Vol. 127, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1985 Pages 15-23

# PERITONEAL MACROPHAGES OF GUINEA PIG POSSIBLY LACK $LTC_4$ SYNTHETASE

Masayoshi ABE*, Yasushi KAWAZOE, Hideaki TSUNEMATSU, and Nobuaki SHIGEMATSU

Research Institute and Clinic for Diseases of the Chest, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

Received December 26, 1984

Peritoneal cells and adherent cells of mice and rats synthesized $LTC_4$ and $LTB_4$ when stimulated with A23187 in vitro. On the other hand, neither peritoneal cells nor adherent cells of guinea pigs generated $LTC_4$, $D_4$, and $E_4$, but did the lower amounts of $LTB_4$. Only generation of $LTB_4$ was potentiated by simultaneous addition of 10 μM A.A. in this species. Enzyme solutions which were extracted from peritoneal cells of these three species were capable of converting DNCB to a colored product in the presence of glutathione and then these potencies were in the following order; guinea pig > mouse > rat. On the other hand, the potencies of converting $LTA_4$ to $LTC_4$ in the presence of glutathione were in the following order; mouse > rat >> guinea pig ≈ 0. These results suggest that macrophages of guinea pigs lack "$LTC_4$ synthetase" and also this enzyme is different from usual GSH S-transferases. 

Leukotrienes are a series of lipoxygenase metabolites of arachidonic acid formed in numerous mammalian tissues and cells (1,2). Sulfidopeptide leukotrienes ($LTC_4$, $D_4$, and $E_4$) elicit enhanced vascular permeability (3) and profound spasm of various smooth muscles (4,5), while $LTB_4$ does chemotaxis for polymorphonuclear leukocyte and monocyte (6).

In contrast to cyclooxygenase, 5-lipoxygenase appears to be less widely distributed (7). Moreover, it has already been reported that the quantity and subclasses of LTs de novo synthesized are remarkably different among species and kinds of leukocytes and stimuli (7).

*To whom correspondence should be addressed.

Abbreviations: LT, Leukotriene; A23187, Calcium ionophore A23187; A.A., Arachidonic acid; DNCB, 2,4-dinitrochlorobenzene; $LTA_4$, Leukotriene $A_4$ (lithium salt); GSH S-transferase, Glutathione S-transferase.

0006-291X/85 $1.50

Synthesis of leukotrienes from peritoneal cells and adherent cells at 3 days after the intraperitoneal injection of thioglycollate medium was compared by using intact cells and supernates of homogenates of peritoneal cells among three species; rat, mouse, and guinea pig. Enzyme activities (supernates of centrifugation at 1,500 g of homogenates of peritoneal cells) were examined for GSH S-transferase and "$LTC_4$ synthetase" by using 2,4-dinitrochlorobenzene (DNCB) and leukotriene $A_4$ (lithium salt) as the substrates, respectively.

## MATERIALS AND METHODS

All organic solvents used were HPLC quality. The water was distilled and filtered using a Milli-Q system (Millipore Corp.). Synthetic leukotriene $C_4$, $D_4$, and $E_4$ were the gifts of Dr. S. Terao (Takeda Pharm. Comp., Osaka) and $LTB_4$ and $LTA_4$ methyl ester of Dr. M. Toda (Ono Pharm. Comp., Osaka). Other chemicals and drugs were commercially of high grade.

### High Performance Liquid Chromatography

The following columns and mobile phases were used (8): (i) Novapak $C_{18}$, 5 μm (Waters Assoc., 0.39 × 15 cm) with the mobile phase of acetonitrile/methanol/water/acetic acid (33.6 : 5.4 : 61 : 1, V/V) adjusted to pH 5.6 with triethylamine, (ii) a straight phase Radial-PAK cartridge, 5 μm (Waters Assoc., 0.8 × 10 cm) with that of hexane /isopropanol/acetic acid (95 : 5 : 0.01, V/V) in RCM-100 system. Flow rate was 1 ml/min for column (i), 4 ml/min for column (ii) and leukotrienes were monitored by ultraviolet absorbance at 280 nm. Retention times of $LTC_4$, $D_4$, $E_4$, and $B_4$ were approximately 6, 8, 10, and 17 min, respectively (on column (i)) and that of $LTB_4$ methyl ester being 8 min (on column (ii)).

