Metabolism of Thromboxane B2 in the Monkey*

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[1H3]Thromboxane B2 was biosynthesized and infused into an unanesthetized monkey. Several urinary metabolites were isolated and their structures elucidated using gas chromatography-mass spectrometry. In addition to the major urinary metabolite, dinor-thromboxane B2, a series of metabolites resulting from dehydrogenation of the alcohol group at C-11 were identified: 11-dehydro-thromboxane B2, 11-dehydro-15-keto 13,14-dihydro-2,3-dinor-thromboxane B2, and 11-dehydro-15-keto-13,14-dihydro-19-carboxyl-2,3,4,5-tetranor-thromboxane B2. 6-(3,3-dihydroxypropyl)-7-hydroxy-10-oxo-3-pentadecenoic acid was also identified. Three mono-O-ethylated metabolites were formed from thromboxane B2, in which this study was infused in an ethanolic solution. A small quantity of thromboxane B2 was excreted unchanged into the urine.

The thromboxanes, thromboxane A2 and TxB2, are a recently described class of compounds shown to be derived from PGF2 (1). The major pathway of PGF2 metabolism in human platelets, guinea pig lung, and rat and guinea pig brain is to the thromboxanes (2-4). In the metabolic sequence, PGF2 is converted to TXA2, which subsequently degrades to TxB2 by the incorporation of a molecule of water with a t1/2 of 32 s at 37°C in aqueous media (1). TXA2 possesses significant biological activity, inducing irreversible platelet aggregation (1) and causing potent contraction of vascular smooth muscle (5). Except for possibly acting as a chemotactic agent (6), TxB2, however, has not been shown to possess any significant biological activity. Because of the biological significance of TXA2, quantification of its synthesis under various conditions has therefore seemed of importance to identify the urinary metabolites of TxB2, the structures of which could provide a basis for future quantitative studies of thromboxane synthesis in vivo. This paper describes the identification of several urinary metabolites of TxB2 in the monkey. The nonhuman primate was used in this initial study because species differences in prostaglandin metabolism have been demonstrated and, at least regarding metabolism of PGF2, the monkey has been found to be the animal species which most closely approximates human prostaglandin metabolism (9). A preliminary report of part of this work describing the identification of the major urinary metabolite as dinor-TxB2 has been published earlier (10).

EXPERIMENTAL PROCEDURES

Materials

Sheep seminal vesicle glands were the generous gift of Dr. John Fike, The Upjohn Co., Kalamazoo, Mich. We are indebted to Dr. N. A. Nelson of The Upjohn Co. for a generous gift of 11-dehydro-thromboxane B2 methyl ester. Arachidonic acid was obtained from Nu-Chek Prep., Inc., Elysian, Minn. (U-13C, 14C). 6-Hydroxy-2,3,4,5-tetradecenoic acid (64 Ci/mmol) from New England Nuclear, Boston, Mass.

Thromboxane B2-[1H3]Arachidonic acid (8.93 pCi/mmol) was prepared and, the prostaglandin endoperoxides (PGH2, PGF2) were biosynthesized using sheep seminal vesicle glands as previously described (11). TxB2 was biosynthesized from the prostaglandin endoperoxides using guinea pig lung microsomes by a procedure recently described in detail (10), yielding a total of 1.25 mg of [1H3]TxB2 (6.90 µCi/mmol).

Methods

Infusion of Thromboxane R-1[1H3]TxB2 (1.25 mg, 6.90 µCi/mmol) was infused into a cynomolgus male monkey as previously described (10).

Amberlite XAD-2 and Silicic Acid Chromatography—Extraction of urine was performed on Amberlite XAD-2 and the crude extract subjected to silicic acid chromatography as previously described (10).

Reversed Phase Partition Chromatography—Reversed phase partition chromatography was performed as previously described (12). Solvent systems used are listed in Table I. Stationary phases were supported on 4.5 g of hydrophobic Hyfla Super-Cel (Fisher Scientific Co., Fair Lawn, N. J.). Fractions (5 ml) were collected.

High Performance Liquid Chromatography—High performance liquid chromatography was performed using a model U6K injector, model 660 solvent programmer, and two model 600A solvent pumps (Waters Associates, Milford, Mass.). A flow rate of 1 ml/min was used, and 1-ml fractions were collected. Chromatography performed on a p-Porasil column (30 cm x 6 mm outer diameter) (Waters Associates) with a gradient of 100% chloroform to 100% methanol/acetone (50:50) in 2 h as previously described (13). Chromatography performed on p-Bondapak...
Eighty per cent of infused radioactivity was recovered in the urine in 8 h, the majority of which was excreted within 2 h. No clinically apparent adverse effects were observed.

Preparation of Derivatives for Gas-Liquid Chromatography and Mass Spectrometry—Methyl ester, ethyl ester, O-methyloxime, and trimethylsilyl ether derivatives were formed as previously described (10). Ethyl ester, deuterated O-methyloxime, and deuterated trimethylsilyl ether derivatives of every metabolite identified were analyzed by mass spectrometry to determine the total number of carboxyl, methoxime, and trimethylsilyl groups present and to aid in the interpretation of structures of various fragment ions in the mass spectra.

Reaction with Ethanolic HCl—An ethanolic HCl solution was prepared by dissolving 50 μl of redistilled acetaldehyde in 1 ml of ethanol. Approximately 20 μg of the ethyl ester of the compound was dissolved in 0.5 ml of ethanol and 0.5 ml of the ethanolic HCl solution added. After 30 min at room temperature, the reaction mixture was evaporated to dryness under a stream of nitrogen, and the residue treated with excess ethereal diazomethane.

Reaction with Sodium Methoxide—A 0.5 M solution of sodium methoxide was prepared by dissolving sodium in methanol. Freshly cut sodium was washed extensively with toluene and then hexane before addition to the methanol. The sodium methoxide solution was then passed through a 0.45 Millipore filter. The solution (100 μl) was added to 1 to 10 μg of the methyl ester of the compound. After 20 min at room temperature, 1 ml of 0.01 M sodium phosphate (pH 5.0) was added and the mixture acidified to pH 5.0 with 0.01 M HCl. The compound was then extracted into ethyl acetate and the solvent evaporated under reduced pressure.

Nomenclature and Carbon-numbering System—Chemical nomenclature and carbon numbering of the TxB2 molecule in the past has usually referred to the acyclic aldehyde-alcohol form of TxB2 instead of the hemiacetal. Several of the TxB2 metabolites identified in this work, however, although retaining a 6-member ring (tetrahydropyran), are not hemiacetals. Although these metabolites can be described by established chemical nomenclature, this nomenclature and the carbon numbering is necessarily totally different than that previously used for TxB2. This complicates the structural comparison of these metabolites with TxB2 and hinders lucid description of the metabolic pathways. These problems could be eliminated if a basic system of nomenclature was adopted for the thromboxanes similar to the prostanoic acid system used for the prostaglandins (15). We, therefore, are proposing that the basic 20-carbon-substituted tetrahydropyran with the carbon-numbering system as illustrated in Fig. 1 be called thrombanoic acid. Using this system, for example, the chemical nomenclature for TxB2 would be 9α,11,15(S)-trihydroxy-thromba-5,13-dienoic acid. Such a system provides both a useful platform for describing the thromboxanes and their metabolites, as well as a nomenclature and carbon-numbering system similar to that used for the prostanoic acids and is used in this paper unless otherwise indicated or obvious.

