

## Metabolism of Thromboxane B<sub>2</sub> in Man

### IDENTIFICATION OF TWENTY URINARY METABOLITES\*

(Received for publication, December 5, 1980, and in revised form, April 23, 1981)

L. Jackson Roberts, II, Brian J. Sweetman, and John A. Oates†

From the Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

[<sup>3</sup>H]<sub>8</sub>Thromboxane B<sub>2</sub> (12.2 Ci/mol) was infused into a healthy adult male. Urinary metabolites of thromboxane B<sub>2</sub> were isolated by reversed phase partition chromatography and high performance liquid chromatography. Structural identification of metabolites was accomplished by gas chromatography-mass spectrometry. Twenty metabolites were identified. Three primary pathways of metabolism of thromboxane B<sub>2</sub> were found. A small quantity of thromboxane B<sub>2</sub> was excreted unchanged, representing 2.5% of total recovered radioactivity. Two additional metabolites retained the original thromboxane B<sub>2</sub> hemiacetal ring; one of these metabolites, 2,3-dinor-thromboxane B<sub>2</sub>, was the major urinary metabolite and represented 23.0% of total recovered radioactivity. The other, 2,3,4,5-tetranor-thromboxane B<sub>2</sub>, represented 5.3% of total recovered radioactivity. Two metabolites representing 1.1% of total recovered radioactivity had initially undergone reduction of the hemiacetal ring and indicated a second but relatively minor pathway of metabolism. A major pathway of metabolism was found to involve dehydrogenation of the hemiacetal alcohol group of thromboxane B<sub>2</sub> resulting in a series of metabolites with a  $\delta$ -lactone ring. Sixteen metabolites representing 29.3% of total recovered radioactivity were identified as products of this pathway of metabolism.

Thromboxane A<sub>2</sub> is a labile metabolite of prostaglandin G<sub>2</sub> with potent biological activity (1). TxA<sub>2</sub><sup>1</sup> contracts vascular smooth muscle including that of the coronary arteries (2) and induces irreversible platelet aggregation (1). A means to assess production of thromboxanes *in vivo* in man should have important biological applications as a tool to investigate the role of thromboxanes in human disease. Since thromboxanes are released in substantial quantities from aggregating platelets, quantification of *in vivo* thromboxane production may provide a means to assess *in vivo* platelet aggregation and

lead to a better understanding of the role of platelets in the pathophysiology of many cardiovascular diseases. It may also provide a means to assess the *in vivo* efficacy of anti-platelet drug therapy.

It is well established that quantification of circulating or urinary prostaglandin metabolites represents a more reliable means of assessing endogenous prostaglandin synthesis *in vivo* than does quantification of the parent compound (3, 4). Therefore, we initially investigated the metabolic fate of TxB<sub>2</sub> in the non-human primate. The major urinary metabolite was found to be 9 $\alpha$ ,11,15(S)-trihydroxy-2,3-dinor-thromboxane-5Z,13E-dienoic acid (2,3-dinor-TxB<sub>2</sub>) (5), as also was reported by Kindahl (6). We found another major pathway of metabolic transformation to involve dehydrogenation of the hemiacetal alcohol group at C-11, resulting in the formation of a series of metabolites with a  $\delta$ -lactone ring structure (7).

Before quantitative studies of thromboxane synthesis *in vivo* in man are possible, however, pathways of human thromboxane metabolism must be defined. This present work describes our investigation into the metabolic fate of TxB<sub>2</sub> in man. A preliminary report of part of this work describing the identification of the major urinary metabolite as 2,3-dinor-TxB<sub>2</sub> has been published earlier (8). The description of identified metabolites in this paper adheres to the recently proposed nomenclature for thromboxanes (9). Although not confirmed, the stereochemistry of identified metabolites has been presumed to have been unaltered by metabolic transformation of TxB<sub>2</sub>.

#### EXPERIMENTAL PROCEDURES<sup>2</sup>

#### RESULTS AND DISCUSSION

At no time during the infusion of TxB<sub>2</sub> were there any significant changes in blood pressure or pulse rate and no clinically apparent adverse effects were observed. Seventy-four % of the infused radioactivity was recovered in the urine within 13 h.

The urine collected was extracted by Amberlite XAD-2 chromatography and the radioactivity was quantitatively eluted with 1600 ml of methanol. The residue obtained after evaporation of the methanol was dissolved in ethyl acetate and applied to a silicic acid column. Ninety-one % of the

\* This work was supported by National Institutes of Health Grant GM15431 and Public Health Service Grant GM00113. This work was presented in part at the 1978 Winter Prostaglandin Conference, Sarasota, FL, and the Seventh International Congress of Pharmacology, Paris, France, 1978. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The Joe and Morris Werthan Professor of Investigative Medicine.

<sup>1</sup> The trivial names and abbreviations used are: TxA<sub>2</sub>, thromboxane A<sub>2</sub>; 9 $\alpha$ ,11 $\alpha$ -epoxy-15(S)-hydroxythromboxane-5Z,13E-dienoic acid; TxB<sub>2</sub>, thromboxane B<sub>2</sub>; 9 $\alpha$ ,11,15(S)-trihydroxythromboxane-5Z,13E-dienoic acid; 11-dehydro-TxB<sub>2</sub>, 9 $\alpha$ ,15(S)-dihydroxy-11-oxothromboxane-5Z,13E-dienoic acid; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; 11 $\alpha$ ,15(S)-dihydroxy-9-ketoprostanoic acid; PGF<sub>2 $\alpha$</sub> , prostaglandin F<sub>2 $\alpha$</sub> ; 9 $\alpha$ ,11 $\alpha$ ,15(S)-trihydroxyprosta-5(Z),13(E)-dienoic acid; Me<sub>3</sub>Si, trimethylsilyl; Me<sub>3</sub>CMe<sub>2</sub>Si, *t*-butyldimethylsilyl.

<sup>2</sup> Portions of this paper (including all of "Experimental Procedures" and most of "Results" which describe chromatographic purification of metabolites and the mass spectral data pertaining to their structural elucidation) are presented in miniprint at the end of this paper. In the standard print section of "Results" are summarized the overall findings of this work. Miniprint is easily read with the aid of standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20814. Request Document No. 80M-2569, cite author(s), and include a check or money order for \$26.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

applied radioactivity was eluted with 1190 ml of ethyl acetate.

Further sample purification and initial compound isolation was effected by reversed phase partition chromatography on a support of 45 g of Hyflo Super-Cel using the solvent system water:*n*-butyl alcohol:acetic acid (300:100:4) (v/v/v). Three polar peaks emerged (Fig. 1) designated Peak A (130 to 160 ml of eluate, 17% of recovered radioactivity), Peak B (370 to 660 ml, 12%), and Peak C (670 to 890 ml, 5%). These were followed by the elution of material not resolved by this solvent system, designated Area D (900 to 2930 ml, 16%) and a large peak of relatively less polar material, designated Peak E (2940 to 4250 ml, 24%). Twenty-six % of the recovered radioactivity remained on the stationary phase and was eluted with 110 ml of methanol and was designated M.

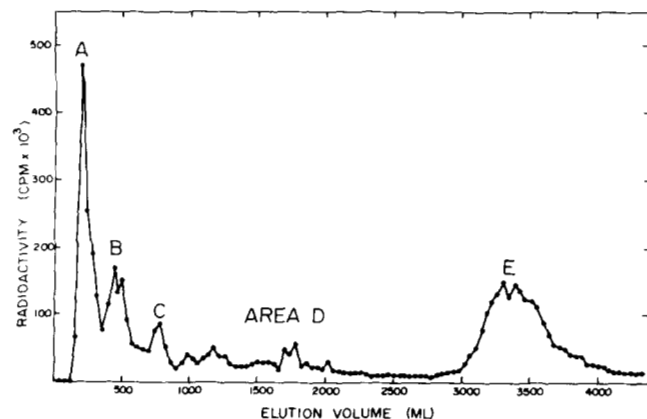


FIG. 1. Reversed phase partition chromatography of material in the Amberlite XAD-2 methanol eluate after purification on silicic acid. Column, 45 g of hydrophobic Hyflo Super-Cel; solvent system, water:*n*-butyl alcohol:acetic acid (300:100:4) (v/v/v); fraction volume, 10 ml.

A flow diagram outlining the urine purification procedures that are described in the miniprint section and the isolation and letter-number designation of identified TxB<sub>2</sub> metabolites are illustrated in Fig. 2. The letter-number designation of each identified metabolite along with its respective chemical name(s) and structure is shown in Fig. 3. Several metabolites were identified from two or more peaks that were completely resolved chromatographically. Explanations for this finding include the possibility of ion-pairing of metabolites with urine impurities and the existence of more than one structural form such as a  $\delta$ -lactone and its acid-alcohol form. Table I lists the relative abundances of each metabolite and the total sum of recovered radioactivity identified.

This study has demonstrated that TxB<sub>2</sub> is transformed by humans into a variety of metabolites which are excreted into the urine. We have previously identified the major urinary metabolite as 2,3-dinor-TxB<sub>2</sub> (8). The present study has described the isolation and structural identification of 19 additional urinary metabolites as well as the excretion of a small quantity of unchanged TxB<sub>2</sub>.

There are several distinctive features in the metabolism of TxB<sub>2</sub> in man which are outlined in Table II. Three separate series of metabolites were categorized, based on ring structure. One series retained the original TxB<sub>2</sub> hemiacetal ring. A major pathway of metabolism involved dehydrogenation of the hemiacetal alcohol group at C-11. The proposed pathways of TxB<sub>2</sub> metabolism in man are illustrated in Fig. 4. Additional detailed studies of sequences and mechanisms of these biochemical transformations are required to define precisely actual metabolic pathways. Compounds in brackets were not isolated but are proposed intermediates in the formation of identified metabolites.

Only two metabolites in addition to a small amount of unchanged TxB<sub>2</sub> were identified with an intact hemiacetal ring, even though these metabolites represented a major por-

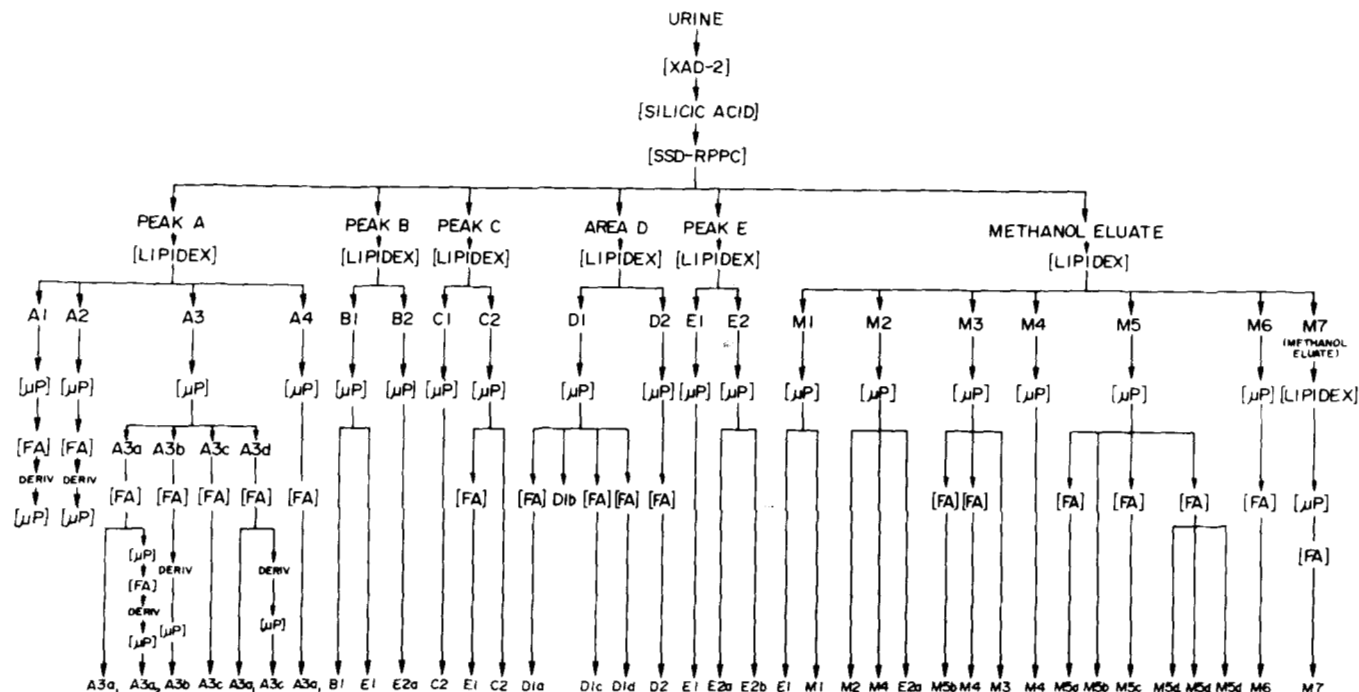


FIG. 2. Flow diagram outlining urine purification procedures and the isolation and letter-number designation of identified metabolites. Brackets denote chromatography procedures. [SSD-RPPC] = Hyflo Super-Cel reversed phase partition chromatography using solvent system D (Table I); [Lipidex] = liquid-gel chromatography on Lipidex-1000; [μP] = high performance liquid chro-

matography on a μPorasil column; [FA] = high performance liquid chromatography on a fatty acid analysis column; DERIV. = conversion of metabolite to either a methyl ester, *t*-butyldimethyldiyl ether or a methyl ester, *O*-methyloxime, *t*-butyldimethylsilyl ether derivative.

