Enzymatic preparation and purification of prostaglandin E_2

MILTON LAPIDUS, NORMAN H. GRANT, and HARVEY E. ALBURN

Research Division, Wyeth Laboratories, Philadelphia, Pennsylvania 19101

ABSTRACT An enzymatic system has been developed for the production of prostaglandin E_2 (PGE_2) from arachidonic acid by extracts of sheep seminal vesicular glands. The presence of glutathione insures high yields.

A new procedure for the purification of PGE_2 was also developed, based on the dialysis of the biosynthesized product at pH 8 and extraction of the dialysate at pH 3 with chloroform. This procedure routinely gives yields of PGE_2 of 25–37% (from arachidonic acid) with a purity of 90–100%.

Additional analytical proof of the identity of PGE_2 was provided by physicochemical characteristics of the crystalline thiosemicarbazide derivative, which can be readily prepared under mild conditions.

KEY WORDS prostaglandin · arachidonic acid · PGE_2 · enzymatic preparation · purification · dialysis · glutathione · PGE_2 thiosemicarbazone · IR spectra · thin-layer chromatography

INDEPENDENT INVESTIGATIONS of Goldblatt (1) and von Euler (2) established that a factor having vasodepressor and smooth muscle-stimulating activity at very low concentrations was present in human seminal plasma and vesicular glands of sheep. An acidic lipid, prostaglandin, was found to be responsible for these activities (3). There followed a period of investigation, recently summarized (4), which culminated in the identification of a family of chemically related prostaglandins that are present in a variety of tissues and species. These C_{20}-carboxylic acids contain a five-membered ring, a keto group, two or three hydroxy groups, and one to three C=C double bonds. With great insight, van Dorp, Beerthuis, Nugteren, and Vonkeman (5) and Bergström, Danielsson, and Samuelsson (6) established the relationship between C_{20} essential fatty acids and prostaglandins. They showed that homogenates of seminal vesicular glands of sheep enzymatically convert arachidonic acid to PGE_2, homo-γ-linolenic acid to PGE_1, and all-*cis*-5,8,11,14,17-eicosapentaenoic acid to PGE_3 (7, 8).

Prostaglandins act at minute concentrations similar to those found for such potent humoral agents as acetyl choline. Comprehensive biological studies have been restricted, however, by the lack of purified material. This paper describes studies on the enzymatic preparation of PGE_2 which led to the development of a simplified isolation scheme that yields relatively pure material and eliminates the need for adsorption chromatography or countercurrent distribution.

MATERIAL

Arachidonic acid was purchased from The Hormel Institute, Austin, Minn.; glutathione from Schwarz BioResearch, Inc., Orangeburg, N.Y.; Adsorbosils 1 and 2 from Applied Science Laboratories Inc., State College, Pa.; Silica Gel G from Brinkmann Instrument Inc., Westbury, N.Y., and glass beads (No. 42850M20) from Arthur H. Thomas Co., Philadelphia, Pa. All solvents and reagents were the best grade commercially available.

EXPERIMENTAL PROCEDURE

Chemical Assay for PGE_2

To a sample of PGE_2 in ethanol was added an equal volume of aqueous 1 N KOH solution. After 15 min at room temperature the absorbance of the chromophore

Abbreviations: PGE_1, 7-[3α-hydroxy-2-(3-hydroxy-1-octenyl)-5-oxocyclopentyl]-heptanoic acid; PGE_2, 7-[3α-hydroxy-2-(3-hydroxy-1-octenyl)-5-oxocyclopentyl]-5-heptenoic acid; PGE_3, 7-[3α-hydroxy-2-(3-hydroxy-1,5-octadienyl)-5-oxocyclopentyl]-5-heptenoic acid; PGA_1, 7-[2-(3-hydroxy-1-octenyl)-5-oxo-1-cyclopentenyl]-heptanoic acid; PGA_2, 7-[2-(3-hydroxy-1,5-octadienyl)-5-oxo-1-cyclopentenyl]-5-heptenoic acid.