RESULTS

At no time during the infusion of TxB2 was there any significant change in blood pressure or pulse rate of the monkey. No clinically apparent adverse effects were observed. Eighty per cent of infused radioactivity was recovered in the urine in 8 h, the majority of which was excreted within 2 h.

A flow diagram outlining the urine purification procedures that are subsequently described and the isolation and letter-number designation of identified TxB2 metabolites is illustrated in Fig. 2.

The urine collected was extracted with Amberlite XAD-2 chromatography. Ninety per cent of the applied radioactivity was eluted with 400 ml of ethanol. The residue obtained after evaporation of the ethanol was dissolved in ethyl acetate and purified by silicic acid chromatography. Radioactivity was eluted quantitatively with 300 ml of ethyl acetate.

Further sample purification and initial compound isolation was achieved by reversed phase partition chromatography using Solvent System D (Table I). The sample was dissolved in 0.5 ml of mobile phase with 1 drop of acetic acid added immediately prior to application to the column. Two polar peaks emerged, designated A and B, followed by elution of a substantial amount of less polar material, designated Area C, that was not resolved by this solvent system (Fig. 3). Additional radioactivity was eluted from the stationary phase with 50 ml of methanol. Total radioactivity recovered from that applied to the column was 65% with 10% in Peak A, 18% in Peak B, 38% in Area C, and 34% was eluted from the stationary phase with methanol. The total radioactivity recovered at this stage from the amount originally infused was 47%.

Identification of Compounds Eluted with Methanol from Stationary Phase—Material eluted with methanol was subjected to reverse phase partition chromatography using Solvent System C-45 (Table I). Four radioactive peaks appeared (Fig. 4) designated M1 (40 to 70 ml of eluate, 9% of recovered radioactivity), M2 (75 ml 115 ml, 25%), M3 (120 to 180 ml, 30%), and M4 (185 to 295 ml, 36%). Material in Peak M1 was subsequently subjected to high performance liquid chromatography on μ-Porasil, but sufficient quantity of material was not present in any radioactive peak to permit identification.

Structures of Compounds in Peak M3—Material in Peak M3 was further purified by high performance liquid chromatography on μ-Porasil. Two prominent peaks emerged, one of which was designated Compound M3 (40 to 45 ml of eluate, 59% of total recovered radioactivity) and the other (31 to 34 ml, 13%) was subsequently determined to be a minor amount of Compound C3 (see below). Compound M3 represented approximately 6% of the total radioactivity present in the urine. Using an identical solvent program, TxB2 elutes from μ-Porasil at 48 to 53 ml. It was, therefore, determined that Compound M3 was less polar than parent TxB2 in this chromatographic system. Gas-liquid chromatographic analysis of the Me3Si ether derivative of the methyl ester of Compound M3 showed a major peak designated M3a and a minor peak.

### Table I

<table>
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**FIG. 1.** Proposed structure and carbon-numbering system for thrombanoic acid.
designated M3b, with C values of 26.5 and 24.0, respectively. Although significantly less polar than parent TxB2 on µ-Porasil chromatography, M3a has a significantly higher C value than the Me:Si ether derivative of the methyl ester of parent TxB2 (23.6) (10). The mass spectrum obtained for the Me:Si ether derivative of the methyl ester of M3b is shown at the top of Fig. 6. Ions were present at m/e 630 (M); 615 (M-15), loss of -CH3; 599 (M-31), loss of -OCH3; 549 (M-90), loss of -MeSiOH: 509 [m/e 509 + 31], 489 [m/e 489 + 60]; 469 [m/e 469 + 90]; 438 [m/e 438 + 120]; 413 [m/e 413 + 150]; 379 [m/e 379 + 180]; 358 [m/e 358 + 210]; 337 [m/e 337 + 240]; 315 [m/e 315 + 270]; 295 [m/e 295 + 300]; 274 [m/e 274 + 330]; 253 [m/e 253 + 360]; 232 [m/e 232 + 390]; 211 [m/e 211 + 420]; 190 [m/e 190 + 450]; 169 [m/e 169 + 480]; 148 [m/e 148 + 510]; 127 [m/e 127 + 540]; 106 [m/e 106 + 570]; 85 [m/e 85 + 600]. A dicarboxylic acid structure was suggested by an increase in C value of 0.9 on gas-liquid chromatographic analysis of the Me:Si ether derivative of the ethyl ester, since a difference of 0.45 to 0.5 C value has been observed for methyl and ethyl esters of monocarboxylic acid TxB2 derivatives (10). This was confirmed by analysis of the ethyl ester by mass spectrometry where the molecular ion of the methyl ester was found to shift from m/e 630 to m/e 658. The location of the second carboxyl group at the original C-11 position was suggested by an increase in C value of 0.9 on gas-liquid chromatographic analysis of the Me:Si ether derivative of the ethyl ester, since a difference of 0.45 to 0.5 C value has been observed for methyl and ethyl esters of monocarboxylic acid TxB2 derivatives (10). This was confirmed by analysis of the ethyl ester by mass spectrometry where the molecular ion of the methyl ester was found to shift from m/e 630 to m/e 658. The location of the second carboxyl group at the original C-11 position was suggested by the prominent ion at m/e 175 in the mass spectrum of the methyl ester and its shift to m/e 189 in the corresponding mass spectrum of the ethyl ester derivative. The δ-lactone, 11-dehydro-TxB2, should be in equilibrium with a dicarboxylic acid structure in the presence of water, and the mass spectral data on M3b indicated that this was the dicarboxylic acid form of M3a or 3-hydroxy-4-(1,4-diroyl-2-nononyl)-6-undecenoic acid. The mass spectrum recorded for the Me:Si ether derivative of the methyl ester of M3b is shown at the top of Fig. 6. Ions were present at m/e 630 (M); 511 (M-15), loss of -CH3; 495 (M-31), loss of -OCH3; 455 (M-71), loss of -(CH3)2CH3; 436 (M-90), loss of MeSiOH: 413 [m/e 413 + 150]; 399 [m/e 399 + 180]; 378 [m/e 378 + 210]; 357 [m/e 357 + 240]; 337 [m/e 337 + 270]; 316 [m/e 316 + 300]; 295 [m/e 295 + 330]; 274 [m/e 274 + 360]; 253 [m/e 253 + 390]; 232 [m/e 232 + 420]; 211 [m/e 211 + 450]; 190 [m/e 190 + 480]; 169 [m/e 169 + 510]; 148 [m/e 148 + 540]; 127 [m/e 127 + 570]; 106 [m/e 106 + 600]. A dicarboxylic acid structure was suggested by an increase in C value of 0.9 on gas-liquid chromatographic analysis of the Me:Si ether derivative of the ethyl ester, since a difference of 0.45 to 0.5 C value has been observed for methyl and ethyl esters of monocarboxylic acid TxB2 derivatives (10). This was confirmed by analysis of the ethyl ester by mass spectrometry where the molecular ion of the methyl ester was found to shift from m/e 630 to m/e 658. The location of the second carboxyl group at the original C-11 position was suggested by the prominent ion at m/e 175 in the mass spectrum of the methyl ester and its shift to m/e 189 in the corresponding mass spectrum of the ethyl ester derivative. The δ-lactone, 11-dehydro-TxB2, should be in equilibrium with a dicarboxylic acid structure in the presence of water, and the mass spectral data on M3b indicated that this was the dicarboxylic acid form of M3a or 3-hydroxy-4-(1,4-diroyl-2-nononyl)-6-undecenoic acid.
FIG. 5. Top, mass spectrum of the methyl ester, Me3Si ether derivative of Metabolite M3a. Bottom, mass spectrum of the methyl ester, Me3Si ether derivative of chemically synthesized 11-dehydro-thromboxane B2.

