

Enzymic Separatory Determination of Prostaglandin E and F<sup>1)</sup>TAKAFUMI ITAMI, KENJI FUKUZAWA, MASUMI TAKAHASHI,  
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A simple and sensitive method is described for separatory determination of prostaglandin E and prostaglandin F. This method is based on three principles: (1) Prostaglandin E and prostaglandin F are oxidized by prostaglandin dehydrogenase, resulting reduced nicotinamide-adenine dinucleotide (NADH). (2) The sensitive determination of reduced NAD is performed by enzymic cycling with resazurin which serves as the terminal electron acceptor. (3) Alkaline treatment converts only prostaglandin E to insensitive material to prostaglandin dehydrogenase.

This method was capable for separatory determination of prostaglandin E and prostaglandin F in the order of  $10^{-11}$  mole.

Prostaglandins have attracted great interest because of their biological effect in a small dosage.<sup>3)</sup> They consist of many groups of compounds and, among them, prostaglandin E and prostaglandin F play main roles and are found in body organs and fluids. Therefore,

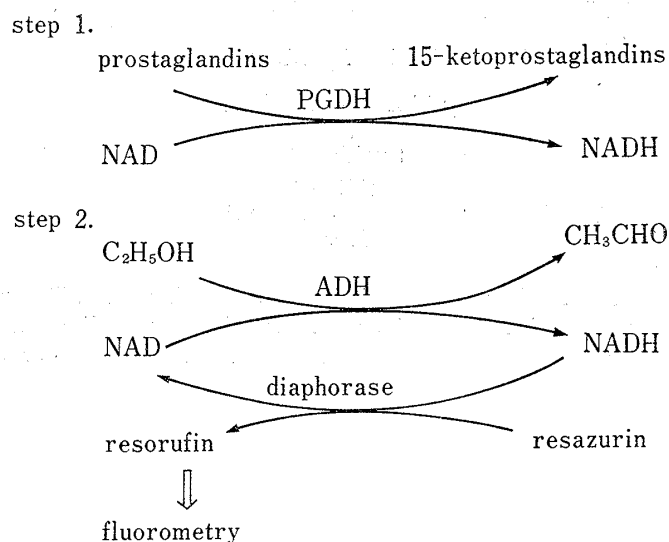


Fig. 1. Principles for the Assay of Prostaglandins by PGDH and Enzymic Cycling of Pyridine Nucleotides

it is necessary to determine prostaglandin E and prostaglandin F respectively. Several reports on pharmacological,<sup>4)</sup> fluorometric<sup>5)</sup> and chromatographic analyses<sup>6)</sup> of prostaglandins have appeared. Recently, a sensitive method for the determination of prostaglandins, which utilized prostaglandin dehydrogenase [nicotinamide-adenine dinucleotide (NAD) 15-hydroxyprostanate oxidoreductase, PGDH] and enzymic cycling of pyridine nucleotides was reported by Ånggård, *et al.*<sup>7)</sup> However, this method cannot distinguish prostaglandin E from prostaglandin F, and takes time and trouble at enzymic cycling process.

The present paper submits a sensitive of  $10^{-11}$  mole and simple method of separatory determination of prostaglandin E and prostaglandin F.

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The proposed method consists of two steps (Fig. 1). In the first step, prostaglandin E and prostaglandin F are oxidized by PGDH to 15-ketoprostaglandins, and NAD used as a cofactor is reduced to reduced NAD (NADH). In the second step, this NADH is used as a cofactor for a pair of dehydrogenases, *i.e.*, NADH is oxidized by diaphorase (1.6.4.3.) and NAD thus formed is reduced by alcohol dehydrogenase (1.1.1.1., ADH). Resorufin, which is the reduced product of resazurin serving as a substrate for diaphorase, can be useful for fluorometric determination, since it is a highly fluorescent compound. The rate of resorufin formation is proportional to the concentration of NADH formed in the first step.