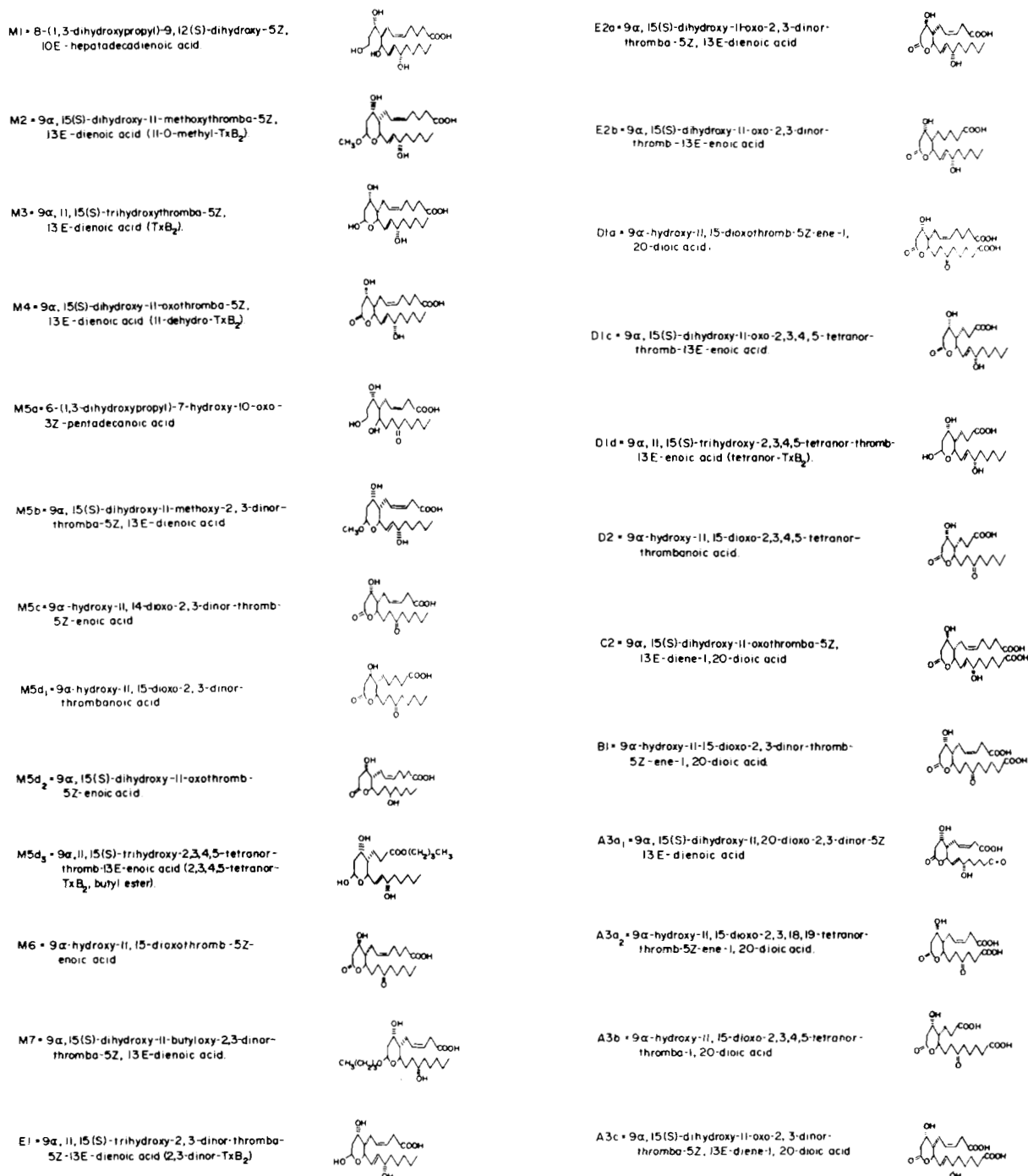


FIG. 3. Letter-number designation of each identified metabolite along with the respective chemical names and structures.

tion of the total radioactivity in the urine. Both metabolites, 2,3-dinor-TxB<sub>2</sub> and 2,3,4,5-tetranor-TxB<sub>2</sub>, were products of β oxidation.

Sensitized guinea pig lungs have been described as capable of converting TxB<sub>2</sub> to 15-keto-13,14-dihydro-TxB<sub>2</sub> (20). The efficiency of this conversion also apparently increases with successive antigenic challenges (21). We have incubated TxB<sub>2</sub> with the 100,000 × *g* supernatant of guinea pig liver with added NAD<sup>+</sup> and have not found any conversion of TxB<sub>2</sub> to 15-keto-13,14-dihydro-TxB<sub>2</sub>. PGE<sub>2</sub> incubated with the same 100,000 × *g* liver supernatant was essentially quantitatively converted to 15-keto-13,14-dihydro-PGE<sub>2</sub>.<sup>3</sup> These data and

<sup>3</sup> L. J. Roberts, II, B. J. Sweetman, and J. A. Oates, unpublished observations.

the present study suggest that in man and in the absence of immunologic sensitization in the guinea pig that TxB<sub>2</sub> is not a good substrate for the 15-hydroxy-prostaglandin dehydrogenase enzyme. We cannot exclude the possibility, however, that 15-keto-13,14-dihydro-TxB<sub>2</sub> was formed in the present study and merely escaped detection since all of the urinary radioactivity was not identified. Alternatively, any 15-keto-13,14-dihydro-TxB<sub>2</sub> formed may not be excreted into the urine due to possible conversion to 11-dehydro-15-keto-13,14-dihydro-TxB<sub>2</sub>. We are presently investigating this latter possibility.

The second major series of compounds, formed as a result of dehydrogenation of the hemiacetal alcohol group of TxB<sub>2</sub>, was comprised of 16 identified metabolites. The second most

TABLE I  
Percentages of total recovered urinary radioactivity represented by identified metabolites

Metabolite	Percentage
E1 + M5b + M7	23.0
M4	7.6
E2a	7.3
D1d + M5d <sub>3</sub>	5.3
D1c	2.6
M3 + M2	2.5
D1a	2.0
D2	1.6
A3c	1.5
A3a <sub>2</sub>	1.3
A3a <sub>1</sub>	1.3
M6	1.0
C2	0.9
M1	0.7
B1	0.6
E2b	0.5
M5c	0.4
M5a	0.4
A3b	0.3
M5d <sub>1</sub>	0.3
M5d <sub>2</sub>	0.1
Total	61.2

TABLE II  
Distribution of types of metabolic transformation among identified metabolites

	Hemiacetal ring		$\delta$ -Lactone ring		Acyclic C-11, C-12 diol		Total	
	%	No.	%	No.	%	No.	%	No.
Ring modifications	50.3	3	47.8	16	1.8	2	100	21
$\beta$ oxidation stages								
C-20	4.1	1	18.9	5	1.1	1	24.1	7
C-18	37.5	1	21.6	8	0.7	1	59.8	10
C-16	8.7	1	7.4	3	0	0	16.1	4
$\omega$ oxidation	0	0	10.8	6	0	0	10.8	6
C-15 hydroxy	50.3	3	35.6	8	1.1	1	87.0	12
C-15 Keto	0	0	12.3	8	0.7	1	13.0	9

abundant metabolite in the urine, 11-dehydro-TxB<sub>2</sub>, was the product of this single metabolic transformation. Additional extensive metabolism of 11-dehydro-TxB<sub>2</sub> occurred by processes of  $\beta$  oxidation,  $\omega$  oxidation, dehydrogenation of the C-15 alcohol group, and reduction of the  $\Delta^5$  and  $\Delta^{13}$  double bonds. The enzyme responsible for dehydrogenation of the hemiacetal alcohol group is not known. We have found that TxB<sub>2</sub> incubated with the 100,000  $\times$  *g* supernatant of guinea pig liver with NAD<sup>+</sup> results in efficient dehydrogenation of the hemiacetal alcohol group. The dehydrogenation was very inefficient in the absence of NAD<sup>+</sup>.<sup>3</sup> Therefore, the enzyme appears to be a soluble, NAD-dependent enzyme.

The several compounds identified that had undergone dehydrogenation of the C-15 alcohol group suggests that the 11-dehydro derivatives are better substrates for the 15-hydroxy-prostaglandin dehydrogenase than compounds with an intact hemiacetal ring. It is assumed that the 15-hydroxy-prostaglandin dehydrogenase enzyme is responsible for the dehydrogenation of the C-15 alcohol group although a different enzyme cannot be excluded.

The third minor series of metabolites were acyclic compounds with alcohol groups at C-11 and C-12. This acyclic structure is the same as the sodium borohydride-reduced product of TxB<sub>2</sub> (13). This biochemical transformation is envisioned to occur by a process of reduction of the C-11 aldehyde group of the aldehyde-alcohol form of the original hemiacetal ring. The enzyme responsible for this conversion

is unknown. However, TxB<sub>2</sub> is converted in part to this acyclic derivative when incubated with 100,000  $\times$  *g* supernatant of guinea pig liver with added NADPH. The conversion is less efficient in the absence of NADPH or in the presence of NADH.<sup>3</sup> It, therefore, appears that this enzyme is a NADPH-dependent soluble enzyme.

Two minor metabolites (E2b and M5d<sub>1</sub>) were identified in which the  $\Delta^5$  double bond has been reduced. Although reduction of the  $\Delta^5$  double bond has been described in the metabolism of PGF<sub>2 $\alpha$</sub>  in the rat (15), this has not been found to occur in the metabolism of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in man, although 5,6-dihydro metabolites may have escaped detection in previous metabolism studies since all urinary radioactivity was not identified (22, 23). The mass spectral data on metabolite A3a<sub>1</sub> suggests the presence of an oxo group attached on one of the carbon atoms from C16-20 and A3a<sub>1</sub> was tentatively identified as either 9 $\alpha$ ,15(S)-dihydroxy-11,19-dioxo-2,3-dinor-thromboxane-5Z,13E-dienoic acid or 9 $\alpha$ ,15(S)-dihydroxy-11,20-dioxo-2,3-dinor-5Z,13E-dienoic acid. Analogous metabolites have not been previously described in the metabolism of prostaglandins. Insufficient material was present to permit further structural analysis. The biochemical mechanisms leading to the formation of this oxo group are unknown. The two most likely sites for the location of the oxo group would seem to be at C-19 or C-20 since metabolites of prostaglandins and TxB<sub>2</sub> with hydroxyl groups at the  $\omega$ -1 and  $\omega$ -2 positions have been described (7, 16-19).

