHPP + I) was made to contain 2,3-butanedione (86.4 mmol/l), Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in the total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in theresulting mixture (200 µl) were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,3-butanedione (21.60 mmol/l), sulfanilamide (0.895

mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 6 (E + R + DHPP + I) was made to contain Hepes buffer (0.24 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were Hepes buffer (0.4 mol/l, pH 8.2), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), 2,3-butanedione (21.60 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.6.1.5 Protection of the Enzyme from Phenylglyoxal

The experiment was done similar to that in the section 3.10.6.1.4. Six tubes were used in the experiment.

The solution in tube No. 1 (E + M) was made to contain phenylglyoxal (29.59 mmol/l), N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and the enzyme

(0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were phenylglyoxal (7.40 mmol/l), N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 2 (E) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), phenylglyoxal (7.40 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 3 (E + M + R + DHPP) was made to contain phenylglyoxal (29.59 mmol/l), N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), phenylglyoxal (7.40 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 4 (E + R + DHPP) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), phenylglyoxal (7.40 mmol/l) and

the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 5 (E + M + R + DHPP + I) was made to contain phenylglyoxal (29.59 mmol/l), N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C) and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), phenylglyoxal (7.40 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37° C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

The solution in tube No. 6 (E + R + DHPP + I) was made to contain N-ethylmorpholine acetate buffer (0.25 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.38 mol/l), sodium dithionite (37.26 mmol/l), DHPP (4.2 mmol/l), sulfanilamide (3.58 mmol/l) and the enzyme (0.05 units) in a total volume of 55 µl. The tube was incubated for 1 hour at 25° C. An aliquot of 50 µl was then transferred into an assay mixture (150 µl, preincubated at 37° C)

and the final volume of 200 µl was obtained. The components in the resulting mixture (200 µl) were N-ethylmorpholine acetate buffer (0.4 mol/l, pH 8.0), magnesium chloride (5 mmol/l), 2-mercaptoethanol (0.114 mol/l), sodium dithionite (11.13 mmol/l), DHPP (1.1 mmol/l), ^{14}C-PABA (328.12 µmol/l), phenylglyoxal (7.40 mmol/l), sulfanilamide (0.895 mmol/l) and the enzyme (0.045 units). After the mixture was incubated at 37^{o} C for 15 minutes, the enzymatic reaction was stopped with 2-mercaptoethanol (80 µl, 1.14 mmol). The enzyme activity was then assayed by the method described in section 3.9.

3.10.6.2 Protection of the Enzyme by DHPP and by Sulfanilamide in the Absence of DHPP

The purpose of the experiment was to determine whether sulfanilamide itself in the absence of DHPP could protect the enzyme from PMB.

The experiment was done in the same way as described in section 3.10.6.1.2 except that DHPP was not added into the incubation mixture in the tube No. 6 and the tube No. 7.

3.11 Study of the General Properties of Dihydropteroate Synthase and Determination of the Inhibitor Constant (K_i) for p-Aminobenzene-sulfonamidoalkanoic Acids

3.11.1 Study of the General Properties of Dihydropteroate Synthase

3.11.1.1 Study of the Effect of the Incubation Time on the Enzyme Activity

The method was done in the same way as in section 3.9 except that the incubation time was varied form 0 to 40 minutes in this experiment. The incubation times were plotted against the enzyme activity.

3.11.1.2 Study of the Effect of the Enzyme Concentration on the Enzyme Activity

The method was done in the same way as that in section 3.9 except that the amount of enzyme was varied from 0.020 to 0.12 units in this experiment. The amounts of the enzyme were plotted against the enzyme activity.

3.11.1.3 Determination of the Optimum pH

The method was done in the same way as described in section 3.9 except that the pH of the buffer was varied from pH 7.8 to pH 8.8 in this experiment. The values of the pH were plotted against the enzyme activity. The optimum pH is a pH at which the enzyme activity is maximal.

3.11.1.4 Determination of the Optimum Temperature

The method was done in the same way as described in section 3.9 except that the incubation temperature was varied from 25 to 55^{o} C in this experiment. The temperatures were plotted against the enzyme activity. The optimum temperature is a temperature at which the enzyme activity is maximal.

3.11.1.5 Determination of the Michaelis Constant (K_m) for PABA

The method was done in the same way as described in section 3.9 except that the concentration of PABA, the pH of the buffer, and the incubation time in this experiment were different from that described in section 3.9. The concentrations of PABA were 0.342, 0.684, 1.026, 1.367, 2.784 and 5.557 µmol/l. The pH of the buffer was 8.55. The incubation time for each PABA concentration was varied from o to 8 minutes. The incubation time for each PABA concentration was plotted against the enzyme activity, and the initial velocity (v) was calculated by using regression analysis. the Km value for PABA was obtained from the Lineweaver-Burk plot (1/v versus 1/[PABA]).

3.11.2 Determination of the Inhibitor Constant (K_i) for p-Aminobenzenesulfonamidoalkanoic Acids and Sulfanilamide

The method was done in the same way as described in section 3.9 except that the concentration of PABA and the pH of the buffer were different, and the inhibitors (p-aminobenzenesulfonamidoalkanoic acids or sulfanilamide) were present in the incubation mixture in this experiment. The concentration of PABA were 11.136, 19.886, and 27.840 µmol/l. The pH of the buffer was 8.55. The concentration of the inhibitor was varied for a particular concentration of PABA. The concentration of sulfanilamide, N-(p-aminobenzenesulfonyl) glycine, N-(p-aminobenzenesulfonyl) tyrosine, N-(p-aminobenzenesulfonyl) alanine, N-(p-aminobenzenesulfonyl) phenylalanine, N-(p-aminobenzenesulfonyl) methionine, N-(p-aminobenzenesulfonyl) leucine, and N-(p-aminobenzenesulfonyl) valine were 0-1.0 mmol/l, 0-360 µmol/l,

0-800 μmol/l, 0-1.0 mmol/l, 0-1.6 mmol/l, 0-1.6 mmol/l, 0-3.0 mmol/l and 0-5.0 mmol/l respectively. The K_i value for each inhibitor was obtained from the Dixon plot in which 1/v was plotted against [I].