was determined by means of a Beckman DU spectrophotometer at 278 mμ. The UV absorption of the chromophore obtained by the treatment of PGE_2 with ethanolic KOH was found to reach a plateau after 5 min at 50°C or 15 min at room temperature. Since the absorbancy was the same at both temperatures, the more convenient room temperature was used. Calculations were based on the known molar absorbancy of PGA_1 (26,800), since PGE_2 is transformed into a chromophore PGA_2 with essentially the same molar absorbancy (9). Crystalline PGE_1 obtained from Dr. B. Samuelsson was converted to PGA_1 in a yield of 94.6% under our assay conditions (both at room temperature and 50°C). Data were correspondingly corrected.

Thin-Layer Chromatography

Thin layers consisting of Adsorbosils 1 and 2 in a ratio of 2:1 were developed with chloroform–methanol–acetic acid–water 360:28:4:3. Best results were obtained when, after application of the sample, the plate was exposed to an atmosphere of vapor from the developing system for 5 min before development was started.

In the argentation system, thin layers of Silica Gel G and $AgNO_3$ in a ratio of 10:1 were developed with the less polar (upper) phase (10) resulting from 3 hr equilibration of the mixture ethyl acetate–water–methanol–acetic acid–2,2,4-trimethylpentane 110:100:35:30:10.

The detecting spray was 10% phosphomolybdic acid and 5% concentrated HCl in absolute ethanol. The blue spots were developed in the oven at 100°C.

Biosynthesis and Purification of PGE_2

After slaughter of sheep, the seminal vesicular glands were immediately removed, frozen, and stored at −18°C or below until use. 1 kg of minced glands was homogenized in a 1 gallon Waring Blendor for 5 min at 4°C with 100 g of glass beads and 2 liters of 0.1 M ammonium chloride, pH 8.5, containing 4 g of glutathione. The slurry was centrifuged at 4000 *g* for 10 min at 4°C and the supernatant solution was collected. The residue was further homogenized with 150 ml of 0.1 M ammonium chloride solution, pH 8.5, and the supernatant fraction was again collected after centrifugation. The pH of the pooled enzyme extract was adjusted to 8.0 before addition to the substrate.

The substrate was prepared by adding 1 g of arachidonic acid to 1 liter of a 0.1 M ammonium chloride solution, pH 8.5, and adjusting the pH to 8.0. The enzyme and substrate were combined and oxygen was bubbled through the mixture during a 1 hr incubation at 37°C. After incubation, the whole mixture was immediately frozen in a thin film and lyophilized. The lyophilized powder was reconstituted to 400 ml with distilled water, adjusted to pH 8, and dialyzed in 12 liters of distilled water at 8°C for 48 hr. The pH of the diffusate was adjusted to pH 3 with dilute sulfuric acid and the PGE_2 was extracted with four 1 liter portions of chloroform. The pooled chloroform was extracted three times with 335-ml portions of 0.2 M potassium phosphate buffer, pH 8.0. The pooled aqueous extract was adjusted to pH 3 with 0.1 M sulfuric acid and the PGE_2 was extracted with three 130 ml portions of methylene chloride. The methylene chloride extract was washed twice with 25 ml of distilled water, the last wash being allowed to separate at 4°C overnight and then removed.

The methylene chloride solution of purified PGE_2 was assayed for PGE_2 by the procedure described above. Total solids were determined by drying an aliquot of the methylene chloride solution at 100°C for 1 hr. The oily residue of PGE_2 resulting from the removal of methylene chloride in vacuo was dissolved in 0.2 M phosphate buffer pH 7.4, diluted with distilled water to 0.06 M phosphate, dispensed into ampules, sealed, and stored at −11°C.