The formation of 11-O-methyl-TxB<sub>2</sub> (M2), 11-O-methyl-2,3-dinor-TxB<sub>2</sub> (M5b), 11-O-butyl-2,3-dinor-TxB<sub>2</sub> (M7), and 2,3,4,5-tetranor-TxB<sub>2</sub> butyl ester (M5d<sub>3</sub>) are all considered to have formed artifactually from TxB<sub>2</sub>, 2,3-dinor-TxB<sub>2</sub>, and 2,3,4,5-tetranor-TxB<sub>2</sub> during chromatography. We had previously found that the hemiacetal alcohol group of the thromboxane ring is highly reactive with an alcohol in the presence of acid and identified several 11-O-ethyl derivatives in our study of the metabolism of TxB<sub>2</sub> in the monkey (7). In that study, the original XAD-2 column was eluted with ethanol and ethanol was frequently used during evaporation procedures to form an azeotrope with water. In the present study, ethanol was purposely avoided throughout urine processing and the XAD-2 column was eluted with methanol. This explains the presence in the present study of 11-O-methyl derivatives and the absence of 11-O-ethyl derivatives. 11-O-butyl-2,3-dinor-TxB<sub>2</sub> undoubtedly formed during the initial reversed phase partition chromatography with the solvent system of butanol/water/acetic acid. The finding of a small quantity of 2,3,4,5-tetranor-TxB<sub>2</sub> butyl ester may be indicative that tetranor-TxB<sub>2</sub> can exist as  $\delta$ -lactone, although this form has not been isolated and identified. We have found that the  $\delta$ -lactone ring of the 11-dehydro metabolites stored in ethanol at -30 °C will esterify with the ethanol whereas no esterification occurs with the upper side chain carboxyl group.<sup>3</sup> We have also observed that 2,3,4,5-tetranor-TxB<sub>2</sub> stored in ethanol will form 2,3,4,5-tetranor-TxB<sub>2</sub> ethyl ester.<sup>3</sup> In contrast, 2,3-dinor-TxB<sub>2</sub> and TxB<sub>2</sub>, which would not be expected to lactonize, when stored in ethanol do not form ethyl esters. This is, therefore, suggestive that the 2,3,4,5-tetranor-TxB<sub>2</sub> butyl ester formed as a result of reaction of its  $\delta$ -lactone form with butanol rather than direct esterification of the upper side chain carboxyl group by butanol.

Of interest was the identification of 2,3-dinor-PGF<sub>2 $\alpha$</sub>  in the urine during the course of analysis of material in peak E2b. This compound was not identified in previous studies of the metabolism of PGF<sub>2 $\alpha$</sub>  in man (22), although we have recently reported 2,3-dinor-PGF<sub>2 $\alpha$</sub>  as the major urinary metabolite of PGD<sub>2</sub> in the non-human primate (16). The fact that a sufficient amount of 2,3-dinor-PGF<sub>2 $\alpha$</sub>  was present in the 13-h urine

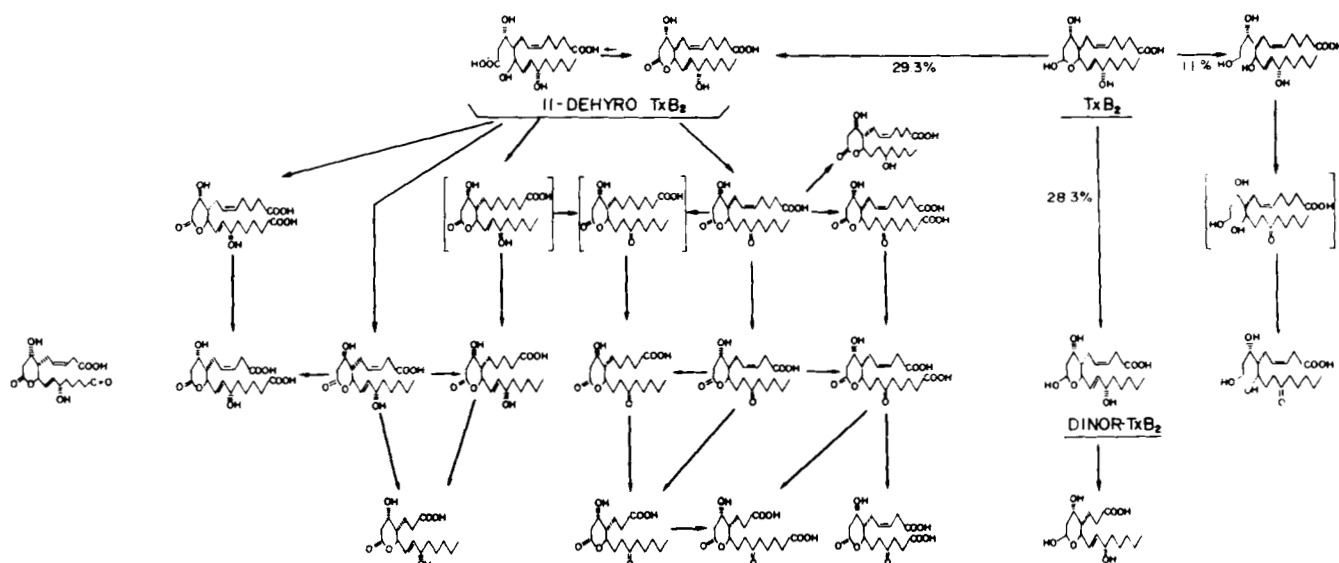


FIG. 4. Proposed pathways of metabolism of thromboxane B<sub>2</sub> in man. Compounds in brackets were not isolated but are proposed intermediates in the formation of identified metabolites. Metabolite A3a<sub>1</sub> (far left) is depicted as a terminal C20 aldehyde although the precise location of the oxo group on carbon atoms C-16 to C-20 has not been defined and the metabolic pathways leading to its formation are unknown.

collection after chromatographic losses to obtain a complete mass spectrum would suggest that several micrograms of the endogenous compound are excreted during a 24-h period.

This study has provided the background biochemical information necessary to begin quantification of urinary metabolites of TxB<sub>2</sub> as a means to assess *in vivo* production of TxB<sub>2</sub> in man. In this regard, we have now developed a stable isotope dilution assay for 2,3-dinor-TxB<sub>2</sub> using combined gas chromatography-mass spectrometry. Six normal adult males have been found to excrete a few hundred picograms/mg of creatinine of 2,3-dinor-TxB<sub>2</sub>.<sup>4</sup> These initial studies document the excretion of endogenous 2,3-dinor-TxB<sub>2</sub> in human urine and indicate the possibility that an assay for urinary 2,3-dinor-TxB<sub>2</sub> may prove to be a useful index of *in vivo* TxB<sub>2</sub> production in man.

**Acknowledgments**—We are grateful to Dr. N. A. Nelson of the Upjohn Co. for the generous gift of chemically synthesized TxB<sub>2</sub>. The skillful technical assistance of N. A. Payne was greatly appreciated.

#### REFERENCES

- Hamberg, M., Svensson, J., and Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 2994-2998
- Ellis, E. F., Oelz, O., Roberts, L. J., II, Payne, N. A., Sweetman, B. J., Nies, A. S., and Oates, J. A. (1976) *Science* **193**, 1135-1137
- Samuelsson, B., and Gr  n, K. (1974) *Biochem. Med.* **11**, 298-303
- Samuelsson, B., Granstr  m, E., Gr  n, K., Hamberg, M., and Hammarstr  m, S. (1975) *Annu. Rev. Biochem.* **44**, 669-695
- Roberts, L. J., II, Sweetman, B. J., Morgan, J. L., Payne, N. A., and Oates, J. A. (1977) *Prostaglandins* **13**, 631-647
- Kindahl, H. (1977) *Prostaglandins* **13**, 619-629
- Roberts, L. J., II, Sweetman, B. J., and Oates, J. A. (1978) *J. Biol. Chem.* **253**, 5305-5318
- Roberts, L. J., II, Sweetman, B. J., Payne, N. A., and Oates, J. A. (1977) *J. Biol. Chem.* **252**, 7415-7417
- Samuelsson, B., Hamberg, M., Roberts, L. J., II, Oates, J. A., and Nelson, N. A. (1978) *Prostaglandins* **16**, 857-860
- Norman, A., and Sj  val, J. (1958) *J. Biol. Chem.* **233**, 872-885
- Nystrom, E., and Sj  val, J. (1973) *Anal. Lett.* **6**, 155-161
- James, A. T., and Martin, A. J. P. (1956) *Biochem. J.* **63**, 144-152
- Hamberg, M., and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 3400-3404
- Svanborg, K., and Bygdeman, M. (1972) *Eur. J. Biochem.* **28**, 127-135
- Gr  n, K. (1972) *Biochim. Biophys. Acta* **231**, 419-444
- Ellis, C. K., Smigel, M. D., Oates, J. A., Oelz, O., and Sweetman, B. J. (1979) *J. Biol. Chem.* **254**, 4152-4163
- Sun, F. F., and Stafford, J. E. (1974) *Biochim. Biophys. Acta* **369**, 95-110
- Taylor, P. L., and Kelly, R. W. (1975) *FEBS Lett.* **57**, 22-25
- Gr  n, K. (1971) *Biochemistry* **10**, 1072-1086
- Dawson, W., Boot, J. R., Cockerill, H. F., Mallen, D. N. B., and Osborne, D. J. (1976) *Nature* **262**, 699-702
- Boot, J. R., Cockerill, A. F., Dawson, W., Mallen, D. N. B., and Osborne, D. J. (1978) *Int. Arch. Allergy Appl. Immunol.* **57**, 159-164
- Granstr  m, E., and Samuelsson, B. (1971) *J. Biol. Chem.* **246**, 7470-7485
- Hamberg, M., and Samuelsson, B. (1977) *J. Biol. Chem.* **246**, 6713-6721

<sup>4</sup> R. Maas, L. J. Roberts, II, and J. A. Oates, unpublished observations.

## SUPPLEMENTAL MATERIAL TO

Metabolism of Thromboxane B<sub>2</sub> in Man

L. Jackson Roberts, II, Brian J. Swetnam, and John A. Oates

This section contains Experimental Procedures and details in Results of chromatographic purification and structural elucidation of isolated metabolites.

## EXPERIMENTAL PROCEDURES

## Materials

Unlabeled  $\text{Ddb}_2$  was a generous gift of Dr. Norman A. Nelson and Dr. John Pike of the Upjohn Company, Kalamazoo, Michigan. [5,6,8,9,11,12,14,15- $^3\text{H}$ ] Arachidonic acid (64 Ci/mmol) was obtained from New England Nuclear, Boston, Massachusetts. [5,6,8,9,11,12,14,15- $^3\text{H}$ ]  $\text{Ddb}_2$  was biosynthesized from [ $^3\text{H}$ ] arachidonic acid using sheep vesicular vesicle microsomes and washed human platelets as previously described (8).

## Methods

**Infusion of Thromboxane B<sub>2</sub>.** - [ $^3\text{H}$ ]  $\text{Ddb}_2$  sodium salt (1.5 mg, 12.2 Ci/mol) was infused into the antecubital vein of a healthy adult male at a rate of 6.4  $\mu\text{g}/\text{min}$ , as previously described (8). Urine was collected from the beginning of the infusion until 64 hours after the infusion was completed. The total time of urine collection was 13 h.

**Acetate- $\text{V}_2\text{O}_5$  and Silicic Acid Chromatography.** - The urine was extracted on Amberlite VAC-2 (Mallinckrodt, Inc., St. Louis, Missouri) chromatography as previously described (8).

**Reversed-Phase Partition Chromatography.** - Reversed-phase partition chromatography was performed on a support of Hyflo Super-Cel (Picher Scientific Co., Fair Lawn, New Jersey) using solvent system D as previously described (8,10).

**Liquid-Liquid Chromatography.** - Reversed phase liquid-liquid chromatography was performed using Lipidex-1000 (Packard Instrument Co., Inc., Downers Grove, Illinois) as previously described (11).

**High Performance Liquid Chromatography.** - High performance liquid chromatography was performed as previously described with a Waters Associates (Milford, Mass.) liquid chromatograph using a  $\mu$ -Porasil and fatty acid analysis column (Waters Associates).

**Chromatography Solvent Systems and Programs.** - Chromatography solvent systems and solvent programs for high performance liquid chromatography are listed in Table I.

**Gas-Liquid Chromatography-Mass Spectrometry.** - Mass spectrometry was performed using a LKB 9000-PDF 12 combined gas-liquid chromatograph-mass spectrometer computer system (5). C-values were obtained using mixtures of methyl esters of normal saturated fatty acids as standards (12). Radioactivity detection of gas-liquid chromatography peaks and C-value determination was accomplished by analyzing a portion of the samples on a Nuclear-Chicago 4740 gas-liquid chromatograph coupled with a series 5000 radioactivity monitor (5). C-values obtained for identified metabolites are listed in Table 2.

**Preparation of Derivatives for Gas-Liquid Chromatography and Mass Spectrometry.** - Methyl esters were formed by treatment with excess diazomethane in methanol for 5 minutes. O-Methylxanthine derivatives were formed by treatment with 2% methanamine NAL in pyridine at room temperature for 12-13 hours. Trimethylsilyl ether derivatives were formed by treatment with N,O-Bis(trimethylsilyl)trifluoroacetamide in pyridine at room temperature for 2 hours. *t*-Butyldimethylsilyl ether derivatives were formed by treatment with *t*-butyldimethylsilylchlorosilane/imidazole in dimethylformamide reagent (Applied Science Laboratories, Inc., State College, PA) at room temperature for 12 hours, followed by addition of water and extraction into hexane.