### Cell Preparations

Male rats (S-D, body weight (b.w.) = 250 - 300g), mice (ICR, b.w. = 20 - 25g), and guinea pigs (Hartley, b.w. = 300 - 400g) were used. Twenty (for rats and guinea pigs) or two milliliters (for mice) of 2.5 % thioglycollate medium was injected into the peritoneal cavity. Animals were sacrificed and exsanguinated at 3 days after the injection. Peritoneal cells were harvested by washing the peritoneal cavity with phosphate buffered saline (PBS) containing 1 % volume of heparin. The harvested cells were gently washed twice with PBS and centrifuged at 220g for 10 min at 4 °C. The cell pellets were suspended in Tyrode's solution (NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.0, glucose 1.0 g/liter) at approximately pH 7.2 and separated in a 9 ml aliquot containing $5 \times 10^7$ cells. Furthermore, to gain adherent cells, a part of peritoneal cells was suspended in Eagle MEM that contained 10 % volume of fetal calf serum and then was poured onto plastic Petri dishes. After incubation for 2 hours at 37 °C in $CO_2$ incubator (95 % air + 5 % $CO_2$), the adherent cells were detached by pouring with cold PBS and gently rubbing the dishes with a rubber policeman. The adherent cells were suspended in Tyrode's solution and separated in a 9 ml aliquot each containing $5 \times 10^7$

cells except for mice. Cell viability examined by Trypan blue was ascertained to be more than 95 %. Cell differentiation was identified using Giemsa's stain.

Preparation of Enzyme Solutions

Peritoneal cell pellets were incubated for 10 min after the addition of 0.83 % $NH_4Cl$/170 mM Tris-HCl (9:1, V/V) for hemolysis of RBC. And then, cells were suspended in the buffer (NaCl 137, KCl 2.6, $NaH_2PO_4$ 0.36, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (Hepes) 10, EDTA 1 mM, pH 7.0) (9) and homogenized by ultrasonication. The suspension was centrifuged at 1,500g for 20 min at 4 °C and the supernate was used as a enzyme solution. The protein content of the enzyme solution was estimated using the Lowry procedure and used bovine serum albumin (BSA) as the standard.

Generation of LTs from intact cells

One milliliter of 50 mM, L-cysteine was added into each aliquot (final concentration, 5 mM) and preincubated at 37 °C for 3 min. After addition of calcium ionophore A23187 (5 μg/ml), each aliquot was further incubated for 20 min. The reaction was terminated by the addition of 4 volumes of cold ethanol into each aliquot and stored for 2 h at 4 °C. After the centrifugation at 900g for 10 min at 4 °C, the supernate was evaporated to dryness under reduced pressure. The residue was incubated for 30 min at 37 °C after the addition of 1 ml of 0.1 N NaOH and thereafter dissolved with 10 ml of distilled water. After neutralization by 1 N HCl, the solution was applied to Amberlite XAD-8 column (1 × 4 cm). The eluate with 80 % ethanol was flash evaporated to dryness and thereafter resuspended in 0.1 ml of the HPLC mobile phase. This (0.02 ml) was injected into the RP-HPLC. Recovery rate of $LTC_4$, $D_4$, $E_4$, and $B_4$ by uv absorbance was approximately 51, 41, 43, and 55 %, respectively. A $LTB_4$ fraction was collected and reinjected into the Novapak column with a mobile phase of acetonitrile/methanol/water/acetic acid (33.6 : 5.4 : 61 : 0.02, V/V), adjusted to pH 5.6 with $NH_4OH$. The $LTB_4$ fraction was recollected and after esterification with ethereal diazomethane, the $LTB_4$ methyl ester was injected into a straight phase Radial-PAK cartridge.

GSH S-transferase activity

GSH S-transferase activity was assayed as described by Habig and Jakoby (10) using 2,4-dinitrochlorobenzene (DNCB) as the substrate in pH 6.5 buffer (50 mM each Hepes and 4-morpholineethanesulfonic acid (Mes)), and 1 mM glutathione (reduced form) and by following the kinetics of the reaction spectrophotometrically (at 340 nm) on a double wavelength, double beam spectrophotometer (Hitachi, 557) at 30 °C.

Saponification of $LTA_4$ methyl ester

An aliquot of the $LTA_4$ methyl ester stock solution (37.5 μg) was stripped of solvent under a stream of $N_2$, treated with 100 μl of tetrahydrofuran and 5 μl of 0.1 M LiOH, and allowed to stand at room temperature for 48 h. $LTA_4$ (lithium salt) was recovered in approximately 60 % yield (personal communication to Dr. M. Toda).