When the methyl ester of M3 was treated with methoxyamine HCl for approximately 18 h at room temperature and then converted to a Me3Si ether derivative and analyzed by gas chromatography, a peak with a C value of 24.5 was present in addition to the two peaks previously noted for M3a and M3b at C values of 26.5 and 24.0, respectively. The M3a peak had also been apparently reduced in relative size proportional to the size of the peak at C 24.5, approximately 30 to 50%. This suggested that M3a had been partially converted to a less polar derivative by treatment with methoxyamine HCl. A known reaction of a lactone with hydroxyamine is the formation of a hydroxamic acid (16). Methoxyamine would be expected to react in a similar fashion. It, therefore, seemed reasonable to suspect that the lactone 11-dehydro-TxB2 had been converted to the acyclic amide derivative illustrated in Fig. 7. The expected molecular ion of this compound as a trimethylsilyl ether derivative is m/e 645. However, when this derivative at C 24.5 was analyzed by mass spectrometry, the highest ion seen was m/e 614, and no deuterium-containing ions were detected in the mass spectrum of the corresponding derivative formed after treatment with trideuteromethoxyamine. These data suggested that the ion at m/e 614 was not the molecular ion. If we assume a molecular ion of m/e 645, the interpretations of the prominent ions seen in the mass spectrum of this derivative are as follows: m/e 614 (M-31), loss of [OCH3]+ 613 (M-32), loss of CH3OH, 586 [M-(32 + 15)], loss of [32 + ·CH3]+ 542 [M-(71 + 32)], loss of [·(CH3)3CH3 + 32]; 523 [M-(90 + 32)], loss of [Me3SiOH + 32]; 382 [M-(141 + 90 + 32)], loss of [·CH2-CH=CH-(CH2)3COOCH3 + 90 + 32]; 365 [M-(190 + 90)], loss of [Me3SiO=CH-CH=CH-C(OH)CH3 + 90]; 350 [M-(173 + 90 + 32)]; 301 (base) [Me3SiO=CH-CH=CH-C(OH)(Me3)CH2]; 211 (301-90), and 173 [Me3SiO=CH-(CH2)3CH3]. This mass spectrum is consistent with the acyclic amide derivative, and we hypothesize that ·OCH3 or CH2OH is very readily eliminated from the amide upon electron impact. This would explain the absence of a visible molecular ion, the presence of many ions formed as a result of the combined loss of methanol plus another moiety, and the absence of deuterium-retaining ions in the derivative formed by treatment with trideuteromethoxyamine. It is interesting that amide derivatives have not been described after methoxyamine treatment of the α-lactone of 6α,7α-dihydroxy-11-keto-tetranorprosta-1,16-dioic acid (17).

To obtain further evidence for the structure of M3a, chemically synthesized 11-dehydro-TxB2 methyl ester was converted to a Me3Si ether derivative and analyzed by gas chromatography-mass spectrometry. This gave a peak with a C value of 26.5 (identical with M3a) and the mass spectrum recorded is seen at the bottom of Fig. 5. It is apparent that this mass spectrum is identical with that of the same derivative of metabolite M3a seen at the top of Fig. 5. When chemically synthesized 11-dehydro-TxB2 methyl ester treated with methoxyamine, converted to a Me3Si ether derivative, and analyzed by gas chromatography-mass spectrometry, an additional peak with a C value of 24.5 was present, and the
FIG. 6. Top, mass spectrum of the methyl ester, Me₂Si ether derivative of Metabolite M3b. Bottom, mass spectrum of the methyl ester, Me₂Si ether derivative of the product formed after treatment of the methyl ester of 11-dehydro-thromboxane B₂ with sodium methoxide.

mass spectrum obtained was identical with that obtained for the derivative of M3 at C 24.5 formed after treatment with methoxyamine.

To obtain additional evidence for the lactone ring structure and the apparent dicarboxylic acid structure of M3b, the chemically synthesized 11-dehydro-TxB₂ methyl ester was treated with sodium methoxide in methanol. This would be expected to convert the lactone to the dicarboxylic acid methyl ester structure as illustrated in Fig. 8. The product of this reaction when converted to a Me₂Si ether derivative and analyzed by gas chromatography-mass spectrometry gave a peak with a C value of 24.0 and the mass spectrum recorded is seen at the bottom of Fig. 6. When compared to the mass spectrum of metabolite M3b at the top of Fig. 6, it is apparent that the two spectra are essentially the same except for minor variation in low mass ions, presumably due to co-eluting impurity.

The additional confirmatory evidence obtained from the analysis of the chemically synthesized compound lead us to conclude that Compound M3a was the δ-lactone, 11-dehydro-TxB₂, and that its corresponding dicarboxylic acid was Compound M3b. Using thromboxanoic acid nomenclature, 11-dehydro-TxB₂ is 9α,15(S)-dihydroxy-11-oxo-thromboxa-5,13-dienoic acid.

It is unlikely that the δ-lactone and the dicarboxylic acid would co-chromatograph throughout all of the chromatographic systems employed during urine processing and only be separated by gas-liquid chromatography. As a free acid or methyl ester the δ-lactone 11-dehydro-TxB₂ would be predicted to be less polar than parent TxB₂ in both the straight phase and reversed phase partition chromatographic systems. The methyl esters of TxB₂ and chemically synthesized 11-dehydro-TxB₂ were subjected to thin layer chromatography with the use of the solvent system benzene/dioxane.
Fig. 8. Conversion of the methyl ester of 11-dehydro-thromboxane B₂ to a dicarboxylic acid methyl ester structure by reaction with 0.5 N sodium methoxide.

Fig. 9. Mass spectrum of the methyl ester, methoxime, trimethylsilyl ether derivative of Compound M4a.

Identification of Compounds Eluting in Peak M4—Material in Peak M4 was further purified by μ-Porasil high performance liquid chromatography, and two discrete peaks emerged. The less polar peak (25 to 28 ml of effluent, 15% of recovered radioactivity) was designated Compound M4a, and the more polar peak (34 to 36 ml, 44%) was designated Compound M4b. M4a represented approximately 1.8% and M4b approximately 5.4% of the total radioactivity in the urine.

The methyl ester of M4a was converted to an O-methyloxime, Me₅Si ether derivative and analyzed by gas chromatography-mass spectrometry. Essentially a single peak was found with a C value of 22.8. The mass spectrum of this derivative of M4a (Fig. 9) showed prominent ions at m/e 603 (M); 588 (M-15), loss of CH₂; 572 (M-31), loss of OCH₃; 513 (M-90), loss of Me₅SiOH; 482 [M-(90 + 57) + 1]; 457 [M-(90 + 57) + 1]; 426 [M-(90 + 31 + 57) + 1]; 400 [M-(113 + 90)], loss of CH₂-CH₂-COCH₃; 371 [M-(142 + 90)], loss of CH₂-C(=NOCH₃)(CH₃)₂CH₃; 360 [M-(153 + 90)], loss of CH₂-C(=NOCH₃)(CH₃)₂CH₃; 298 [Me₅SiO(CH₂)₃CH₃], 258 [Me₅SiO(CH₂)₃CH₃], and 219 [Me₅SiO(CH₂)₃CH₃]. Clues to the structure of M4a were found in the base ion m/e 258 and the prominent ion at m/e 219. Confirmation that the ion at m/e 219 contained two Me₅Si groups and the ion at m/e 258 contained one Me₅Si and one O-methyloxime group was obtained by analysis of [Me₅Si]₂Me₅Si and trideuteromethoxime derivatives. A base ion at m/e 258 is also found in the mass spectrum of the O-methyloxime, Me₅Si ether derivative of the methyl ester of 15-keto-13,14-dihydro-TxB₂ (18), and, therefore, suggested that M4a was an acyclic compound in which dehydrogenation of the alcohol group at the original C 15 position and reduction of the original Δ₁³ double bond had occurred. The conspicuous absence of a prominent ion at m/e 174 suggested that an acyclic aldehyde-alcohol structure was not present as is typically seen after formation of O-methyloxime, Me₅Si ether derivative of TxB₂ and TxB₂ metabolite with a hemiacetal ring (cf. Compound C1) (10, 18). However, a prominent ion at m/e 219 is also found in the mass spectrum of the methyl ester, Me₅Si ether derivative of the product formed by treatment of TxB₂ with sodium borohydride and indicates an acyclic structure with Me₅Si groups attached at the original C-11 and C-12 positions. The ion at m/e 400 formed in part by the loss of 113 mass units from the carboxyl side chain also suggested that M4a had undergone one step of β oxidation to a C18 derivative.