## RESULTS

**Further Purification and Identification of Compounds Eluted with Methanol from Stationary Phase (Solvent System D Column).** - The material eluted with methanol from the stationary phase was subjected to further chromatography on a 2.6 x 28.3 cm column of Lipidex-1000, solvent system B, flow rate 40 ml/h, 6 ml fractions (Fig. 1). Six peaks emerged designated M1 (45-270 ml of eluate, 13% of recovered radioactivity), M2 (276-342 ml, 57), M3 (348-468 ml, 16%), M4 (454-594 ml, 30%), M5 (600-792 ml, 11%), and M6 (798-948 ml, 5%). Fifteen percent of the radioactivity was eluted with 84 ml of methanol and designated M7.

Table 2  
C-Values Obtained on 15, Deasil  
for Identified Thromboxane B<sub>2</sub> Metabolites

Metabolite	Me-TMS	Me-HD-TMS	Me-TBMS	Me-HD-TBMS
M1	23.5			
M2	22.5			
M3	23.6	23.7		
M4 (6-lactone)	26.7			
M4 (acid-alcohol)	23.8			
M5a		22.5		
M5b	21.7			
M5c (6-lactone)		25.2		
M5c (acid-alcohol)		22.9		
M5d (6-lactone)		25.5		
M5d (acid-alcohol)		22.5		
M5e (6-lactone)	26.8			
M5f		22.5		
M6 (6-lactone)		26.8		
M6 (acid-alcohol)		22.9		
M7	23.0			
E1	22.1	22.2		
E2a (6-lactone)	25.1			
E2a (acid-alcohol)	22.3			
E2b (6-lactone)	24.9			
E2b (acid-alcohol)	22.1			
D1a (6-lactone)		29.8		
D1a (acid-alcohol)		27.3		
D1c (6-lactone)	23.2			
D1d	20.6			
D2 (6-lactone)	20.5	23.45		
C2 (6-lactone)	28.7		33.1	
C2 (acid-alcohol)	26.7		32.0	
B1 (6-lactone)		27.8 & 28.8		
A3a <sub>1</sub> (6-lactone)		27.3		26.0
A3a <sub>2</sub> (6-lactone)				28.6
A3b (6-lactone)		26.5		
A3c	28.0		32.3	

Me-TMS = methyl ester, trimethylsilyl ether

Me-HD-TMS = methyl ester, O-methylxanthine, trimethylsilyl ether

Me-TBMS = methyl ester, tert-butyldimethylsilyl ether

Me-HD-TBMS = methyl ester, O-methylxanthine, tert-butyldimethylsilyl ether

**Purification of M1.** - Material in M1 was further purified by high performance liquid chromatography on a  $\mu$ -Porasil column with solvent system F. In addition to multiple small peaks, two prominent peaks emerged. The less polar peak (55-61 ml of eluate, 27% of recovered radioactivity) was subsequently determined to be a minor amount of compound E1 (see below). The more polar peak (74-77 ml, 19%) was designated Metabolite M1.

**Structure of Metabolite M1.** - The methyl ester of M1 was converted to a Me<sub>3</sub>Si ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum was identical to that obtained for the product formed after treatment of  $\text{Ddb}_2$  with sodium borohydride (13). It was therefore concluded that Metabolite M1 was 8-(1,3-dihydroxypropyl)-9,12(5)-dihydroxy-32,13E-heptadecadienoic acid.

**Purification of M2.** - Material in Peak M2 was further purified by high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Four prominent peaks emerged. The least polar peak (28-29 ml of eluate, 7% of recovered radioactivity) was designated M2. Another peak (38-39 ml, 24%) was subsequently determined to be a minor amount of Metabolite M4 (see below). A more polar peak (42-44 ml, 19%) was subsequently determined to be a minor amount of Metabolite E2a (see below). A broad peak of more polar material eluted (44-50 ml, 55%) from which no compound was subsequently identified.

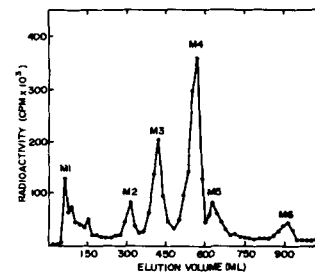


Fig. 1. Liquid-gel chromatography on Lipidex-1000 of material eluted with methanol from the stationary phase of the Hyflo Super-Cel Solvent System D chromatography (Fig. 1 in regular print section). Column 2.6 x 28.3 cm; Solvent B (Table 1); flow rate: 40 ml/h; fraction volume: 6 ml.

**Structure of Compound M2.** - The methyl ester of M2 was converted to a Me<sub>3</sub>Si ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained was identical to that obtained for 11-O-methyl- $\text{Ddb}_2$  formed by reacting  $\text{Ddb}_2$  with methanol (1). We, therefore, concluded that Compound M2 is 9a,15(5)-dihydroxy-11-methoxythromboxane-52,13E-dienoic acid. In our previous study of  $\text{Ddb}_2$  metabolism in the monkey (7), among the urinary compounds identified were 11-O-ethyl-2,3-dinor- $\text{Ddb}_2$ , 11-O-ethyl-2,3,4,5-tetra-nor- $\text{Ddb}_2$ , and 11-O-ethyl-19-hydroxy-2,3-dinor- $\text{Ddb}_2$ . We also demonstrated that  $\text{Ddb}_2$  when reacted with acetyl chloride in ether or ethanol was converted to the respective 11-O-acetyl or 11-O-ethyl derivative. Ethanol was used in the monkey study to elute compounds from XAD-2 and also frequently used to form an azeotrope with water. In the present study, ethanol was deliberately avoided throughout the purification procedures. Methanol was used to elute compounds from XAD-2. An additional 11-O-methyl compound as well as a 11-O-butyl derivative have been identified in this study (see Compounds M5b and M7). 11-O-Ethyl derivatives have not been detected in this study. This data indicates that these compounds most likely are formed as a result of exposure of the hemiacetal alcohol group to an alcohol in the presence of acid. The 11-O-butyl derivative was apparently formed as a result of exposure to butanol under acidic conditions during the initial reversed phase partition chromatography using solvent system D (butanol/water/acetic acid).

**Purification of M3.** - Material in Peak M3 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Three prominent peaks emerged. The less polar peak (44 ml of eluate, 7% of recovered radioactivity), was subsequently found to be a lesser quantity of Compound M5b (see below). A peak of intermediate polarity (46-47 ml, 19%) was subsequently found to be a lesser quantity of Metabolite M4 (see below). The most polar peak was designated Compound M3 (51-55 ml, 55%). Compounds M5b and M4 in peak M3 were each further purified by high performance liquid chromatography on a fatty acid analysis column using solvent system H. M5b eluted quantitatively at 36 ml of eluate and M4 at 28-29 ml.

**Structure of Compound M3.** - The methyl ester of M3 was converted to a trimethylsilyl ether derivative and a O-methylxanthine, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. Mass spectra obtained for both derivatives were identical to those previously reported for  $\text{Ddb}_2$  (13) and it was therefore concluded that Compound M3 was  $\text{Ddb}_2$ .

**Purification of M4.** - Material in M4 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. A single prominent peak emerged (51-52 ml of eluate, 82% of recovered radioactivity) that was designated Metabolite M4.

**Structure of Metabolite M4.** - The methyl ester of M4 was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained was identical to that previously reported by us for the same derivative of 11-dehydro- $\text{Ddb}_2$  (7). It was therefore concluded that M4 was the 6-lactone 9a,15(5)-dihydroxy-11-oxothromboxane-52,13E-dienoic acid.

**Purification of M5.** - Material in M5 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Four prominent peaks emerged designated M5a (28-31 ml of eluate, 13% of recovered radioactivity), M5b (32 ml, 29%), M5c (34-36 ml, 23%), and M5d (39-42 ml, 23%). M5a, M5c, and M5d were each subjected to additional purification by high performance liquid chromatography on a fatty acid analysis column using solvent systems H, I, and H, respectively. M5a eluted from this column in a single peak at 24-30 ml of eluate (100% of recovered radioactivity) and was designated Metabolite M5a. M5c eluted from the column in a single prominent peak at 29 ml eluate (61%) along with other small peaks, and was designated Metabolite M5c. Material in M5d eluted from the column in three prominent peaks designated Compound M5d<sub>1</sub> (32-33 ml, 43%), M5d<sub>2</sub> (34-36 ml, 30%), and M5d<sub>3</sub> (37-41 ml, 27%).

**Structure of Metabolite M5a.** - The methyl ester of M5a was converted to a O-methylxanthine, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. It was concluded that M5a was 6-(1,3-dihydroxypropyl)-7-hydroxy-10-oxo-12-pentadecanoic acid on the basis of a mass spectrum identical to one previously published by us for this compound (7).

**Structure of Compound M5b.** - The methyl ester of M5b was converted to a Me<sub>3</sub>Si ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained contained prominent ions at m/e 514 (6), 499 (9-15), loss of  $\text{CH}_3$  483 (4-11), loss of  $\text{OCH}_3$  482 (4-12), loss of  $\text{CH}_2\text{OH}$  467 (4-32), 463 (15-17), loss of  $\text{CH}_2\text{CH}_2\text{OH}$  424 (4-40), loss of  $\text{H}_2\text{SiO}_3$  411 (4-32-71), 401 (4-11), loss of  $\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$  353 (4-30-71), 321 (4-30-71-32); 301 ( $\text{H}_2\text{SiO}_3\text{-CH}_2\text{CH}_2\text{CH}_2\text{OH}$ ), formed by rearrangement; 228 (base) ( $\text{H}_2\text{SiO}_3\text{-CH}_2\text{CH}_2\text{CH}_2\text{OH}$ ), 159 ( $\text{H}_2\text{SiO}_3\text{-CH}_2\text{CH}_2\text{OH}$ ), and 129 ( $\text{H}_2\text{SiO}_3\text{-CH}_2\text{CH}_2\text{OH}$ ). This mass spectrum is similar to that obtained for Compound M2 in that loss whose formation involved loss of the carbonyl side chain has identical m/e values. The spectra differ, however, in that loss in M5b whose formation did not involve loss of the carbonyl side chain are 28 mass units less than the corresponding loss in M2. This data indicated that M5b differed from M2 by the loss of 2 carbons from the carbonyl side chain as a result of one step of  $\beta$ -oxidation. It was therefore concluded that M5b was 9a,15(5)-dihydroxy-11-methoxy-2,3-dinor-thromboxane-52,13E-dienoic acid. As previously discussed (see Structure of Compound M2), the formation of this compound most likely arose artifactually by exposure of 2,3-dinor- $\text{Ddb}_2$  to methanol under acidic conditions during urine processing.

**Structure of Metabolite M5c.** - The methyl ester of M5c was converted to O-methylxanthine, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The structure of M5c was determined to be 9a-hydroxy-11,15-dioxo-2,3-dinor-thromboxane-52-monoic acid on the basis of a mass spectrum identical to that previously published by us for this compound (7).