For the separatory determination of prostaglandin F, the same procedure is carried out as above after alkaline treatment, with which only prostaglandin E is converted to prostaglandin B, which is not oxidized with PGDH and prostaglandin F remains unchanged. The amount of prostaglandin E is estimated from the difference in the values of prostaglandin F (alkaline treatment) and total prostaglandins (non-treatment).

If a sample contains other groups of prostaglandins, such as prostaglandin A or its derivatives, which are not usually found in most organs and fluids, these groups may be removed by a very simple silicic acid chromatography.<sup>8)</sup>

### Experimental

**Materials**—Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), prostaglandin F<sub>1α</sub> (PGF<sub>1α</sub>), and PGDH from swine lung<sup>9)</sup> were presented from Ono Pharmaceutical Industries, LTD. ADH was obtained from Böhringer Mannheim, NAD, NADH and diaphorase from Sigma Chemical Co., and resazurin from Wako Pure Chemical Industries, LTD.

**PGDH Reagent**—8 unit of PGDH was dissolved in 1 ml of 0.1M Tris-HCl buffer of pH 8.0, containing 0.2mM NAD, 1 mM mercaptoethanol and 0.03% bovine serum albumin.

**Enzymic Cycling Medium**—10 unit of ADH and 0.1 unit of diaphorase were dissolved in 1 ml of 0.1M phosphate buffer of pH 7.4, containing 10 μM of resazurin, 0.5M of ethanol and 1 mM of nicotinamide.

**Alkaline Treatment**—The sample solution which contains prostaglandin E and prostaglandin F was treated at 35° for 30 min in 50% ethanol containing 0.1M KOH, then neutralized with same volume of 0.08N acetic acid.

**Determination of Prostaglandin**—Step-1: 20 μl of PGDH reagent was added to 5 μl of the alkaline treated or non-treated reaction mixture. This mixture was incubated at 44° for 3 hr. After incubation, 25 μl of 0.6N NaOH was added and heated at 60° for 30 min to destroy remained NAD, and then this reaction mixture was neutralized by addition of 50 μl of 0.3N acetic acid.

Step-2: 150 μl of enzymic cycling medium was added to the 100 μl of neutralized reaction mixture and incubated at 37° for 60 min. The resorufin produced was measured at excitation and emission wavelength of 560 and 580 mμ respectively in Hitachi MPF-2A fluorescence spectrophotometer.

### Result and Discussion

The condition of alkaline treatment for the conversion of PGE<sub>1</sub> to prostaglandin B<sub>1</sub> was studied in accordance with the method of Nugteren, *et al.*<sup>10)</sup> The reaction was followed by measuring the rise in absorbancy at 278 mμ (production of prostaglandin B<sub>1</sub>). The result was shown in Fig. 2. PGE<sub>1</sub> was completely converted to prostaglandin B<sub>1</sub> by incubation at 35° for 30 min with 50% ethanol containing 0.1M KOH.

The rate of resorufin formation at various pH was studied to know the optimum pH in enzymic cycling. As shown in Fig. 3, maximum rate of resorufin formation was found at pH 7.4. Therefore, in subsequent studies the cycling medium was buffered at pH 7.4.

On the basis of these findings described above, the sensitive method for the separatory determination of prostaglandin E and prostaglandin F was prepared. As shown in Fig. 4 and Fig. 5, a linear relationship was found between the intensity of resorufin fluorescence and

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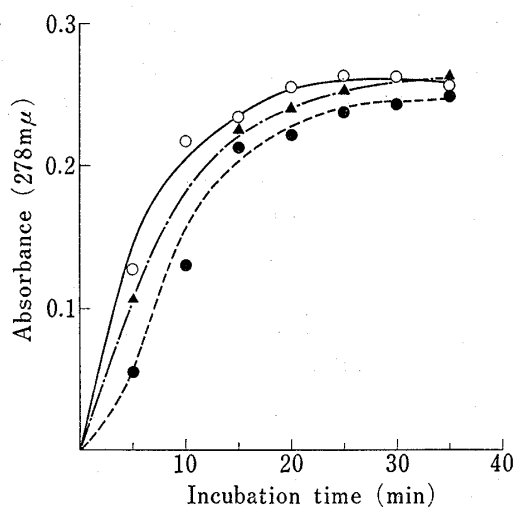