Incubation conditions

Enzyme solution (500 μl) and 15 mM GSH (100 μl) were preincubated for 1 min at 37 °C (in duplicate). After the addition of 50 μl

(150 μM) of $LTA_4$ (lithium salt) in suspending buffer containing 10 mg/ml of BSA (11,12), each aliquot was further incubated for 10 min. The reaction was terminated by addition of 4 volumes of cold ethanol and stored for 2 h at 4 °C. After centrifugated at 1,500g for 10 min at 4 °C, evaporated to dryness, and suspended in 0.1 ml of the HPLC mobile phase, this (0.02 ml) was injected into the RP-HPLC.

Spasmogenic activity of the HPLC eluate was axamined at 37 °C by using a guinea pig ileum in 5 ml organ bath with Krebs-Henseleit solution (NaCl 122, KCl 6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, glucose 11.5 in mM). Administration of the RP-HPLC mobile phase in 0.5 % or less into the organ bath did not elicit any significant contraction of the ileum.

## RESULTS AND DISCUSSION

Peritoneal cells harvested from mice, rats, and guinea pigs at 3 days after the intraperitoneal injection of 2.5 % thioglycollate medium were composed of macrophages in more than 80 %. Adherent cells recovered after incubation of peritoneal cells for 2 hours were composed of macrophages in more than 90 %. The peritoneal cells and macrophages of mice generated $LTC_4$, $D_4$, and $B_4$ when stimulated with calcium ionophore A23187 (5 μg/ml), as shown in Figure 1. These three peaks were manually collected and then their uv spectra were monitored. The fractions of $LTC_4$, $LTD_4$, and $LTB_4$ showed uv maxima at 270,281, 290 nm for the former two and 260, 270, 282 nm for the latter one. Moreover, $LTC_4$ and $LTD_4$ fractions elicited a characteristic contraction of guinea pig ileum by administration into organ bathes in 0.5 % and completely antagonized by FPL 55712 (1 μM or less). $LTB_4$ fraction was further analyzed on straight phase HPLC after methyl esterification. This chromatogram exhibited the identical retention time with synthetic $LTB_4$ methyl ester and almost a single peak.

Peritoneal cells and macrophages of rats generated $LTC_4$, $LTD_4$, and $LTB_4$. $LTB_4$ was generated in as large a quantity as $LTC_4$ (Fig. 2). Amounts of $LTC_4$ were more abundant in the macrophages than the peritoneal cells in comparison with equal cell numbers. $LTB_4$ fraction consisted of almost a single peak on the straight phase HPLC, being the same as mice.

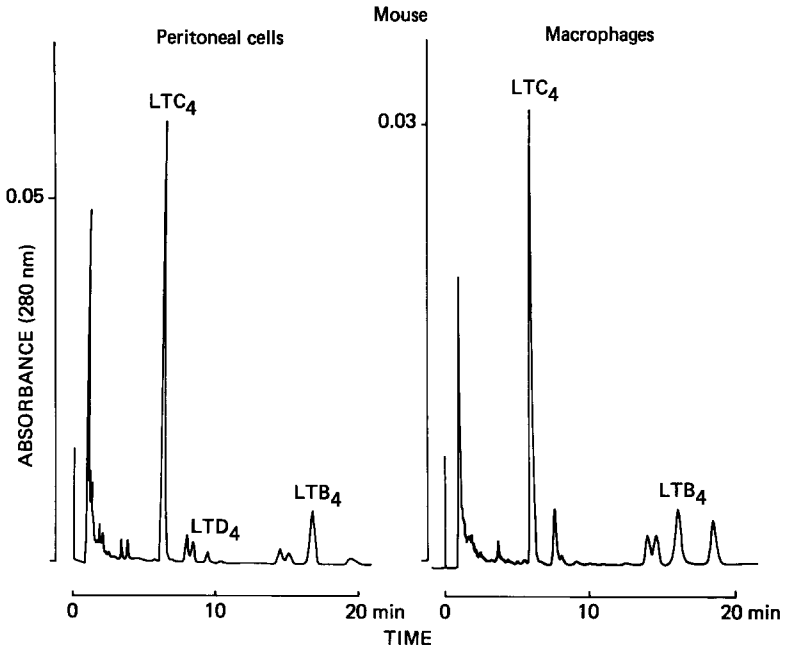


Fig. 1. Reversed-phase HPLC profiles of LTs generated from the peritoneal cells ($5 \times 10^7$ cells) and the macrophages ($2.5 \times 10^7$ cells) from the mice when stimulated with A23187 (5 μg/ml). The column used was Novapak, $C_{18}$ and the solvent system was acetonitrile/methanol/water/acetic acid (33.6 : 5.4 : 61 : 1, V/V) adjusted to pH 5.6 with triethylamine. The flow rate was 1 ml/min and uv absorbance was monitored at 280 nm.