TABLE 1

Chromatography Solvent Systems and Solvent Programs

Chromatography	Solvent System	Components	Program
1. Hyflo Super-Cel Reversed Phase Partition Chromatography	D	Mobile phase - water (300) Stationary phase - n-butyl Alcohol (100) Acetic acid (4)	Isocratic
2. Lipidex-1000 Liquid-Liquid Chromatography	A	Water/methanol/n-butyl alcohol/chloroform/acetic acid (40:60:5:5:0.12)	Isocratic
	B	Water/methanol/n-butyl alcohol/chloroform/acetic acid (50:50:5:5:0.11)	Isocratic
	C	Water/methanol/n-butyl alcohol/chloroform/acetic acid (60:40:7:3:0.11)	Isocratic
	E	Water/methanol/n-butyl alcohol/chloroform/acetic acid (70:30:9:1:0.11)	Isocratic
3. High Performance Liquid Chromatography- $\mu$ -Porasil Column	F	Solvent 1 - Chloroform Solvent 2 - Chloroform/methanol/acetic acid (500:50:1)	Linear gradient of Solvent 1 to Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
	G	Solvent 1 - Hexane Solvent 2 - Chloroform	Linear gradient of Solvent 1 to Solvent 2 over 1 hour, 1 ml/min, 1 ml fractions
4. High Performance Liquid Chromatography-Fatty Acid Analysis Column	H	Solvent 1 - Acetonitrile/water/benzene/acetic acid (100:400:1:0.5) Solvent 2 - Acetonitrile/benzene/acetic acid (500:1:0.5)	Linear gradient Solvent 1 to 70% Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
	I	Same as H	Linear gradient Solvent 1 to Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
	J	Same as H	Linear gradient Solvent 1 to 40% Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
4. (continued)	K	Solvent 1 - Acetonitrile/water/benzene/acetic acid (125:375:1:0.5) Solvent 2 - Acetonitrile/benzene/acetic acid (500:1:0.5)	Linear gradient Solvent 1 to 50% Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
	L	Solvent 1 - Acetonitrile/water/benzene/acetic acid (200:300:1:0.5) Solvent 2 - Acetonitrile/benzene/acetic acid (500:1:0.5)	Linear gradient Solvent 1 to Solvent 2 over 2 hrs, 1 ml/min, 1 ml fractions
	M	Acetonitrile/water/benzene/acetic acid (50:400:1:0.5)	Isocratic, 2 ml/min, 2 ml fractions
	N	Acetonitrile/water/benzene/acetic acid (100:400:1:0.5)	Isocratic, 2 ml/min, 2 ml fractions





**Purification and Structure of Metabolite E1**—Further purification and mass spectral analysis of Metabolite E1 has been previously published (8). Metabolite E1 was found to be the major urinary metabolite 9 $\alpha$ ,11,15-(*s*-trihydroxy-2,3-dinor-thromba-5 $\alpha$ ,13 $\beta$ -dienoic acid, 2,3-dinor-Thb<sub>2</sub>).

**Purification of E2**—Material in E2 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Two peaks emerged designated Metabolites E2a (38–40 ml of eluate, 82% of recovered radioactivity) and E2b (43–45 ml, 11%).

**Structure of Metabolite E2a**—The methyl ester of E2a was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 7) showed prominent ions at *m/z* 496 (90, 483 (M-15), loss of  $\cdot\text{CH}_3$ ; 467 (M-31), loss of  $\cdot\text{OCH}_3$ ; 427 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 408 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 385 (M-113), either formed by loss of  $\cdot(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{COOCH}_3$  or (M-71 + 42), loss of (71 + elimination of O- $\text{CH}_2$  from the ring); 377 (M-90 + 31); 343, rearrangement ion of unknown structure; 337 (M-90 + 71); 323 (M-160 + 15); loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring + 15; 318 (M-2 + 90); 295 (M-90 + 71 + 42); 247 (M-2 + 90 + 11); 235 (M-2 + 90 + 31); 199  $[\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2]$ , possibly in a four-membered ring form involving C13-15 and charged oxygen; 161 (Fig. 5); and 129  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2]$ . This mass spectrum is very similar to that previously published for 11-dehydro-Thb<sub>2</sub> (7) in that ions not containing the carboxyl side chain are identical but the mass spectra differ in that ions containing the carboxyl side chain are 28 mass units lower in the mass spectrum of E2a than the corresponding ions in the mass spectrum of 11-dehydro-Thb<sub>2</sub>. This data indicated that E2a differed from 11-dehydro-Thb<sub>2</sub> by the loss of 2 carbons from the carboxyl side chain as a result of one step of  $\beta$ -oxidation. This structure was also supported by obtaining mass spectra for the dicarboxylic acid form of this 6-lactone and the amide derivative when reacted with methanamine-HCl (c.f. ref. 7). It was therefore concluded that E2a was 9 $\alpha$ ,15(S)-dihydroxy-11-oxo-2,3-dinor-thromba-5 $\alpha$ ,13 $\beta$ -dienoic acid.

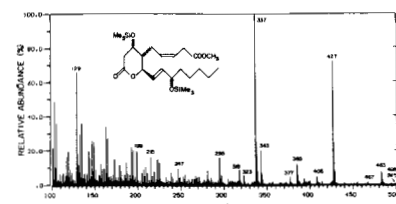


Fig. 7—Mass spectrum of the methyl ester, trimethylsilyl ether derivative of Metabolite E2a.

**Structure of Metabolite E2b**—The methyl ester of material in E2b was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained as a methyl ester, trimethylsilyl ether derivative (Fig. 8) showed prominent ions at *m/z* 500 (90; 485 (M-15), loss of  $\cdot\text{CH}_3$ ; 469 (M-31), loss of  $\cdot\text{OCH}_3$ ; 429 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 410 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 387 (M-71 + 42), loss of (71 + O- $\text{CH}_2$ ); 379 (M-90 + 31); loss of (90 +  $\cdot\text{OCH}_3$ ); 345 (M-155), rearrangement ion of unknown structure; 339, formed either by (M-90 + 71), or (M-160 + 1), loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring + 1; 325 (M-160 + 15); 249 (M-2 + 90 + 71); 217 (M-2 + 90 + 71 + 32); loss of (2 + 90 + 71 +  $\text{CH}_2\text{O}$ ); 199  $[\text{C}_6\text{H}_9\text{O}^+(\text{SiMe}_3)_2]$ , possibly in a four-membered ring form involving C13-15 and charged oxygen; and 129  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2]$ . Analysis of this mass spectrum and comparison with the mass spectrum of E2a indicated that E2b was a dihydro analogue of E2a. The data indicated that the double bond that had been reduced in E2b was  $\alpha^2$  since all ions in E2b that retained the upper side chain were 2 mass units higher than the corresponding ions in E2a. The only ion in the mass spectra of both compounds that did not retain the upper side chain in its structure was identical (m/z 199), indicating that the original  $\Delta^{13}$  double bond in each compound had not been reduced.

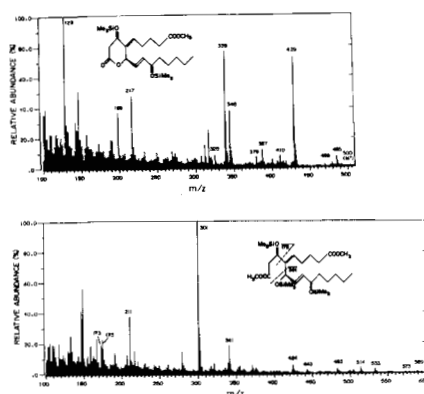


Fig. 8—Mass spectrum of the methyl ester, trimethylsilyl ether derivative of Metabolite E2b.

In addition to the mass spectrum recorded above for the 6-lactone form of E2b, another radioactive peak was also present on gas chromatography at a shorter retention time which gave a mass spectrum consistent with the dicarboxylic acid methyl ester form of the 6-lactone of E2b (c.f. ref. 7). This mass spectrum provided additional evidence for the proposed structure of E2b, that the original  $\alpha^2$  double bond had been reduced, and that the original  $\Delta^{13}$  double bond remained intact. This mass spectrum as a methyl ester, trimethylsilyl ether derivative (bottom, Fig. 9) showed prominent ions at *m/z* 589 (M-15), loss of  $\cdot\text{CH}_3$ ; 573 (M-31), loss of  $\cdot\text{OCH}_3$ ; 533 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 514 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 483 (M-90 + 31); 447 (M-90 + 71); 424 (M-2 +

90); 341 (M-173 + 90), loss of  $[\cdot\text{CH}(\text{SiMe}_3)_2]$ ; 301 (base)  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}(\text{OSiMe}_3)]$ ; 211 (301-90); 175  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{COOCH}_3]$ ; and 173  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}_2]$ . The base ion *m/z* 301 whose structure consists of the entire lower side chain (original C12-C20) confirms that the original  $\Delta^{13}$  double bond is intact.

Further evidence for the proposed structure of E2b was also obtained by analysis of the mass spectrum of the methyl ester, tert-butyldimethylsilyl ether derivative of the 6-lactone form of E2b. This mass spectrum showed prominent ions at *m/z* 569 (M-15), loss of  $\cdot\text{CH}_3$ ; 553 (M-31), loss of  $\cdot\text{OCH}_3$ ; 527 (base), (M-57), loss of  $\cdot\text{CH}_3$ ; 513 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 495 (M-57 + 32); loss of (57 +  $\text{CH}_2\text{O}$ ); 453 (M-131), loss of  $\cdot\text{OSiMe}_2\text{CH}_2$ ; 427 (M-115 + 42); loss of  $[\cdot\text{CH}_2\text{CH}_2\text{COOCH}_3 + \text{O}-\text{CH}_2]$ ; 421, either (M-131 + 31), loss of  $[\text{MeSiO}^+-\text{Si}(\text{CH}_3)_2]$  or (M-131 + 32); 411 (M-131 + 42); 395 (M-132 + 57); 361 (M-132 + 71); 363 (M-132 + 57 + 32); 337 (M-132 + 115); 325 (M-132 + 57 + 71); 293 (325-32); 251 (M-2 + 131 + 71); and 219 (M-131 + 132 + 71 + 31). The mass spectrum of this derivative of E2b was also consistent with the proposed structure and it was therefore concluded that E2b was 9 $\alpha$ ,15(S)-dihydroxy-11-oxo-2,3-dinor-thromba-13 $\beta$ -enoic acid.

**Further Purification and Identification of Compounds Eluting in Area D** Material in Area D was subjected to liquid gel chromatography on a 2.6  $\times$  30 cm column of Lipidex-1000 using solvent system E at a flow rate of 40 ml/hr, 6 ml fractions. Two broad unresolved peaks emerged (Figure 9) designated D1 (366–696 ml of eluate, 72% of recovered radioactivity) and D2 (702–858 ml, 23%).

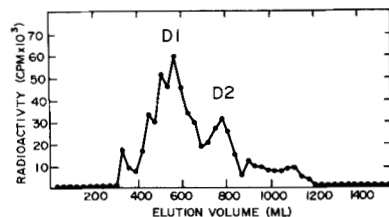


Fig. 9—Liquid-gel chromatography on Lipidex-1000 of material in Area D (Fig. 2). Column: 2.6  $\times$  30 cm; Solvent System E (Table 1); Flow rate: 40 ml/hr; fraction volume: 6 ml.

**Further Purification of D1**—Material in D1 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Four peaks emerged designated D1a (46–46 ml of eluate, 23% of recovered radioactivity), D1b (53–56 ml, 15%), D1c (57–58 ml, 22%), and D1d (61–63 ml, 46%). Co-eluting impurities prevented identification of a metabolite in peak in D1b.

**Further Purification of D1a**—D1a was further purified by high performance liquid chromatography on a fatty acid analysis column using solvent system J. Two peaks emerged. A minor peak at 29–31 ml of eluate (27% of recovered radioactivity) could not be subsequently identified. The major peak at 55–56 ml (73%) was designated Metabolite D1a.

**Structure of Metabolite D1a**—The methyl ester of D1a was converted to an O-methylxime, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 10) showed prominent ions at *m/z* 527 (90); 512 (M-15), loss of  $\cdot\text{CH}_3$ ; 496 (M-31), loss of  $\cdot\text{OCH}_3$ ; 454 (M-73), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 440 (M-57), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 427 (M-100), loss of  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ , following abstraction of a proton by nitrogen; 412 (M-115), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 406 (M-90 + 31), loss of  $[\text{MeSiOH} + 31]$ ; 396 (M-100 + 31); 386 (M-141), loss of  $\cdot(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ ; 366 (M-160 + 1), loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring + 1; 341 (M-186), loss of  $\cdot(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ ; 336 (M-160 + 31); 326 (M-160 + 90 + 31); 296 (M-141 + 90); 275 (M-2 + 31 + 300 + 90); 264 (M-160 + 100 + 1); 213 and 212 (unknown origin); 187 (base)  $[\text{CH}_2\text{CH}(\text{MeSiO})_2]$ ; 173  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}_2]$ ; and 115 (Fig. 5). Ions involving the loss of 146 mass units indicated a 6-lactone ring structure. The base ion *m/z* 187 indicated that  $\alpha$ -oxidation, dehydrogenation of the alcohol group at C-15, and reduction of the  $\Delta^{13}$  double bond had occurred. Ions involving the loss of 141 mass units indicated that  $\beta$ -oxidation had not occurred on the upper side chain. It was therefore concluded that D1a was 9 $\alpha$ ,15(S)-dihydroxy-11-oxo-2,3,4,5-tetra-nor-thromba-13 $\beta$ -enoic acid.