Fig. 2. Time Course of Conversion of  $\text{PGE}_1$  to Prostaglandin  $\text{B}_1$  by Alkaline Treatment

$\text{PGE}_1$  (10  $\mu\text{g}$ ) was incubated at  $35^\circ$  in 10 ml of 50% ethanol containing 0.1M KOH (—○—), of 50% ethanol containing 0.05M KOH (—●—) or of  $\text{H}_2\text{O}$  containing 0.1M KOH (—▲—).

The spectra of resulting reaction medium was determined at 278  $m\mu$ .

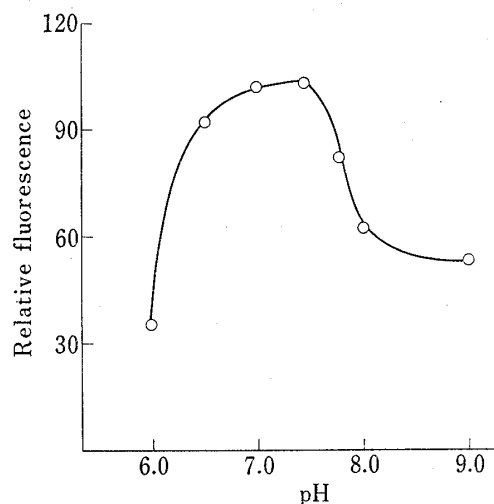


Fig. 3. Effect of pH in Enzymic Cycling Medium on the Formation Rate of Resorufin

NADH (50 ng/25  $\mu\text{l}$ ) was incubated at  $37^\circ$  for 1 hr in mixture of 25  $\mu\text{l}$  of 0.6N NaOH, 50  $\mu\text{l}$  of 0.3N acetic acid and 150  $\mu\text{l}$  of enzymic cycling medium.

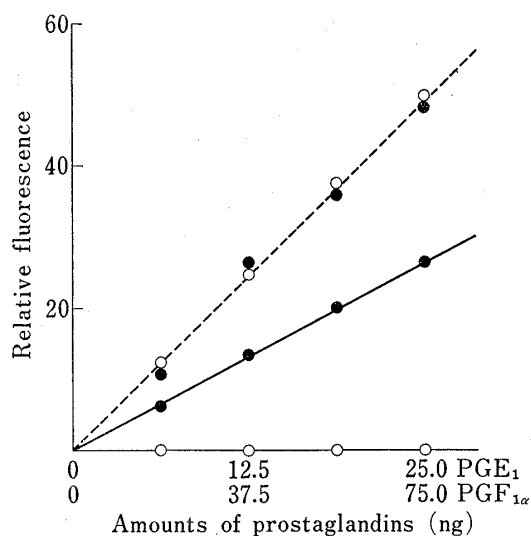


Fig. 4. Determination of the Amounts of  $\text{PGE}_1$  and  $\text{PGF}_{1\alpha}$

—●—:  $\text{PGE}_1$ ; —○—:  $\text{PGE}_1$  (alkaline treatment); —●—:  $\text{PGF}_{1\alpha}$ ; —○—:  $\text{PGF}_{1\alpha}$  (alkaline treatment)

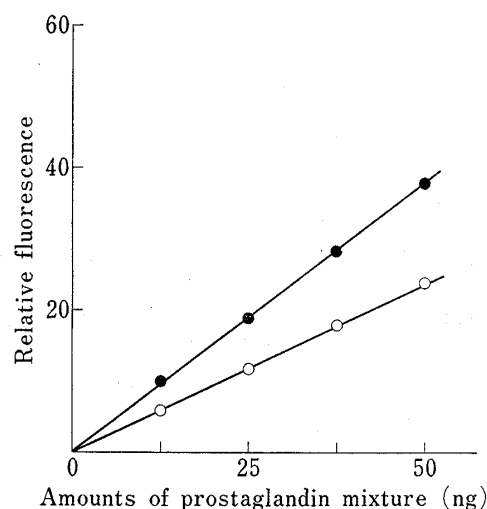


Fig. 5. Separatory Determination of the Amounts of  $\text{PGE}_1$  and  $\text{PGF}_{1\alpha}$