From these data it was, therefore, concluded that Compound M4a was 6-(1,3-dihydroxypropyl)-7-hydroxy-10-oxo-3-pentadecenoic acid.

The methyl ester of M4b was then converted to a Me₅Si ether derivative and analyzed by gas chromatography-mass spectrometry. A single radioactive peak was present with a C value of 22.2. The mass spectrum of this derivative of M4b

\[
\text{M₅SiO(CH₂)₃CH₃} \quad \text{Me₅SiO(CH₂)₃CH₃} 
\]
FIG. 10. Mass spectrum of the methyl ester, Me3Si ether derivative of Compound M4b.

(Fig. 10) showed prominent ions at m/e 528 (M); 513 (M-15), loss of CH2=CH2; 482 (M-46), loss of CH3CHOH; 457 (M-71), loss of CH2=CH2, 438 (M-90), loss of MeSiOH; 392 (M-(46 + 71)); 367 (M-190), loss of [OCH(OCH2CH3)CH2]2CH=OSiMe3, eliminated from the ring; 321 (M-90 + 71 + 46); 301, probably [Me3SiO'CH-C=CH=CH(OSiMe3)(CH2)2CH2], formed by rearrangement; 295; 228 +[MeSiO-CH=CH=CH-CH(OSiMe3)CH2]; and 173, probably derived from two sources: (a) [Me3SiO'CH=CH=CH(OSiMe3)(CH2)2CH2], and (b) Me3SiO'CH=CH=CH(OSiMe3)CH2]2CH2-CH=CH2-COOCH3, which is eliminated from the ring; 217, probably derived from the ring; 203, probably derived from the ring. The base ion at m/e 228 suggested that this compound had in part been formed by one step of $\beta$ oxidation resulting in the loss of 2 carbons from the upper side chain (see structure of Compound C1). Two Me3Si groups were indicated by analysis of the [1H]Me3Si ether derivative and were assigned locations at C-7 by a shift of m/e 228 to m/e 237 and at C-13 by a shift of m/e 217 to 216. The absence of a trimethylsilyl group at C-9 was suggested by the absence of the ion m/e 217 which is prominent in the mass spectrum of the Me3Si ether derivative of the methyl ester of dinor-TxB2. Dehydration of the hydroxyl group at C-9 would account for the loss of the C-9 hydroxyl group and would render the molecule less polar than dinor-TxB2. However, the expected molecular ion for the Me3Si ether derivative of the methyl ester of this compound would be m/e 482. The molecular ion seen in the mass spectrum of M4 was m/e 528. These data suggested, therefore, that instead there was a relatively nonpolar group comprising 43 mass units attached to C-9. A reasonable structure that would satisfy these requirements would be 9-O-ethyl-dinor-TxB2.

To obtain additional evidence for this proposed structure, TxB2 was converted to an ethyl ester and treated with ethanolic HCl (see "Experimental Procedures"). This treatment would be expected to convert the TxB2 ethyl ester to the two epimers of the mono-O-ethylated derivative as illustrated in Fig. 11. The product of this reaction was purified by $\mu$-Porasil high performance liquid chromatography, and two discrete radioactive peaks were found with elution volumes of 16 to 19 and 23 to 26 ml, comprising 20% and 25% of the recovered radioactivity, respectively, as well as a more polar peak at 39 to 47 ml (54%), which was subsequently determined to be unchanged TxB2. The two less polar compounds were converted to Me3Si ether derivatives and analyzed by gas chromatography-mass spectrometry. The peak at 16 to 19 ml had a C value of 24.1 and the 23- to 26-ml peak a C value of 24.3. The mass spectra recorded for these two compounds were essentially the same except for very minor variations in ion intensities and were interpreted to be the two epimers of mono-O-ethyl-TxB2 ethyl ester (cf. Ref. 1). When these mass spectra were compared to the mass spectrum of the Me3Si ether derivative of the ethyl ester of M4b (C22.8), all ions not containing the carboxyl side chain were identical, but ions containing the carboxyl side chain were 28 mass units less in M4b than in mono-O-ethyl-TxB2.

These data indicated that M4b was a Cl8 derivative of mono-O-ethyl-TxB2, and it was, therefore, concluded that the methyl ester of the mono-O-ethyl-TxB2 obtained by this procedure was compared to the spectrum of the same derivative of M4b, it was again apparent that the patterns of fragmentation were identical, but ions containing the carboxyl side chain were 28 mass units less in M4b than in mono-O-ethyl-TxB2.

These data indicated that M4b was a Cl8 derivative of mono-O-ethyl-TxB2, and it was, therefore, concluded that M4b was 11-O-ethyl-2,3-dinor-TxB2, 2-(3-hydroxy-1-octenyl)-3-(4-carboxy-2-butenyl)-4-hydroxy-6-ethoxy-tetrahydropyran. Using thrombanoic acid nomenclature, M4b is 9a,15(S)-dihydroxy-11-ethoxy-2,3-dinorthromba-5,13-dienoic acid.

Initially it was considered that this compound might have
been generated artificially in vitro from dinor-TxB2 in the presence of ethanol under acidic conditions during the urine purification procedures. To investigate this possibility, 200 μg of [1H]TxB2 (6.9 μCi/μmol) were dissolved in a 20% solution of ethanol in 0.9% NaCl solution (saline), kept at room temperature for 3 h, added to 100 ml of human urine, and then subjected to identical urine purification procedures as employed in the processing of the monkey urine. This resulted only in the recovery of unchanged TxB2. We were, therefore, not able to reproduce the formation of a mono-O-ethylated derivative from TxB2 by our urine purification procedures.

The other predominant ring structure found in several metabolites, δ-lactone, would not be expected to form a mono-O-ethylated derivative at the original C-11 position in the presence of ethanol and acid. To confirm this, however, 11-dehydro-TxB2 methyl ester was reacted with methanolic HCl analogous to the ethanolic HCl treatment of TxB2. Methanolic HCl was used because of the presence of the methyl ester in our chemically synthesized compound. We have shown that methanolic HCl is as equally effective in converting TxB2 to mono-O-methyl-TxB2 as ethanolic HCl is in forming the mono-O-ethyl derivative. When the products of this reaction were converted to Me₃Si ether derivatives and analyzed by gas chromatography-mass spectrometry, 11-dehydro-TxB2 was found to have been converted to two major compounds. The identification of these compounds was not rigorously pursued, but neither had a retention time or mass spectrum compatible with 11-O-methyl-TxB2.