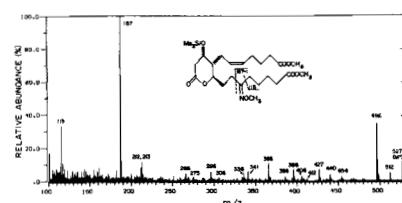


Fig. 10—Mass spectrum of the methyl ester, O-methylxime, trimethylsilyl ether derivative of Metabolite D1a.

**Further Purification and Structure of Metabolite D1c**—D1c was further purified by chromatography on a fatty acid analysis column using solvent system J. A single peak emerged at 41–44 ml of eluate (100% of recovered radioactivity) designated Metabolite D1c. The methyl ester of D1c was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Figure 11) showed prominent ions at *m/z* 457 (M-15), loss of  $\cdot\text{CH}_3$ ; 441 (M-31), loss of  $\cdot\text{OCH}_3$ ; 401 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 382 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 372, unknown origin; 359 (M-71 + 42); loss of (71 + O- $\text{CH}_2$ ); 351 (M-90 + 31); 317, rearrangement ion; 311 (base) (M-90 + 71); and (M-160 + 1), loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring + 1; 284; unknown origin; 269 (M-90 + 71 + 42); 264, either (M-90 + 71 + 32); loss of (90 + 71 +  $\text{CH}_2\text{O}$ ); or (M-160 + 32 + 1); 241 (M-160 + 71); 199  $[\cdot\text{CH}(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2)]$ ; 161 (Fig. 5); and 129  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2]$ . This mass spectrum was similar to the mass spectrum of the same derivative of 11-dehydro-Thb<sub>2</sub> except that ions containing the carboxyl side chain in D1c are 54 mass units less than corresponding ions in 11-dehydro-Thb<sub>2</sub> and the base ion in D1c is the (M-90 + 31) ion instead of *m/z* 129. It was therefore concluded that D1c was 9 $\alpha$ ,15(S)-dihydroxy-11-oxo-2,3,4,5-tetra-nor-thromba-13 $\beta$ -enoic acid.

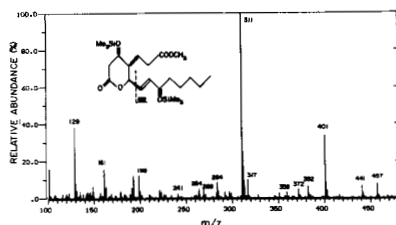


Fig. 11—Mass spectrum of the methyl ester, trimethylsilyl ether derivative of D1c.

**Further Purification and Structure of Metabolite D1d**—D1d was further purified by high performance liquid chromatography on a fatty acid analysis column using solvent system K. A single peak emerged at 12–17 ml of eluate (100% of recovered radioactivity) that was designated Metabolite D1d. The methyl ester of D1d was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 12) showed prominent ions at *m/z* 546 (90), 531 (M-15), loss of  $\cdot\text{CH}_3$ ; 515 (M-31), loss of  $\cdot\text{OCH}_3$ ; 475 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 456 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 385 (M-90 + 71); 366 (M-2 + 90); 312 (M-2 + 90 + 31); loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring; 301,  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}(\text{OSiMe}_3)]$ ; 241  $[\text{CH}_2\text{CH}(\text{CH}_2)_2\text{CH}_2]$ , formed by rearrangement; 235 (M-2 + 90 + 71); 269 (M-90 + 187); loss of (90 +  $\cdot(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ ); 241 (M-234 + 71); 217  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}_2]$ ; 202 (base)  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}_2]$ ; and 173  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2]$ . This mass spectrum was very similar to the mass spectrum of the same derivative of Thb<sub>2</sub> except that ions in D1d containing the carboxyl side chain are 54 mass units less than corresponding ions in the mass spectrum of Thb<sub>2</sub>. This data indicated that D1d differed from Thb<sub>2</sub> by the loss of 4 carbons from the carboxyl side chain as a result of two steps of  $\beta$ -oxidation. Additional evidence was obtained by analysis of D1d as a methyl ester, O-methylxime, trimethylsilyl ether derivative. The mass spectrum of this derivative of D1d showed prominent ions at *m/z* 575 (90, 560 (M-15), loss of  $\cdot\text{CH}_3$ ; 544 (M-31), loss of  $\cdot\text{OCH}_3$ ; 529 (M-31 + 15); 528 (M-32 + 15); loss of  $[\text{CH}_2\text{O} + 15]$ ; 504 (M-71), loss of  $\cdot(\text{CH}_2)_4$ ; 485 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 472 (M-71 + 32); 470 (M-90 + 15); 454 (M-90 + 71); 438 (M-2 + 90 + 31); 414 (M-90 + 71); 393 (M-2 + 90); 382 (M-90 + 71 + 32); 364 (M-2 + 90 + 31); 312 (M-173 + 90); loss of  $[\cdot\text{CH}(\text{OSiMe}_3)(\text{CH}_2)_2\text{CH}_2]$ ; 301 (base)  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}(\text{OSiMe}_3)]$ ; 211 (301-90); 174  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2\text{CH}_2]$ ; and 173  $[\text{MeSiO}^+-\text{CH}(\text{CH}_2)_2]$ . This mass spectrum was consistent with the side-chain-alcohol form of the proposed structure of D1d. It was therefore concluded that the structure of Metabolite D1d was 9 $\alpha$ ,11,15-(*s*-trihydroxy-2,3,4,5-tetra-nor-thromba-13 $\beta$ -enoic acid (tetra-nor-Thb<sub>2</sub>).

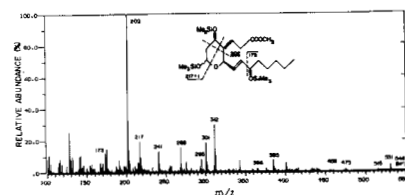


Fig. 12—Mass spectrum of the methyl ester, trimethylsilyl ether derivative of Metabolite D1d.

**Further Purification of D2**—Material in D2 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. A single prominent peak emerged designated Metabolite D2 (31–32 ml of eluate, 43% of recovered radioactivity) along with two smaller peaks (40–41 ml, 14%, and 45 ml, 5%). Insufficient material was present in the two smaller peaks to permit structural identification.

**Further Purification and Structure of Metabolite D2**—D2 was further purified by high performance liquid chromatography on a fatty acid analysis column using solvent system K. A single peak emerged at 41–42 ml (100% of recovered radioactivity) and was designated Metabolite D2. The methyl ester of D2 was converted to an O-methylxime, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained showed prominent ions at *m/z* 429 (90; 398 (M-31), loss of  $\cdot\text{OCH}_3$ ; 373 (M-56), loss of  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  following abstraction of a proton by nitrogen; 368 (M-90 + 31); loss of  $\text{H}_2\text{SiOH} + 31$ ; 156,  $[\text{CH}_2\text{CH}(\text{CH}_2)_2\text{CH}_2]$ ; 143  $[\cdot\text{CH}_2\text{C}(\text{CH}_2)_2\text{CH}_2]$ ; and 128, either  $[\text{CH}_2\text{CH}(\text{CH}_2)_2\text{CH}_2]$  or  $[\text{CH}_2\text{CH}(\text{CH}_2)_2\text{CH}_2]$ . This mass spectrum was similar to the mass spectrum of the same derivative of M6 except that ions containing the carboxyl side chain in D2 are 56 mass units lower than the corresponding ions in M6. This indicated that D2 differed from M6 by the loss of 4 carbons from the upper side chain as a result of two steps of  $\beta$ -oxidation. It was therefore concluded that the structure of D2 was 9 $\alpha$ -hydroxy-11,15-dioxo-2,3,4,5-tetra-nor-thromba-13 $\beta$ -enoic acid.

**Further Purification and Identification of Compounds Eluting in Peak C** Material in Peak C was converted to a methyl ester and subjected to liquid-gel chromatography on a 2.2  $\times$  30 cm column of Lipidex-1000 using solvent system C at a flow rate of 70 ml/hour collecting 6 ml fractions. The prominent peaks emerged (Figure 13) designated C1 (192–240 ml of eluate, 15.4% of recovered radioactivity) and C2 (312–384 ml, 23%).

**Further Purification of C2**—Material in C2 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Two peaks emerged designated C2 (36 ml of eluate, 56% of recovered radioactivity) and a peak at 38–41 ml (18%). It was subsequently determined after additional purification on a fatty acid analysis column and analysis by gas chromatography-mass spectrometry that the compound at 38–41 ml of eluate was a lesser quantity of Metabolite E1.

**Structure of Metabolite C2**—The methyl ester of C2 was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 14) showed prominent ions at *m/z* 570 (90; 555 (M-15), loss of  $\cdot\text{CH}_3$ ; 539 (M-31), loss of  $\cdot\text{OCH}_3$ ; 480 (M-90), loss of  $\text{H}_2\text{SiOH}$ ; 455 (M-115), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 449 (M-90 + 31); 429 (M-141), loss of  $\cdot\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ ; 413 (M-115 + 42); loss of (115 + O- $\text{CH}_2$ ); 409 (M-160 + 15); loss of the elements of  $\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  from the ring + 1; 395 (M-160 + 15); 379 (M-160 + 31); 371, rearrangement ion; 365 (M-115 + 50); 340, possibly (371-31); 323 (M-115 + 90 + 42); 275 (M-2 + 90 + 115);



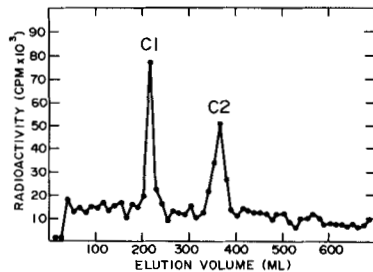


Fig. 13 - Liquid-gel chromatography on Lipidex-1000 of the methyl ester of material in Peak C (Fig. 2). Column: 2.2 x 30 cm; Solvent System C (Table 1); flow rate: 70 mL/h; fraction volume: 6 mL.

243 [M-2 x 90 + 115 + 32]; 217 [M<sub>2</sub>SiO<sub>2</sub>-OH(CH<sub>2</sub>)<sub>2</sub>COOCH<sub>3</sub>]; and 129 [M<sub>2</sub>SiO<sub>2</sub>-OH-CH(CH<sub>3</sub>)<sub>2</sub>]. This mass spectrum was very similar to that of the same derivative of 11-dehydro-Tb<sub>2</sub> except that ions formed as a result of the loss of 71 mass units in the mass spectrum of 11-dehydro-Tb<sub>2</sub> had analogous ions in the mass spectrum of C2 that involved the loss of 115 mass units. This indicated that C2 was apparently formed as a result of  $\omega$ -oxidation of 11-dehydro-Tb<sub>2</sub>. Additional evidence was obtained by obtaining a mass spectrum of the methyl ester, tert-butylidimethylsilyl ether derivative of C2. The mass spectrum of this derivative showed prominent ions at m/z 639 (M-15), loss of -CH<sub>3</sub>; 623 (M-31), loss of -OCH<sub>3</sub>; 597 (base) (M-57), loss of -CH<sub>3</sub>; 570 (M-115), loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 465 [M-(132 + 57)], loss of (M<sub>2</sub>SiO<sub>2</sub>SiOH + 57); 395, either (M-259), loss of -CH(OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>)COOCH<sub>3</sub>, or [M-(202 + 57)], loss of the elements of (OC(O)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> from the ring + 57); 321 [M-(202 + 131)], loss of (202 + 132 + 31); 257, unknown origin; and 101, C<sub>10</sub>H<sub>17</sub>O<sub>2</sub>Si<sub>2</sub>. This mass spectrum of this derivative of C2 was judged consistent with the proposed structure of C2. In addition, a mass spectrum of an earlier gas chromatographic peak as a methyl ester, tert-butylidimethylsilyl ether derivative provided further support for the proposed structure. The mass spectrum obtained indicated that this earlier peak represented a small quantity of the acid-alcohol form of the 6-lactone and showed prominent ions at m/z 743 (M-57), loss of -CH<sub>3</sub>; 711 [M-(57 + 32)], loss of (57 + CH<sub>2</sub>O); 637, either [M-(132 + 31)], loss of (M<sub>2</sub>SiO<sub>2</sub>SiOH + OCH<sub>3</sub>), or [M-(131 + 32)], loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 612 [M-(131 + 57)], 537 [M-(131 + 132)]; 505, either [M-(131 + 132 + 32)], or [M-(2 + 132 + 31)]; 429 (base) [M<sub>2</sub>SiO<sub>2</sub>-OH-CH(CH<sub>3</sub>)<sub>2</sub>COOCH<sub>3</sub>]; 297 (429-132), 217 [M<sub>2</sub>SiO<sub>2</sub>-OH-CH(CH<sub>3</sub>)<sub>2</sub>], and 159, possibly (217-57). The ion at m/z 217 indicated that this was the acid-alcohol form of the 6-lactone (7). The ion m/z 429 indicated that  $\omega$ -oxidation had occurred. From interpretation of these mass spectra, it was therefore concluded that the structure of C2 was 9a,15(S)-dihydroxy-11-oxo-thromboxane-32,13E-dioxo-1,20-diolic acid.