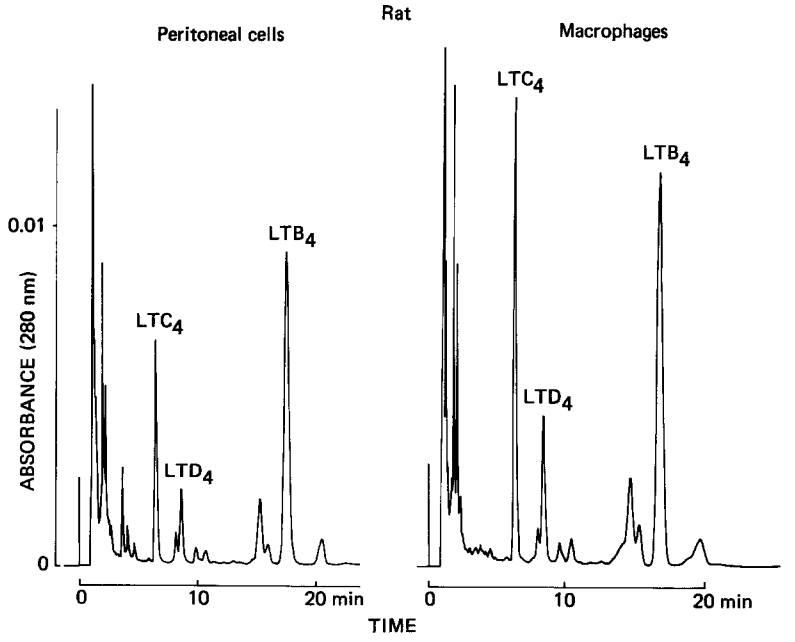


Fig. 2. RP-HPLC profiles of LTs generated from the peritoneal cells ($5 \times 10^7$ cells) and the macrophages ($5 \times 10^7$ cells) from the rats. Others are the same as those in Fig. 1.

On the contrary, peritoneal cells and macrophages harvested from guinea pigs did not generate detectable peaks of $LTC_4$, $D_4$, and $E_4$, but did show a small amount of $LTB_4$ (Fig. 3, (A)). After simultaneous addition of arachidonic acid (10 μM), generation of $LTB_4$ was significantly potentiated, but those of $LTC_4$, $D_4$, and $B_4$ were still undetectable (Fig. 3, (B)). Neither endotoxin (10 μg/ml) nor muramyl dipeptide (MDP, 10 μg/ml) could stimulate detectable generation of $LTC_4$, $D_4$, $E_4$, and $B_4$ (not shown). On the basis of the combined data, it is suggested that there are significant differences among the species in the generation of LTs and, furthermore, macrophages of guinea pigs generate $LTB_4$, but not sulfidopeptide LTs. When one considers that guinea pigs have been utilized for such a long time as experimental models for immediate-type hypersensitivity and acute inflammation (13), this was an unexpected result. Such a defect could be contributed to two enzyme levels, either a defect of 5-lipoxygenase and/or glutathione S-transferase (14). Since $LTB_4$ was generated with and without addition of exogeneous arachidonic acid, the existence of 5-lipoxygenase was demonstrated in this species.