It appears that M4b was formed prior to the first reversed phase partition chromatographic step (Solvent System D) since mono-O-ethyl-dinor TxB2 (M4b) was separated from dinor-TxB2 (C1) by this chromatography. If mono-O-ethyl-dinor-TxB2 was formed in vitro prior to this chromatography, the only earlier procedure where compounds were possibly exposed to ethanol in the presence of acid is XAD-2 chromatography. To evaluate this possibility, TxB2 as well as ll-dehydro-TxB2 was exposed to ethanol in the presence of acid is XAD-2 chromatography. To investigate this possibility, 200 pg of [1H]TxB2 (6.9 μCi/μmol) were dissolved in a 20% solution of ethanol in 0.9% NaCl solution (saline), kept at room temperature for 3 h, added to 100 ml of human urine, and then subjected to identical urine purification procedures as employed in the processing of the monkey urine. This resulted only in the recovery of unchanged TxB2. We were, therefore, not able to reproduce the formation of a mono-O-ethylated derivative from TxB2 by our urine purification procedures.

In contrast, as described above, in the presence of ethanol and acid, the two mono-O-ethyl epimers are formed in approximately equal abundance and are separated by high performance liquid chromatography and gas-liquid chromatography.

Structures of Compounds in Peak M2—Material in Peak M2 was further purified by μ-Porasil high performance liquid chromatography. Two prominent peaks emerged and were designated Compound M2a (40 to 43 ml of eluate, 18% of recovered radioactivity) and Compound M2b (48 to 53 ml, 41%). M2a represented about 1.5% and M2b about 3.5% of the total radioactivity in the urine.

M2b had an identical elution volume on μ-Porasil chromatography as parent TxB2. The methyl ester of M2b was converted to a O-methyloxime, Me₃Si ether derivative, and analyzed by gas chromatography-mass spectrometry. A single radioactive peak was present with an assigned C value of 23.7, identical with that of TxB2. The mass spectrum recorded for this derivative of M2b showed ions at m/e 629 (M), 614, 598, 558, 539, 526, 508, 468, 448, 436, 418, 366, 301 (base), 211, 174, and 173. This mass spectrum was identical with that of the O-methyloxime, Me₃Si ether derivative of the methyl ester of authentic TxB2. In addition, when the Me₃Si ether derivative of the methyl ester of M2b was analyzed by gas chromatography-mass spectrometry, a mass spectrum was recorded that was identical with that obtained for the same derivative of TxB2 (10, 18). It was, therefore, concluded that M2b was unchanged TxB2.

The methyl ester of M2a was converted to a Me₃Si ether derivative and analyzed by gas chromatography-mass spectrometry. A prominent peak was found with a C value of 20.2 and the mass spectrum recorded for this derivative of M2a (Fig. 12) showed prominent ions at m/e 502 (M), 487 (M-15), loss of CH₂=CH₂ (M-31), loss of OCH₃; 456 (M-46), loss of CH₂=CH=CH₂ (M-90), loss of Me₃SiOH; 449 [M-(90 + 71)]; 431 (M-190), loss of [OCH(OCH₂CH₂)CH₂=CH=OSiMe₃], eliminated from the ring: 301, probably [Me₃SiO=CH=CH=CH(OSiMe₃)](CH₂)₄CH₃, formed by rearrangement; 295 [M-(90 + 71 + 46)]; 292 [Me₃SiO=CH=CH-(CH₂)₄COOCH₃] and 173, probably derived from two sources: (a) Me₃SiO=CH-(CH₂)₄CH₃, and (b) [Me₃SiO=CH=CH-(CH₂)₄=CH(OH)CH₃] − H. The base ion m/e 202 suggested that this metabolite had been formed in part by two steps of β oxidation resulting in the loss of 4 carbons from the carboxyl side chain. Clues to other structural modifications were found in the ions m/e 456 and 295, the formation of which involved...
the loss of 46 mass units, and the ion \( m/e \) 312, formed by the loss of 190 mass units. This pattern of fragmentation is analogous to that resulting in the formation of ions \( m/e \) 482, 321, and 338, respectively, in the mass spectrum of the MeSi ether derivative of the methyl ester of mono-\( O \)-ethyl-dinor TxB\(_2\) (see structure of M4). These data and other reasoning analogous to that outlined for the determination of the structure of M4 suggested that this metabolite had an ethoxy group attached at C-7.

It was, therefore, concluded that Compound M2a was 11-\( O \)-ethyl 2,3,4,5-tetranor TxB\(_2\), or 2 (3 hydroxyl 1 octenyl) 3 (2-carboxyethyl)-4-hydroxy-6-ethoxy-tetrahydropyran. Using thrombanoic acid nomenclature, M2a is 9a,15(S)-dihydroxy-11-ethoxy-2,3,4,5-tetranorthromb-13-enio acid.

Identification of Compounds Eluting in Area C—Material that eluted in Area C (Fig. 3) was subjected to reverse phase partition chromatography using Solvent System C-45 (Table I). Three radioactive peaks were present (Fig. 13), C1 (5 to 85 ml of effluent, 67% of recovered radioactivity), C2 (90 to 135 ml, 18%), and C3 (140 to 225 ml, 15%).

Structure of Compound C1—Material in Peak C1 was further purified by \( \mu \)-Porasil high performance liquid chromatography. A single prominent peak emerged (50 to 56 ml of effluent, 45% of recovered radioactivity), designated Compound C1, along with several less polar unresolved peaks. Compound C1 isolated from this chromatography represented approximately 12% of the total radioactivity present in the urine. An additional amount of Compound C1, 2.5% of the total radioactivity in the urine, was also isolated from Peak B (see “Structure of Compounds in Peak B2”). The total amount of C1, therefore, represented approximately 14.5% of the total radioactivity present in the urine. The identification of Compound C1 was recently reported as dinor-TxB\(_2\), the hemiacetal derivative of 6-(1-hydroxy-3-oxopropyl)-7,10(S)-dihydroxy-3,9,15-trihydroxy-2,3-dinorthromb-5,13-enio acid. Identification was based on mass spectral analysis of C1 and its reduced product and the formation of an identical compound from TxB\(_2\) in vitro by incubation with rat liver mitochondria.

Material in Peak C2 could not be sufficiently purified by subsequent chromatographic procedures to permit identification.

Structure of Compound C3—Material in Peak C3 was further purified by \( \mu \)-Porasil high performance liquid chromatography. A single prominent peak was present (31 to 35 ml of effluent, 65% of recovered radioactivity) which was designated Compound C3. C3 represented approximately 4% of the total radioactivity present in the urine.