PGE_2 Thiosemicarbazone

A solution of 250 mg of thiosemicarbazide in 10 ml of water was added to 429 mg of PGE_2 dissolved in 10 ml of methanol. A drop of glacial acetic acid was added and the solution was warmed to 40°C, filtered, and allowed to stand at 25°C for 2 days. The addition of 10 ml of water initiated crystal formation. After a further addition of 10 ml of water and cooling in an ice bath for 1 hr, the crystals were collected. The derivative was recrystallized from methanol–water 1:3 and dried in vacuo at 56°C, yielding 313 mg, mp 126–128°C.

Analysis: $C_{21}H_{35}N_3O_4S$;

calculated: C, 59.3; H, 8.2; S, 7.5.

found: C, 59.2; H, 8.3; S, 7.5.

Methyl Ester of PGE_2

PGE_2 (112 mg) was esterified with diazomethane (11) in methanol–ether 1:10 for 15 min and the solvents were removed in a rotary evaporator. The oily residue dissolved in chloroform was analyzed by thin-layer chromatography and found to be homogeneous.

RESULTS AND DISCUSSION

The success of the procedure for the isolation of PGE_2 was based on the experimental observation that it is readily dialyzable. Thus the necessary steps are (*a*) extraction of the enzyme from sheep seminal vesicular glands with an ammonium chloride solution containing glutathione, (*b*) incubation of the extract with arachidonic acid, (*c*) concentration of the reaction mixture by lyophilization, (*d*) reconstitution of the powder into a small aqueous volume, (*e*) dialysis against water, and (*f*) extraction of the PGE_2 from the diffusate into organic

TABLE 1 YIELDS OF PURIFIED PGE_2

Glutathione	Purified PGE_2* Yield	Purity
g/liter	%	%
0	13.1	
1.1	31.5	99.3
1.1	30.1	99.9
1.1	35.9	80.3
1.1	39.1	98.3
1.1	35.7	98.3
1.1	29.4	88.8
1.3	26.8	100.0

Arachidonic acid (1 g) was incubated with enzyme prepared from 1 kg of seminal vesicular glands in the presence of the indicated amount of glutathione at pH 8.0 and 37°C for 1 hr; total volume 3.5 liter.

* Based on spectrophotometry of the alkaline chromophore.

solvents such as chloroform or methylene chloride. By using this procedure we have eliminated manipulations such as precipitation with large volumes of acetone, filtration to remove precipitated protein, extraction with hexane to remove lipids, evaporation in vacuo to remove acetone, countercurrent distribution, and column chromatography (12). The dialysis procedure usually affords PGE_2 in a yield of 25–37% from arachidonic acid, with high purity (Table 1). If glutathione is not added to the enzyme system, however, the biosynthesis of PGE_2 is limited.

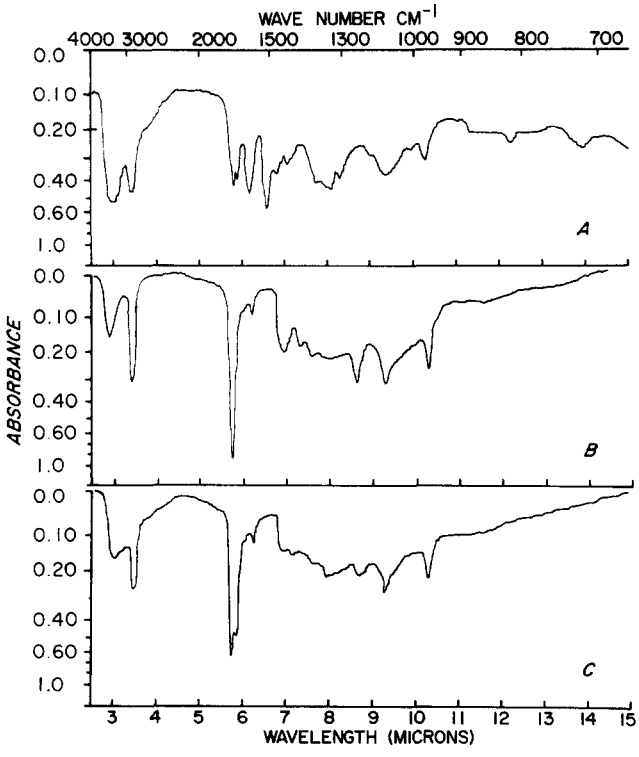

FIG. 1. IR spectra of *A*, PGE_2 thiosemicarbazone (KBr); *B*, PGE_2 methyl ester ($CHCl_3$); and *C*, PGE_2 ($CHCl_3$).