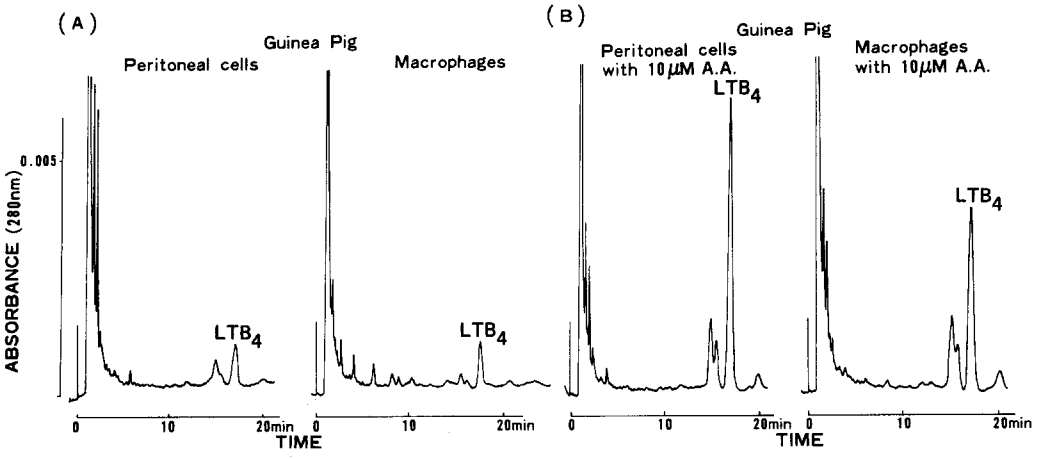


Fig. 3. RP-HPLC profiles of LTs generated from the peritoneal cells ($5 \times 10^7$ cells) and the macrophages ($5 \times 10^7$ cells) from the guinea pigs. (A) without addition of arachidonic acid and (B) with simultaneous addition of arachidonic acid (10 μM). Others are the same as those in Fig. 1.

Table 1. Comparison of GSH S-transferase and " $LTC_4$ synthetase" activity among mouse, rat, and guinea pig

| Species | GSH S-transferase (nmol/mg protein/min) | $LTC_4$ synthetase (pmol $LTC_4$/mg protein/min) |
|---|---|---|
| Mouse | 42.5 ± 1.9 (3) | 19.7 (2) |
| Rat | 9.93 ± 0.74 (3) | 1.57 (2) |
| Guinea Pig | 52.0 ± 1.7 (3) | not detected (2) |

Values are expressed as mean ± S.E.M. for GSH S-transferase and the mean of the two aliquots for "$LTC_4$ synthetase". Number in parentheses indicates number of aliquots. Procedures of enzyme preparations, saponification of $LTA_4$ methyl ester, and assay for GSH S-transferase and "$LTC_4$ synthetase" activities are mentioned in Materials and Methods.

A further experiment using enzyme solutions from these three species was done next. Supernates of centrifugation at 1,500 g of homogenates of peritoneal cells, which were harvested from the peritoneal cavities at 3 days after the intraperitoneal injection of thioglycollate medium, were utilized as the enzyme solutions. So, the glutathione S-transferase activities were compared among these three species by using $LTA_4$ (lithium salt) and 2,4-dinitrochlorobenzene (DNCB) as the substrates, respectively. The enzyme solutions of these three species effectively converted DNCB to a colored product (at 340 nm) in the presence of glutathione and the orders of its potencies were as follows: guinea pig $>$ mouse $>$ rat (Table 1). On the other hand, the enzyme solutions of mice effectively converted $LTA_4$ to $LTC_4$ during the incubation for 10 min and its efficiency of conversion was approximately 2.5 % per mg protein (mean of two aliquots), as shown in Fig. 4. The converted $LTC_4$ was identified by uv spectrum of the $LTC_4$ fraction on the RP-HPLC and contractility of guinea pig ileum by administration of its fraction into the organ bath and its antagonism by FPL 55712 (the inserts in Fig. 4). The enzyme solution of rats also converted $LTA_4$ to $LTC_4$, but the efficiency of conversion was lower (approximately 0.2 % as