The methyl ester of C3 was converted to an O-methyloxime, Me\(_2\)Si ether derivative and analyzed by gas chromatography-mass spectrometry. Two peaks were present with \( C \) values of 25.0 and 25.2. The mass spectra recorded for these two peaks were essentially the same except for minor variation in ion intensities and most likely represented syn-anti isomerism of the O-methyloxime groups. The mass spectrum of the less polar isomer is shown in Fig. 14. Ions of high intensity were found at \( m/e \) 455 (M); 440 (M-15), loss of \( \cdot \)CH\(_2\); 424 (M-31), loss of \( \cdot \)OCH\(_3\); 412 (M-43), loss of \( \cdot \)(CH\(_2\))\(_2\)CH\(_2\)OH; 399 (M-56), loss of \( \cdot \)(CH\(_2\))\(_2\)CH\(_2\)OH - H; 388 [M-(31 + 56)]; 342 (M-113), loss of \( \cdot \)(CH\(_2\))\(_2\)CH\(_2\)OH - 29H; 340 (M-115), loss of \( \cdot \)OCH\(_3\); 313 (M-142), loss of \( \cdot \)CH\(_2\)-C(=NOCH\(_3\))\(_2\)(CH\(_2\))\(_2\); 294, formed either by M-(90 + 71), loss of [Me\(_2\)SiOH + \( \cdot \)CH\(_2\)CH\(_2\)OH], or M-(160 + 1), loss of [\( \cdot \)OCOCH\(_2\)CH\(_3\)=OSiMe\(_3\)] + H, eliminated from the ring; 238 [M-(160 + 56 + 1)], loss of [\( \cdot \)OCOCH\(_2\)CH\(_3\)=OSiMe\(_3\)] + 56 + H; 219 (M-(90 + 142)); 156 \( \cdot \)[(CH\(_2\))\(_2\)-C(=NO-
CH(3)(CH2)(5CH3), 1437 [CI(3-)C(=NOCH3)(CH2)=CH2] - 11, and 1287 [C(=NOCH3)(CH2)=CH2]. From the behavior of C3 on μ-Porasil and gas-liquid chromatography systems C3 was judged significantly less polar than TxBz on the former and more polar than TxBz on the latter. This apparent disparity regarding polarities in these systems suggested that C3 may be a lactone (cf. Compound M3a). The presence of a methoxime group and its assignment to the various fragmentation ions was confirmed by mass spectral analysis of the triuteromethoxime derivative. The ion at m/e 342, formed by the loss of 113 mass units from the carboxyl side chain, indicated that the formation of C3 had probably involved one step of β oxidation, resulting in the loss of 2 carbons from the upper side chain. These data, therefore, suggested that C3 had been formed from TxBz by dehydrogenation of the alcohol group at C 11, reduction of the Δ^1 double bond, dehydrogenation of the alcohol group at C-15, and β oxidation to a C-18 derivative.

To obtain additional evidence for the proposed structure of C3, the methyl ester was treated with sodium methoxide in methanol, converted to an O-methoxime, MeSi ether derivative, and analyzed by gas chromatography-mass spectrometry. This treatment would be expected to convert the δ-lactone structure to a dicarboxylic acid methyl ester form (cf. Fig. 8). When the product of this reaction was analyzed by gas chromatography, a peak with a C value of 23.0 was present, and the mass spectrum showed ions of high intensity at m/e 559 (M); 544 (M-15), loss of -CH3; 528 (M-31), loss of -OCH3; 494 (M-90), loss of Me,SOH; 438 (M-[M-90+31]); 258 (base) [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2] 175 [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2]; and 1/5 [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2]. The difference in C values of C3 and the product formed after treatment with sodium methoxide (Δδ) is compatible with C3 being converted from a δ-lactone to a dicarboxylic acid methyl ester form (cf. Compounds M3a and M3b). The base ion at m/e 258 was predicted since the same major fragment ion is seen in the mass spectrum of the O-methoxime, MeSi ether derivative of the methyl ester of 15-keto-13,14-dihydro-TxBz, which has a structurally identical lower side chain, and also indicates that the original Δ^1 double bond has been reduced (18). The ion at m/e 175 was also expected and is indicative of a methyl ester group at C-9 (cf. Compound M3b).

From these data it was therefore concluded that Compound C3 was the δ-lactone of 3-hydroxy-4-(1-hydroxy-4-oxononyl)-5-nonenodiic acid or 11-dehydro-15-keto-13,14-dihydro-TxBz. When thionomanoic acid nomenclature, Compound C3 is 9α-hydroxy-11,15-dioxo-2,3-dinor-thromboxane-5-enoic acid.

Identification of Compounds Eluting in Peak B—Fifty per cent of the material eluting in Peak B was converted to a methyl ester and 50% to an ethyl ester. The methyl and ethyl esters were separately subjected to reverse phase partition chromatography using Solvent System F-50 (Table I). Analogous chromatographic results were obtained for both esters. Two prominent peaks emerged in the chromatography of the methyl esters, designated B1 (20 to 35 ml of effluent, 20% of recovered radioactivity) and B2 (40 to 70 ml, 30%) (Fig. 14). Thirty-three per cent of recovered radioactivity remained on the alcohol group at C-15, and p oxidation to a C-18 derivative. The elution of a relatively small quantity of dinor-TxBa in the alcohol group at C-15, reduction of the Δ^1 double bond, dehydrogenation of the alcohol group at C-15, and β oxidation to a C-18 derivative.

To obtain additional evidence for the proposed structure of C3, the methyl ester was treated with sodium methoxide in methanol, converted to an O-methoxime, MeSi ether derivative, and analyzed by gas chromatography-mass spectrometry. This treatment would be expected to convert the δ-lactone structure to a dicarboxylic acid methyl ester form (cf. Fig. 8). When the product of this reaction was analyzed by gas chromatography, a peak with a C value of 23.0 was present, and the mass spectrum showed ions of high intensity at m/e 559 (M); 544 (M-15), loss of -CH3; 528 (M-31), loss of -OCH3; 494 (M-90), loss of Me,SOH; 438 (M-[M-90+31]); 258 (base) [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2] 175 [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2]; and 1/5 [Me3SiO'=CH(CH2)=OCH(OCH2CH3)CH2]. The difference in C values of C3 and the product formed after treatment with sodium methoxide (Δδ) is compatible with C3 being converted from a δ-lactone to a dicarboxylic acid methyl ester form (cf. Compounds M3a and M3b). The base ion at m/e 258 was predicted since the same major fragment ion is seen in the mass spectrum of the O-methoxime, MeSi ether derivative of the methyl ester of 15-keto-13,14-dihydro-TxBz, which has a structurally identical lower side chain, and also indicates that the original Δ^1 double bond has been reduced (18). The ion at m/e 175 was also expected and is indicative of a methyl ester group at C-9 (cf. Compound M3b).

From these data it was therefore concluded that Compound C3 was the δ-lactone of 3-hydroxy-4-(1-hydroxy-4-oxononyl)-5-nonenodiic acid or 11-dehydro-15-keto-13,14-dihydro-TxBz. When thionomanoic acid nomenclature, Compound C3 is 9α-hydroxy-11,15-dioxo-2,3-dinor-thromboxane-5-enoic acid.

Identification of Compounds Eluting in Peak B—Fifty per cent of the material eluting in Peak B was converted to a methyl ester and 50% to an ethyl ester. The methyl and ethyl esters were separately subjected to reverse phase partition chromatography using Solvent System F-50 (Table I). Analogous chromatographic results were obtained for both esters. Two prominent peaks emerged in the chromatography of the methyl esters, designated B1 (20 to 35 ml of effluent, 20% of recovered radioactivity) and B2 (40 to 70 ml, 30%) (Fig. 14). Thirty-three per cent of recovered radioactivity remained on the alcohol group at C-15, and p oxidation to a C-18 derivative. The elution of a relatively small quantity of dinor-TxBa in the alcohol group at C-15, reduction of the Δ^1 double bond, dehydrogenation of the alcohol group at C-15, and β oxidation to a C-18 derivative.