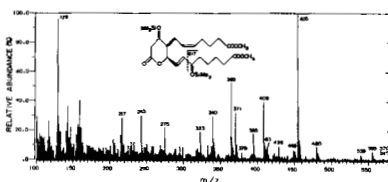


Fig. 14 - Mass spectrum of the methyl ester, trimethylsilyl ether derivative of Metabolite C2.

#### Further Purification and Structure of Metabolites in C1

Material in C1 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Two peaks emerged at 36-37 ml of eluate (48% of recovered radioactivity) and 55-57 ml (21%). From subsequent mass spectral analysis of the compound at 36-37 ml, it was determined that this metabolite was a lesser quantity of Metabolite C2. Co-eluting impurities prevented identification of the metabolite at 55-57 ml.

#### Further Purification and Identification of Compounds Eluting in Peak B

Material eluting in Peak B was methylated and subjected to chromatography on a 2.2 x 30 cm column of Lipidex-1000 using solvent system C at a flow rate of 60 mL/hour; 6 mL fractions. Two prominent peaks emerged (Fig. 15) designated B1 (596-660 ml of eluate, 34% of recovered radioactivity) and B2 (794-900 ml, 37%), 14% of recovered radioactivity remained on the stationary phase.

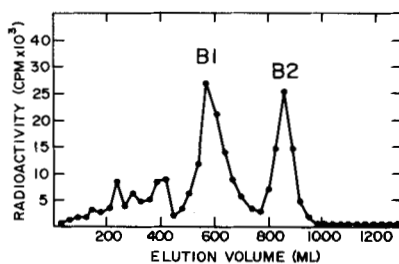


Fig. 15 - Liquid-gel chromatography on Lipidex-1000 of the methyl ester of material in Peak B (Fig. 2). Column: 2.2 x 30 cm; Solvent System C (Table 1); flow rate: 60 mL/h; fraction volume: 6 mL.

**Further Purification of B1** - Material in B1 was subjected to further purification by high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Two prominent peaks emerged. The less polar peak (32-33 ml of eluate, 13% of recovered radioactivity) was designated Metabolite B1. A mass peak (42-43 ml, 70%) was subsequently determined by mass spectral analysis to be a lesser quantity of Metabolite B1.

**Structure of Metabolite B1** - The methyl ester of B1 was converted to an O-methylolone, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained was interpreted to be a mixed spectrum of Metabolite B1 and a co-eluting impurity. Ions interpreted to originate from the co-eluting impurity were m/z 420, 396, 317, 217, 191, and 159. Loss of high intensity attributed to fragmentation of Metabolite B1 were m/z 499 (M), 485 (M-15), loss of -CH<sub>3</sub>; 468 (M-31), loss of -OCH<sub>3</sub>; 412 (M-87), loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 399 (M-100), loss of (CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub> following abstraction of a proton by nitrogen; 388 [M-(160 + 11)], loss of the elements of (OC(O)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> from the ring + R); 369 [M-(160 + 31)]; 157 (base), -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 115 (Fig. 2). When this mass spectrum was compared to that obtained for Metabolite B1a, it was apparent that Metabolite B1 was a C18 analog of Metabolite B1a. A base ion of m/z 187 in both spectra indicated that  $\omega$ -oxidation had occurred on the upper side of Metabolite B1. It was, therefore, concluded that Metabolite B1 was 9a-hydroxy-11,15-dioxo-2,3,18,19-tetrahydro-52-ene-1,20-diolic acid.

**Further Purification and Structure of the Metabolite in B2** - Material in B2 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. A single prominent peak emerged at 37-38 ml of eluate (85% of recovered radioactivity). From mass spectral analysis of this compound, it was determined that this compound was a lesser quantity of Metabolite B2a.

#### Further Purification and Identification of Compounds Eluting in Peak A

Material in Peak A was converted to a methyl ester and subjected to additional chromatography on a 2.6 x 30 cm column of Lipidex-1000, solvent system B, at a flow rate of 40 mL/h, 6 mL fractions. Three distinct peaks emerged along with a less resolved peak (Fig. 16) designated Peak A1 (138-174 ml of eluate, 8% of recovered radioactivity), Peak A2 (180-252 ml, 24%), Peak A3 (258-348 ml, 31%), and Peak A4 (344-480 ml, 11%). A negligible amount of radioactivity remained on the stationary phase. Multiple additional chromatographic procedures did not result in sufficient purification of compounds in Peak A1 and A2 to permit identification by gas chromatography-mass spectrometry.

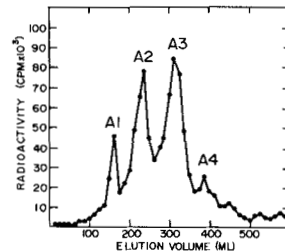


Fig. 16 - Liquid gel chromatography on Lipidex-1000 of the methyl ester of material in Peak A (Fig. 2). Column: 2.6 x 30 cm; Solvent System B (Table 1); flow rate: 40 mL/h; fraction volume: 6 mL.

**Further Purification of A1** - Material in A1 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. Four prominent peaks emerged designated A1a (22-25 ml of eluate, 34% of recovered radioactivity), A1b (26-28 ml, 11%), A1c (32-34 ml, 22%), and A1d (35-39 ml, 14%).

**Further Purification of A1a** - Material in A1a was subjected to high performance liquid chromatography on a fatty acid analysis column using solvent system L. Two prominent peaks emerged designated A1a1 (15-17 ml, 23%) and A1a2 (24-29 ml, 71%). A1a2 had to be further purified to permit analysis. A1a2 was therefore re-chromatographed on a  $\mu$ -Porasil column with a linear program of solvent 1 to 23% solvent 2 over 1 hour (solvent 1 = chloroform, solvent 2 = chloroform/methanol/acetic acid (900:50:11)), 1 mL fractions. A single peak eluted at 46-48 ml of eluate. A1a2 was also re-chromatographed on a fatty acid analysis column using solvent system K. A single peak eluted at 31-35 ml of eluate. In spite of these extensive attempts at purification, when a portion of the methyl ester of A1a2 was converted to an O-methylolone, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry, adequate purity of A1a2 had not been attained to permit identification. Therefore, the remainder of A1a2 was converted to a methyl ester, O-methylolone, tert-butylidimethylsilyl ether derivative and re-chromatographed on a  $\mu$ -Porasil column using solvent system G. A single peak emerged at 40-41 ml of eluate, 100% of recovered radioactivity.

**Structure of Metabolite A1a2** - The methyl ester of A1a2 was converted to an O-methylolone, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained was interpreted to be a mixed spectrum of Metabolite A1a2 plus a co-eluting impurity. Insufficient quantity of this compound prevented additional purification. A small quantity of A1a2 was also recovered from Peak A3d and Peak A4 (see below). Ions from co-eluting impurities were also present in the spectra obtained for compound A1a2 recovered from Peaks A3d and A4 although different than the impurity ions in Peak A1a2. From comparison of these spectra, it was therefore possible to ascertain which ions were due to co-eluting impurities and which ions were generated as a result of fragmentations of Metabolite A1a2 (Fig. 17). The mass spectrum of A1a2 obtained from Peak A1a2 is shown at the top of the figure and the mass spectrum of A1a2 obtained from Peak A4 is shown at the bottom. Intense ions apparently due to fragmentation of Metabolite A1a2 were m/z 541 (M), 526 (M-15), loss of -CH<sub>3</sub>; 510 (M-31), loss of -OCH<sub>3</sub>; 451 (M-90), loss of M<sub>2</sub>SiO<sub>2</sub>SiOH; 427 (M-114), loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 420 [M-(50 + 31)]; 398 (M-143), unknown origin; 380 [M-(160 + 1)], loss of the elements of (OC(O)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> from the ring + R); 343 (M-198), rearrangement ion; 337 [M-(114 + 90)], 259, either [M-(90 + 31 + 160 + 1)], or [M-(90 + 160 + 32)]; loss (90 + 160 + CH<sub>2</sub>O); and 129 [M<sub>2</sub>SiO<sub>2</sub>-OH-CH(CH<sub>3</sub>)<sub>2</sub>]. A mass spectrum obtained of the (Tb<sub>2</sub>)<sub>2</sub>-methylolone, trimethylsilyl ether derivative indicated the presence of two trimethylsilyl groups and one O-methylolone group and supported the structural assignments of the various fragment ions. The location of the hydroxyl group was suggested by the ion at m/z 380 involving the loss of 160 mass units and the base ion of m/z 129. The elimination of 160 mass units has been found in the mass spectra of all 11-dehydro-Tb<sub>2</sub> metabolites, indicating a 6-lactone ring structure. A base ion at m/z 129 is also the base ion of all 11-dehydro-Tb<sub>2</sub> metabolites with a hydroxyl group at C-15 but is absent in 15-keto-11-dehydro-Tb<sub>2</sub> metabolites. In addition, the mass spectrum of the (Tb<sub>2</sub>)<sub>2</sub>-trimethylsilyl ether derivative indicated that the ion at m/z 343 contained two trimethylsilyl groups. This ion is considered to be analogous to the rearrangement ion seen in all 11-dehydro-Tb<sub>2</sub> metabolites with a hydroxyl group at C-15. Fragmentation resulting in the elimination of 114 mass units containing the O-methylolone group provided a clue to the location of the O-methylolone group. The loss of 114 mass units suggests that the O-methylolone group is attached to one of the C16-C20 carbons on the lower side chain. Prostaglandin metabolites with hydroxy

groups at the  $\omega$ -1 and  $\omega$ -2 position have been described (16-19) and a C-19 hydroxylated Tb<sub>2</sub> metabolite was found in the monkey (7). Therefore, although the biochemical mechanism of formation is unknown, it would seem most likely that either a keto group is attached at C-19 or an aldehyde at C-20. This data, therefore, suggested that the structure of A1a2 was either 9a,15(S)-dihydroxy-11,19-dioxo-2,3,18-dioxo-52,13E-diolic acid or 9a,15(S)-dihydroxy-11,20-dioxo-2,3,18-dioxo-52,13E-diolic acid.

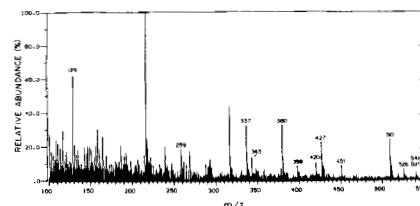
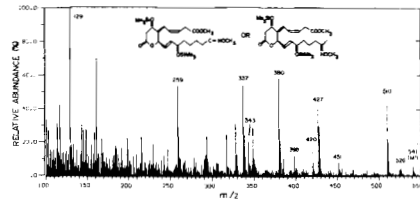


Fig. 17 - Mass spectra of the methyl ester, O-methylolone, trimethylsilyl ether derivative of Metabolite A1a2 and co-eluting impurities isolated from Peak A1a2 (top) and Peak A1d (bottom).

**Structure of Metabolite A1a2** - The methyl ester of A1a2 was converted to an O-methylolone, tert-butylidimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 18) showed prominent ions at m/z 515 (M), 498 (M-15), loss of -CH<sub>3</sub>; 482 (M-31), loss of -OCH<sub>3</sub>; 456 (M-57), loss of -CH<sub>3</sub>; 426 [M-(57 + 32)], loss of (57 + CH<sub>2</sub>O); 414 [M-(57 + 42)], loss of (57 + O=CH<sub>2</sub>); 400 (M-113), loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 350, either [M-(132 + 31)], loss of (M<sub>2</sub>SiO<sub>2</sub>SiOH + OCH<sub>3</sub>), or [M-(131 + 32)], loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 312 [M-(113 + 57 + 31)]; 311 (M-202), loss of the elements of (OC(O)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> from the ring); 310 [M-(202 + 1)]; 280 [M-(202 + 31)]; 159 (base) [-(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>]; 145 (Fig. 5) or [-(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>]; and 101 [Tb<sub>2</sub>SiO<sub>2</sub>SiOH]. Ions involving the loss of 113 mass units indicated that one step of  $\omega$ -oxidation had occurred with the loss of two carbons from the upper side chain. The ion at m/z 159 indicated that in addition to  $\omega$ -oxidation,  $\beta$ -oxidation had also occurred with the loss of 2 carbons from the lower side chain. It was therefore concluded that A1a2 was 9a-hydroxy-11,15-dioxo-2,3,18,19-tetrahydro-52-ene-1,20-diolic acid.