Prepared and purified as described, PGE_2 gave a dominant spot at R_f 0.42 on the Absorbosil thin-layer plate. IR spectra of the purified PGE_2 showed absorption at 3333 (OH), 2881 and 2840 (CH_2, CH_3), 2702 to 2272 (acid OH), 1739 (cyclopentyl C=O), 1709 (acid C=O), 1600 (*cis* C=C), and 970 cm^{-1} (*trans* C=C) (Fig. 1 *C*). Mass spectrographic analysis indicated a molecular weight of 334 (PGE_2 minus one molecule of H_2O). On a thin layer of Silica Gel G–$AgNO_3$ the methyl ester of PGE_2 showed as a single spot with R_f = 0.85. The IR spectrum of PGE_2 methyl ester showed absorption at 3367 (OH), 2932 and 2840 (CH_2, CH_3), 1736 (ester and cyclopentyl C=O), 1602 (*cis* C=C), and 970 cm^{-1} (*trans* C=C) (Fig. 1 *B*). IR spectra of PGE_2 thiosemicarbazone showed absorption at 3344 and 3225 (NH, NH_2), 3154 (OH), 2890 and 2825 (CH_2, CH_3), 2702 to 2040 (acid OH), 1706 (acid C=O), 1677 (C=N), 1602 (NH_2), 1510 (NH-C=S), and 970 cm^{-1} (*trans* C=C) (Fig. 1 *A*).

The PGE_2 obtained from our isolation procedure was identified by the chemical and physical analyses of the PGE_2 thiosemicarbazone. This derivative can be conveniently made under rather mild reaction conditions.

The authors thank Miss Marian McGettigan for expert technical assistance; Dr. D. DeJongh, Chemistry Department, Wayne State University, Detroit, Mich., for mass spectrum analysis; and Dr. S. Schrader for the interpretation of the mass spectrum.

Manuscript received 24 October 1967; accepted 13 February 1968.

REFERENCES

1. Goldblatt, M. W. 1933. *Chem. Ind. (London).* **52:** 1056.
2. von Euler, U. S. 1934. *Naunyn-Schmiedebergs Arch. Exptl. Pathol. Pharmakol.* **175:** 78.
3. von Euler, U. S. 1936. *J. Physiol.* **88:** 213.
4. Samuelsson, B. 1965. *Angew. Chem. Intern. Ed. Engl.* **4:** 410.
5. van Dorp, D. A., R. K. Beerthuis, D. H. Nugteren, and H. Vonkeman. 1964. *Biochim. Biophys. Acta.* **90:** 204.
6. Bergström, S., H. Danielsson, and B. Samuelsson. 1964. *Biochim. Biophys. Acta.* **90:** 207.
7. Bergström, S., H. Danielsson, D. Klenberg, and B. Samuelsson. 1964. *J. Biol. Chem.* **239:** PC4006.
8. van Dorp, D. A., R. K. Beerthuis, D. H. Nugteren, and H. Vonkeman. 1964. *Nature.* **203:** 839.
9. Bygdeman, M., and B. Samuelsson. 1966. *Clin. Chim. Acta.* **13:** 465.
10. Green, K., and B. Samuelsson. 1964. *J. Lipid Res.* **5:** 117.
11. Schlenk, H., and J. L. Gellerman. 1960. *Anal. Chem.* **32:** 1412.
12. Daniels, E. G., J. W. Hinman, B. A. Johnson, F. P. Kupiecki, J. W. Nelson, and J. E. Pike. 1965. *Biochem. Biophy. Res. Commun.* **21:** 413.