The methyl ester of Compound B2 was converted to a MeSi ether derivative and analyzed by gas chromatography-mass spectrometry. A single radioactive peak was present with a C value of 23.9. The mass spectrum recorded for this derivative of B2 (Fig. 16) showed prominent ions at m/e 616 (M), 601 (M-15), loss of -CH3; 570 (M-46), loss of CH3CH2OH; 526 (M-90), loss of Me,SOH; 480 (M-[M-90+46]); 457 (M-159), loss of -CH3)-CH(OSiMe3)CH2; 426 (M-190), loss of -(OCH2CH2CH2CH2OH)-CH(OSiMe3); 411 (M-[M-159+46]); 390 (M-[2 x 90 + 46]); 367 (M-[159 + 90]); 321 (M-[159 + 90 + 46]); 317; 295; 228 [(Me3SiO)-CH-CH-OCH2CH2CH2]; 173 [Me3SiO'-CH-CH-OCH2CH2CH2]; and 177 [Me3SiO'-CH-CH-OCH2CH2CH2]. The base ion m/e 228 suggested that the formation of B2 had involved one step of β oxidation resulting in a C16 derivative. Mass spectral analysis of the [H3]MeSi ether derivative indicated that three Me3Si groups were present by a shift of m/e 616 to m/e 643. However, the lack of a prominent ion at m/e 217, the presence of several ions whose formation involved the loss of 46 mass units, and the ion at m/e 426 formed by the loss of 190 mass units suggested that a MeSi group was not attached to C9 but that B2 was a mono-O-ethylated derivative at C9 (see under “Structure of M4 and M2a”). In addition to the Me3Si groups apparently attached at C-7 and C-13, a clue to the location of the third Me3Si group was afforded by the prominent ion m/e 117. This ion is typical of prostaglandin metabolites with a hydroxyl group at the ω-2 position which have been previously identified in monkey urine, human semen, and rat urine (9, 20, 21). The ion at m/e 117 is conspicuously absent in ω-1, hydroxylated prostaglandin metabolites. The formation of several ions by or in part by the elimination of the terminal 5 carbons from the lower side chain with an attached Me3Si group is also typical of both ω-
1 and ω-2 hydroxylated metabolites (20, 21).

We, therefore, concluded that compound B2 is 11-O-ethyl-19-hydroxy-2,3-dinor-TxB2, or 2-(3,7-dihydroxy-1-octenyl)-3-(4-carboxy-2-butenyl)-4-hydroxy-6-ethoxy-tetrahydropyran. Using thrombanoic acid nomenclature, Compound B2 is 9α, 15(S),19-trihydroxy-11-ethoxy-2,3-dinor-thromboxa-5,13-dienoic acid.

Identification of Compounds Eluting in Peak A—Sixty per cent of the material in Peak A was converted to a methyl ester and 40% to an ethyl ester before subsequent purification. The methyl and ethyl esters were purified separately by identical procedures with analogous chromatographic results. The methyl ester fraction was initially subjected to high performance liquid chromatography using a μ-Bondapak column (see under “Experimental Procedures”). A single prominent peak emerged, designated A1 (29 to 33 ml of effluent, 20% of recovered radioactivity), along with more polar unresolved material (Fig. 17). Material in Peak A1 was further purified by μ-Porasil high performance liquid chromatography. A single prominent peak was present which was designated Compound A1 (25 to 26 ml of effluent, 70% of recovered radioactivity). Compound A1 represented approximately 1.5% of the total radioactivity present in the urine.

Structure of Compound A1—The methyl ester of A1 was converted to an O-methyloxime, Me3Si ether derivative, and analyzed by gas chromatography-mass spectrometry. A radioactive peak was present with a C value of C 26.2. As noted previously, the apparent disparity in polarity characteristics of A1 as judged by its behavior on μ-Porasil and gas-liquid chromatography again suggested the possibility of a lactone ring structure. The mass spectrum recorded for this derivative (Fig. 18) showed ions of high intensity at m/e 473 (M); 458 (M-15), loss of -CH3; 442 (M-31), loss of -OH; 386 (M-87), loss of -(CH2)5COOCH3; from either upper or lower side chain; 373 (M-100), loss of -(CH2)5COOH - H; 352 [M-(90 + 31)], loss of [Me3SiOH + 31]; 312 [M-(160 + 1)], loss of [(OCOCH3)CH=O(SiMe3) + H], eliminated from ring; 296 [M-(90 + 87)]; 22 [M-(160 + 101)], loss of [160 + -(CH2)5COOCH3]; 187 [CH4=O(NOCH3)(CH2)5COOCH3 + H] and 115 [CH2=O(CH2)5COOCH3]. The presence of one methoxime group was confirmed by analysis of the trideuteromethoxime derivative. A dicarboxylic acid structure was suggested by an increase in C value of 1.0 on gas-liquid chromatography of the ethyl ester compared to the methyl ester and was confirmed by mass spectrometry where the molecular ion of the methyl ester, m/e 473, was found to shift 28 mass units to m/e 501 in the mass spectrum of the ethyl ester. The formation of ions at m/e 312 and m/e 212 by elimination in part of 160 mass units is characteristic of the δ-lactone ring structure (see identification of structures of M3a and C3). It was concluded from these data that Metabolite A1 had been formed from TxB2 by a process of dehydrogenation of the alcohol group at C-11, reduction of the Δ13 double bond, dehydrogenation of the alcohol group at C-15, ω oxidation, and δ oxidation to a C16 derivative. A1 is, therefore, the δ-lactone of 3,5-dihydroxy-4-(2-carboxyethyl)-8-oxo-tridecane-dioic acid or 11-dehydro-15-keto-13,14-dihydro-19-carboxyl-2,3,4,5-tetranor-TxB2. Using thrombanoic acid nomenclature, A1 is 9α-hydroxy-11,15-dioxo-2,3,4,5-tetranorthromba-1,20-dioic acid.

DISCUSSION

This study has demonstrated that TxB2 in the monkey is converted to a variety of metabolites that are rapidly excreted into the urine. We had previously identified the major urinary metabolite as dinor-TxB2 (10) as has also been found by Kindahl (22). The present paper has described the isolation and structural identification of seven additional urinary metabolites as well as the excretion of a small amount of unchanged TxB2.

One major pathway of metabolism of TxB2 is via one step of β oxidation resulting in the formation of dinor-TxB2, the most abundant metabolite. The second major pathway of metabolism results from dehydrogenation of the alcohol group at C-11. This yields 11-dehydro-TxB2, which undergoes further metabolic transformation by processes of β oxidation, ω oxidation, dehydrogenation of the alcohol group at C-15, and Δ13
double bond reduction. A minor metabolite reflects that metabolic transformation of TxB2 also occurs by a process of reduction, resulting in an acyclic compound with hydroxyl groups attached at the original C-11 and C-12 positions. This metabolite has also undergone one step of \( \beta \) oxidation, dehydration of the alcohol group at the original C-15 position, and reduction of the original \( \Delta^{12} \) double bond. Three additional metabolites were also identified that were mono-O-ethylated derivatives at the original C-11 position of TxB2. Table II lists the percentage of total recovered radioactivity represented by each compound identified.

The origin and significance of the three mono-O-ethylated derivatives is not clear at this time. It is not entirely certain whether the ethoxy group was adducted \textit{in vivo} or \textit{in vitro}. If the derivatives had been formed \textit{in vitro}, one might expect to find the same ratio of parent compound to mono-O-ethylated compound for all three compounds as was found for dinor TxB2 and mono-O-ethyl-dinor-TxB2 (approximately 2.5:1). However, no parent compounds with a hydroxyl group at the original C-11 position were found corresponding to 11-O-ethyl-tetranor-TxB2 or 11-O-ethyl-19-hydroxy-dinor-TxB2. In addition, as previously outlined, because we were not able to reproduce the formation of a mono-O-ethylated derivative by our urine purification procedures, because there is evidence they were present before the initial reversed phase partition chromatography, and the fact that only one epimer of each compound was isolated all strongly suggest that these derivatives were not formed \textit{in vitro}. The other remaining possibility is that an ethoxy group was adducted \textit{in vivo}, either from the small amount of ethanol (1 ml) used to make a 20% solution of ethanol in saline as a vehicle for infusion of TxB2 or from some endogenous source. Future experiments are being planned to investigate these various possibilities.