$\text{PGE}_1$  and  $\text{PGF}_{1\alpha}$  were mixed at the rate of one to three in weight. —●—:  $\text{PGE}_1 + \text{PGF}_{1\alpha}$ ; —○—:  $\text{PGE}_1 + \text{PGF}_{1\alpha}$  (alkaline treatment)

concentration of  $\text{PGE}_1$ ,  $\text{PGF}_{1\alpha}$  and their mixture ( $\text{PGE}_1 : \text{PGF}_{1\alpha} = 1 : 3$ ), up to 25 ng of  $\text{PGE}_1$ , 75 ng of  $\text{PGF}_{1\alpha}$  and 50 ng of their mixture.

The activity of PGDH used for  $\text{PGF}_{1\alpha}$  was about 60% of that for  $\text{PGE}_1$ . This result was a little different from that (70%) of Ånggård, *et al.*<sup>7)</sup> While the quantitative formation of resorufin with  $\text{PGE}_{1\alpha}$  was unchanged even in the case of alkaline treatment, the resorufin formation with  $\text{PGE}_1$  was entirely absent when treated with alkali (Fig. 4).

Resorufin fluorescence obtained by alkaline treatment of the mixture of  $\text{PGE}_1$  and  $\text{PGF}_{1\alpha}$  decreased quantitatively by the amount due to  $\text{PGE}_1$ , compared to the mixture not treated with an alkali (Fig. 5).

The prostaglandin B compounds formed by alkaline treatment were found to inhibit PGDH, noncompetitively.<sup>11)</sup> These inhibitor constants were fairly high, about 210  $\mu\text{M}$ , which is about 25 times that of  $K_m$  of PGE<sub>1</sub>. In our method, it was preferable to measure the total prostaglandin (prostaglandin E + prostaglandin F) and each prostaglandin ranging from 10 ng to 50 ng. In this range, the maximum amount of prostaglandin B, formed from prostaglandin E by alkaline treatment, may be about 40 ng. Calculating from the inhibitor constant of prostaglandin B, the prostaglandin B in this concentration may not hinder this enzymic assay, practically.

The proposed method is a modified one for the determination of prostaglandins by the combination of the method of Änggård, *et al.*<sup>7)</sup> with the enzymic assay of NADH by Guilbault and Kramer.<sup>12)</sup> In their method, the continuous reduction of NAD in the presence of an excess of ethanol and ADH was coupled with resazurin and diaphorase. Their method is more sensitive than the determination of NADH from spectrophotometric measurement of reduced cytochrome c<sup>13)</sup> or of dichlorophenol-indophenol.<sup>14)</sup>

Compared with the method of Änggård, *et al.*,<sup>7)</sup> the present method is more convenient because of simplifying an enzymic cycling system, and more sensitive since the fluorescence of final product, resorufin, is more intensive than of NADH. Further, it is capable for separatory determination of PGE and PGF.

**Acknowledgements** The authors wish to express their appreciation to Ono Pharmaceutical Industries, LTD. for presentation of prostaglandins and PGDH.

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## Reductive Cyclization of *o*-Nitrobenzylideneacetylacetone Analogues

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Reductions of *o*-nitrobenzylideneacetylacetone analogues (I, II and III) by means of stannous chloride in hydrochloric acid or catalytic hydrogenation of 5% palladium carbon as catalyst gave the mixture of 3-acylquinaldines and these N-oxides, analysed by gas chromatographies.

Cyclization between aromatic nitro group and ortho side chain to synthesize heterocyclic compounds has widely been investigated by many workers.<sup>2)</sup> Among them, Loudon and Tennant<sup>3-5)</sup> reported that the *o*-nitrobenzylidene derivatives in inert solvent using hydrogen

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