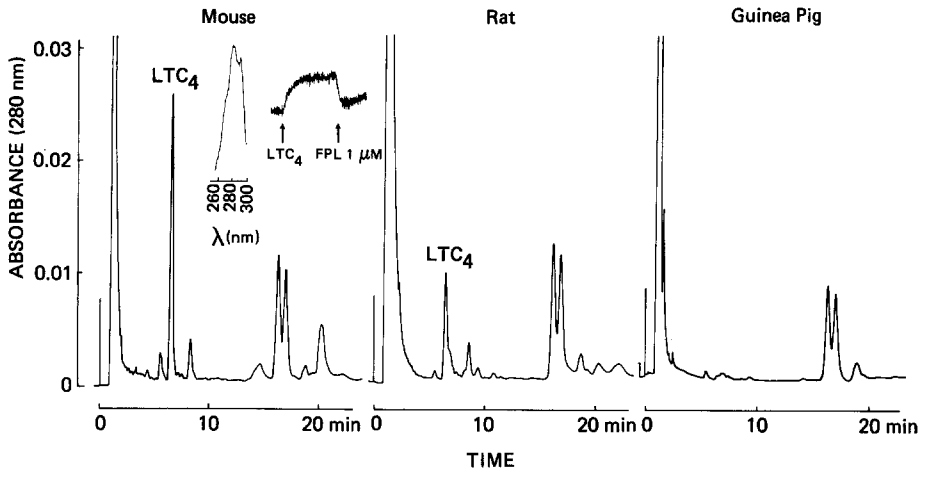


Fig. 4. RP-HPLC profiles of the products from the incubation of enzyme solutions from mice, rats, and guinea pigs with GSH and $LTA_4$. Incubation conditions and enzyme preparations were described in Materials and Methods. The inserts show a uv spectrum of the collected fraction corresponding to $LTC_4$ after the elimination of absorbance of the HPLC mobile phase and a contraction of a guinea pig ileum induced by the administration of this fraction in 0.5 % into an organ bath. This contraction was antagonized by FPL 55712 (1 μM). Others are the same as those in Fig. 1.

the mean of the two aliquots). On the other hand, enzyme solution of guinea pigs did not convert $LTA_4$ to $LTC_4$ at all. Therefore, the potencies of converting $LTA_4$ to $LTC_4$ were in the following order; mouse $>$ rat $\gg$ guinea pig $\approx 0$.

On the basis of this combined data, it is suggested that peritoneal macrophages of guinea pigs lack an enzyme which catalyzes a reaction of conjugation of glutathione with epoxide-base of $LTA_4$, tentatively named "$LTC_4$ synthetase". Moreover, the remarkable discrepancy in potencies of enzyme activities between GSH S-transferase using DNCB as the substrate and "$LTC_4$ synthetase" using $LTA_4$ as the substrate among these three species suggest that "$LTC_4$ synthetase" may be a unique enzyme, as pointed out by Bach et al. (9). The possibility that the enzyme activity of "$LTC_4$ synthetase" of guinea pigs may be inhibited by some inhibitors cannot be ruled out. An experiment to elucidate this point is in progress.

ACKNOWLEDGMENTS

We are grateful to Dr. Y. Koshihara for critical discussion.

This work has been presented at Kyoto Conference on Prostaglandins, Kyoto, Nov. 25-28, 1984

REFERENCES

1. Samuelsson, B., Borgeat, P., Hammarstrom, S., and Murphy, R.C. (1979) Prostaglandins 17: 785-787.
2. Samuelsson, B. and Hammarstrom, S. (1980) Prostaglandins 19: 645-648.
3. Dahlen, S.E., Bjork, J., Hedqvist, P., Arfors, K.E., Hammarstrom, S., Lindgren, J.A., and Samuelsson, B. (1981) Proc. Natl. Acad. Sci. USA. 78: 3887-3891.
4. Hedqvist, P., Dahlen, S-E, Gustafsson, L., Hammarstrom, S., and Samuelsson, B. (1980) Acta Physiol. Scand. 110: 331-333.
5. Sirois, P., Roy, S., Tetrault, J.P., Borgeat, P., Picard, S. and Corey, E.J. (1981) Prostaglandins Med. 7: 327-340.
6. Goetzl, E.J. and Pickett, W.C. (1981) J. Exp. Med. 153: 482-487.
7. Lewis, R.A. and Austen, K.F. (1984) J. Clin. Invest. 73: 889-897.
8. Abe, M., Kawazoe, Y., and Shigematsu, N. (1985) Anal. Biochem. 144: in press.
9. Bach, M.K., Brashler, J.R., and Morton, D.R. (1984) Arch. Biochem. Biophys. 230: 455-465.
10. Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974) J. Biol. Chem. 249: 7130-7139.
11. Fitzpatrick, F.A., Morton, D.R., and Wynalda, M.A. (1982) J. Biol. Chem. 257: 4680-4683.
12. Maycock, A.L., Anderson, M.S., DeSousa, D.M., and Kuehl, F.A. (1982) J. Biol. Chem. 257: 13911-13914.
13. Brocklehurst, W.E. (1960) J. Physiol. 151: 416-435.
14. Samuelsson, B. (1983) Science 220: 568-575.