It is assumed that the double bond in the dinor metabolites is in the \( \Delta^{1} \) position. Although the amount of these metabolites did not permit chemical degradation to confirm this assumption, it seems unlikely that double bond isomerization from the \( \Delta^{2} \) to the \( \Delta^{1} \) position by action of the enzyme \( \Delta^{2,3}\)-cis-\( \Delta^{2}\)-trans-enoyl-CoA isomerase (23) has occurred, as such isomerization has not been described for dinor prostaglandin metabolites previously identified (24, 25).

The proposed pathways of TxB2 metabolism in the monkey are illustrated in Fig. 19. A small quantity of unchanged TxB2 was recovered from the urine. The three mono-O-ethylated metabolites have been excluded from the figure because of their somewhat obscure mechanism of formation. The major pathway of metabolism is through \( \beta \) oxidation to dinor-TxB2 (C1). Although we have demonstrated that \textit{in vitro} TxB2 can in part be additionally \( \beta \)-oxidized to a C16 derivative\(^{3}\) and have identified two minor C16 urinary metabolites (A1, M2a), in contrast to PGE2 and PGF2\(_\alpha\), the formation of C16 metabolites apparently is not a major pathway of thromboxane metabolism \textit{in vivo} in the monkey.

The other major pathway of thromboxane metabolism involves dehydrogenation of the alcohol group at the C-11 position. The second most abundant metabolite isolated was the 6-lactone 11-dehydro-TxB2 (M3). The other two metabolites identified with the 6-lactone ring structure had been degraded by additional processes of \( \beta \) oxidation, dehydrogenation of the alcohol group at the original C-15 position, and reduction of the \( \Delta^{12} \) double bond. The C16 compound had also undergone \( \omega \) oxidation. Since \( \beta \)-oxidized prostaglandins have been shown to be poor substrates for the prostaglandin dehydrogenase enzyme (26), it seems reasonable to propose that 11-dehydro-TxB2 initially undergoes dehydrogenation of the alcohol group at C-15 and \( \Delta^{12} \) double bond reduction as an intermediate step prior to subsequent \( \beta \) oxidation to a C18 derivative (C3). C3 is probably further degraded by an additional step of \( \beta \) oxidation to a C16 derivative as well as \( \omega \) oxidation, resulting in the formation of metabolite A1. As indicated in Fig. 19, it is suggested that this second step of \( \beta \) oxidation occurs prior to \( \omega \) oxidation, although the reverse sequence of events cannot be excluded.


### Table II

<table>
<thead>
<tr>
<th>Compound</th>
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<tr>
<td>A1</td>
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**FIG. 18.** Mass spectrum of the methyl ester, methoxime, Me3Si ether derivative of Metabolite A1.
A minor pathway of metabolism was indicated by metabolite M4a. This compound had been formed by a process of reduction resulting in an acyclic structure with hydroxyl groups attached at the original C-11 and C-12 positions. This is envisioned to occur by reduction of the aldehyde group in the acyclic aldehyde-alcohol form of TxB2. In addition, dehydrogenation of the original C-15 alcohol group and reduction of the original Δ15 double bond had occurred. As indicated in Fig. 19, we have proposed that reduction of the aldehyde group occurs prior to dehydrogenation of the C-15 alcohol group and Δ15 double bond reduction. This seems reasonable since there were no 15-keto-13,14-dihydro metabolites identified with an intact hemiacetal ring structure, suggesting that structural modification of the ring improves the ability of the compound to act as a substrate for dehydrogenation of the C-15 alcohol group. The 15-keto-13,14-dihydro derivative is also proposed as being formed prior to β oxidation to the C18 derivative, since β-oxidized prostaglandins have been shown to be poor substrates for the prostaglandin dehydrogenase (26), and also because we have demonstrated that TxB2 can be converted in vitro to 8-(1,3-dihydroxypropyl)-9-hydroxy-12-oxo-5-heptadecenoic acid, the C20 derivative of M4a, by incubation of TxB2 with the 100,000 × g supernatant of guinea pig liver.3

As mentioned above, it is interesting that the only metabolites identified which were formed in part by dehydrogenation of the alcohol group at the original C-15 position and reduction of the Δ15 double bond, in addition had undergone structural modification of the hemiacetal ring structure. These data suggest that the Δ-lactone ring structure of 11-dehydro-TxB2 and the acyclic structure of M4a allow these metabolites to be better substrates for the prostaglandin dehydrogenase enzyme than parent TxB2. Further in vitro studies will be necessary, however, to confirm these assumptions. In addition, although 15-keto-13,14-dihydro-TxB2 has been shown to be formed by the immune-challenged guinea pig lung (19), formation of this metabolite in vitro by the nonimmunologically challenged monkey apparently is not a significant pathway of metabolism.

β oxidation, ω oxidation, dehydrogenation of the alcohol group at C-15, and reduction of the Δ15 double bond have all been described as common processes in the metabolism of prostaglandins (24, 25). Although TxB2 is not a prostaglandin because of the hemiacetal ring, dehydrogenation of the alcohol group at C-15 has not been previously described in the metabolism of prostaglandins. Therefore, the enzyme that may be responsible for this transformation of TxB2 is at present unknown. In addition the enzyme responsible for reduction of the aldehyde group in the acyclic aldehyde-alcohol form of TxB2 is at present unknown. In addition to conversion to 8-(1,3-dihydroxypropyl)-9-hydroxy-12-oxo-5-heptadecenoic acid, we have also been able to demonstrate conversion of TxB2 to 11-dehydro-TxB2 by incubation of TxB2 with the 100,000 × g supernatant of guinea pig liver.3 A boiled control resulted in no conversion. These initial in vitro experiments, therefore, suggest that 11-dehydrogenation and aldehyde reduction are at least enzymatic processes involving soluble enzymes.

The structural identification of the TxB2 metabolites described in the present study has provided the initial background chemical information necessary for future detailed studies of the sequences and mechanisms of these biochemical transformations and has provided a basis for the eventual quantification of the excretion of TxB2 metabolites as an index of TxB2 synthesis in vivo.

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Addendum—Since submission of this manuscript additional data has been obtained that has helped to clarify the somewhat uncertain origin of the three mono- O-ethylated derivatives described in the present work. We have now performed a similar study of TxB2 metabolism in man. In this study, however, TxB2 was infused as a sodium salt instead of dissolved in ethanol/saline and wherever ethanol had been used in the isolation and purification of metabolites from the monkey, methanol was substituted. Compounds identified...
in the study in man have included mono-O-methyl-TxB2, mono-O-methyl-dinor-TxB2, and mono-O-butyl-dinor-TxB2. No mono-O-
ethylated derivatives have been found. These data imply that the
formation of these compounds in the study in man occurred in vitro
from the exposure of the hemiacetal ring to methanol or butanol
(Solvent System D) under acidic conditions and strongly suggest that
the mono-O-ethylated compounds isolated from monkey urine were
not formed in vivo but were formed in an analogous fashion in vitro
from exposure of dinor-TxB2, tetranor-TxB2, and 19-hydroxy-dinor-
TxB2 to ethanol under acidic conditions during urine processing.

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