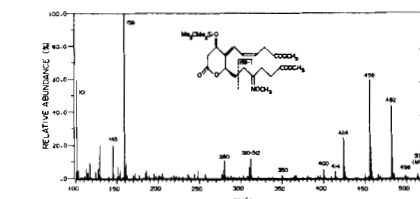


Fig. 18 - Mass spectrum of the methyl ester, O-methylolone, tert-butylidimethylsilyl ether derivative of Metabolite A1a2.

**Further Purification of A1b** - A1b was subjected to high performance liquid chromatography on a fatty acid analysis column using solvent system L. A single prominent peak emerged (27-30 ml of eluate, 51% of recovered radioactivity) along with other more polar unresolved material. Analysis of a portion of A1b as a methyl ester, O-methylolone, trimethylsilyl ether derivative by gas chromatography-mass spectrometry did not permit identification due to co-eluting impurities. The remainder, therefore, was converted to an O-methylolone, tert-butylidimethylsilyl ether derivative and re-chromatographed on a  $\mu$ -Porasil column using solvent system G. Two peaks emerged at 45 ml of eluate (53%) and 47 ml (47%). These were considered to be most likely syn and anti O-methylolone isomers and were combined for analysis by gas chromatography-mass spectrometry.

**Structure of Metabolite A1b** - The methyl ester, O-methylolone, tert-butylidimethylsilyl ether derivative of A1b was analyzed by gas chromatography-mass spectrometry. The resolved peaks were obtained which were interpreted as consistent with syn and anti O-methylolone isomers. The mass spectrum obtained (Fig. 19) showed prominent ions at m/z 515 (M), 500 (M-15), loss of -CH<sub>3</sub>; 486 (M-31), loss of -OCH<sub>3</sub>; 458 (M-57), loss of -CH<sub>3</sub>; 428 (M-87), loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 426 [M-(57 + 32)], loss of (57 + CH<sub>2</sub>O); 415 (M-100), loss of (CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>; 384 (M-131), either loss of -(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub> or [M-(57 + 32 + 42)], loss of (57 + 32 + O=CH<sub>2</sub>); 358 [M-(100 + 57)]; 352 [M-(132 + 31)], loss of (M<sub>2</sub>SiO<sub>2</sub>SiOH + 31); 340 [M-(87 + 57 + 31)]; 314, either [M-(132 + 31 + 42)], or [M-(131 + 32 + 42)]; 312 [M-(202 + 31)], loss of the elements of (OC(O)CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> from the ring + R); 286 [M-(132 + 87)]; 282 either [M-(202 + 31)], or [M-(132 + 101)], loss of [132 + (CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>]; 212 [M-(202 + 101)]; 187 [-(CH<sub>2</sub>)<sub>4</sub>COOCH<sub>3</sub>]; 145 and 115 (base) (Fig. 5), and 101 [Tb<sub>2</sub>SiO<sub>2</sub>SiOH]. Ions involving the loss of 202 mass units indicated a 6-lactone ring structure. The prominent ions at m/z 115 and 187 indicated that  $\omega$ -oxidation had occurred and an O-methylolone group was attached at C-15. M/z 187 is also a very intense ion in m/z 115 the base ion in the mass spectrum of the methyl ester, O-methylolone, trimethylsilyl ether derivative of 9a-hydroxy-11,15-dioxo-2,3,4,5-tetrahydro-52-ene-1,20-diolic acid (7). This mass spectrum of this derivative of A1b was therefore interpreted to be consistent with the structure 9a-hydroxy-11,15-dioxo-2,3,4,5-tetrahydro-52-ene-1,20-diolic acid.

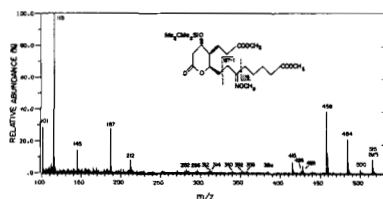


Fig. 19 - Mass spectrum of the methyl ester, 0-methylthio, tert-butyltrimethylsilyl ether derivative of Metabolite A3b.

**Further Purification of A3c** - Material in A3c was subjected to high performance liquid chromatography on a fatty acid analysis column using solvent system K. A single peak emerged at 42-46 eluate (100% of recovered radioactivity) that was designated Metabolite A3c.

**Structure of Metabolite A3c** - The methyl ester of A3c was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained (Fig. 20) showed prominent ions at  $m/z$  342 (90), 527 (90), loss of  $\cdot\text{CH}_3$ ; 511 (8-31), loss of  $\cdot\text{OCH}_3$ ; 427 (8-115), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 421 [M-(90 + 31)], loss of  $\text{O}(\text{CH}_2\text{OH} + 31)$ ; 412 [M-(115 + 15)]; 385 [M-(115 + 42)], loss of  $(115 + \text{O}=\text{CCH}_3)$ ; 381 [M-(160 + 1)], loss of the elements of  $[\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}(\text{OSiMe}_3)_2]$  from the ring + R; 367 [M-(160 + 15)]; 362 [M-(2 x 90)]; 343, rearrangement ion; 337 [M-(115 + 90)]; and 129, (base)  $[\text{Me}_3\text{SiO}^+-\text{CH}=\text{CH}-\text{CH}_3]$ . The ions involving the loss of 160 mass units indicated a 6-lactone ring structure for A3c. The several ions involving the loss of 115 mass units suggested that  $\omega$ -oxidation had occurred with a hydroxyl group attached at C-15. The mass spectrum of an earlier radioactive gas chromatographic peak in A3c provided additional information for the structure of A3c. The mass spectrum obtained for this peak in addition to ions apparently arising from co-eluting impurities showed intense ions at  $m/z$  614 (8-32), loss of  $\text{CH}_2\text{CH}_2$ ; 556 (8-90), loss of  $\text{Me}_3\text{SiOH}$ ; 541 [M-(90 + 15)], loss of (90 +  $\cdot\text{CH}_2$ ); 531 (8-115), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 525 [M-(90 + 31)], loss of (90 +  $\cdot\text{OCH}_3$ ); 510 [M-(90 + 31 + 15)]; 466 [M-(2 x 90)]; 441 [M-(115 + 90)]; 345 (base),  $[\text{Me}_3\text{SiO}^+-\text{CH}=\text{CH}-\text{CH}_3]$   $[\text{OSiMe}_3](\text{CH}_2)_4\text{COOCH}_3$ ; 339 [M-(217 + 90)], loss of  $\cdot[\text{CH}(\text{OSiMe}_3)(\text{CH}_2)_4\text{COOCH}_3 + 90]$ ; 255 (345-90); 217  $[\text{Me}_3\text{SiO}^+-\text{CH}(\text{CH}_2)_4\text{COOCH}_3]$ ; and 175  $[\text{Me}_3\text{SiO}^+-\text{CHCH}_2\text{COOCH}_3]$ .

As mentioned above, the mass spectrum of the longer retention time peak indicated a 6-lactone ring structure. The mass spectrum of this shorter retention time peak was consistent with the acid-alcohol form of the 6-lactone (7). The base ion of 345 indicated that  $\omega$ -oxidation had occurred and a hydroxyl group was attached at C-15. From this mass spectrum it was therefore concluded that the structure of A3c was 9a,15(n)-dihydroxy-11-oxo-2,3-dinor-thromboxane-5Z, 13E-diene-1,20-diolate acid.

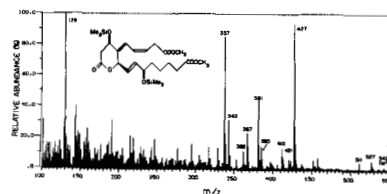


Fig. 20 - Mass spectrum of the methyl ester, trimethylsilyl ether derivative of Metabolite A3c.

**Further Purification and Structure of Metabolite in A3d** - Material in A3d was subjected to high performance liquid chromatography on a fatty acid analysis column using solvent system K. Three discrete peaks emerged at 17-20 ml of eluate (26% of recovered radioactivity), 37-41 ml (45%) and 43-46 ml (29%). No metabolite could be identified on subsequent mass spectral analysis of material eluting at 43-46 ml. Mass spectral analysis of the compound eluting at 17-20 ml as a methyl ester, 0-methylthio, trimethylsilyl ether derivative gave a mass spectrum identical to that described for metabolite A3a<sub>1</sub>. The recovery of the same compound from two widely separated peaks from the same  $\mu$ -Porasil chromatographic strip (peaks A3a and A3d) could possibly be explained by existence of both the 6-lactone and the acid-alcohol form of A3a<sub>1</sub>. The form chromatographed in peak A3a being the 6-lactone and the form chromatographed in A3d being the more polar acid-alcohol form which prior to derivatization for mass spectral analysis underwent lactonization.

Identification by mass spectroscopy of the metabolite eluting at 37-41 ml was not possible as a methyl ester, trimethylsilyl ether derivative due to co-eluting impurities. The methyl ester of material eluting at 37-41 ml was therefore converted to a tert-butyltrimethylsilyl ether derivative and

subjected to chromatography on a  $\mu$ -Porasil column using solvent system G. A single prominent peak emerged at 30-32 ml of eluate (100% of recovered radioactivity). Mass spectral analysis of this derivative gave a mass spectrum with prominent ions at  $m/z$  611 (8-15), loss of  $\cdot\text{CH}_3$ ; 595 (8-31), loss of  $\cdot\text{OCH}_3$ ; 569 (base) (8-57), loss of  $\cdot\text{OCH}_3$ ; 537 [M-(57 + 32)], loss of (57 +  $\text{CH}_2\text{OH}$ ); 511 [M-(115), loss of  $\cdot(\text{CH}_2)_4\text{COOCH}_3$ ; 463, either [M-(131 + 32)], loss of  $\cdot(\text{OSiMe}_3)_2$ ; 32), or [M-(132 + 31)], loss of  $(\text{OSiMe}_3)_2$ ; 31); 437 [M-(132 + 57)]; 419 (8-207), unknown origin; 405 [M-(132 + 57 + 32)]; 379 [M-(132 + 115)]; 367 [M-(202 + 57)], loss of the elements of  $[\text{O}(\text{C}=\text{O})\text{CH}_2\text{CH}(\text{OSiMe}_3)_2]$  from the ring + 57]; 293 [M-(202 + 131)]; 261 either [M-(202 + 132 + 31)], or [M-(202 + 131 + 32)]; 229, unknown origin; and 101  $[\text{SiMe}_3\text{O}^+-\text{CH}=\text{CH}-\text{CH}_3]$ . The prominent ions involving the loss of 115 mass units indicated that  $\omega$ -oxidation had occurred. Ions involving the loss of 202 mass units indicated a 6-lactone ring structure. Interpretation of this mass spectrum indicated that the structure of this compound was the same as A3c. It was, therefore, concluded that this compound was a lesser quantity of Metabolite A3c.

**Further Purification and Structure of Metabolite in A4** - Material in A4 was subjected to high performance liquid chromatography on a  $\mu$ -Porasil column using solvent system F. A single prominent broad unresolved peak emerged at 17-25 ml of eluate (51% of recovered radioactivity). This peak was further subjected to chromatography on a fatty acid analysis column using solvent system L. Three prominent peaks emerged at 16-18 ml of eluate (27%), 28-32 ml (38%), and 42-45 ml (18%). Identification of a metabolite from the peaks at 28-32 ml and 42-45 ml was not possible after additional purification and analysis by gas chromatography-mass spectrometry. The methyl ester of material in the peak at 16-18 ml was converted to a 0-methylthio, trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry. The mass spectrum obtained and C-value was identical to that recorded for Metabolite A3a<sub>1</sub>. It was, therefore, concluded that this compound was a lesser quantity of A3a<sub>1</sub>.

**Isolation and Identification of 2,3-Dinor-PGF<sub>2</sub> as a Human Urinary Metabolite** - When the methyl ester of material in Peak E2b (see above) was converted to a trimethylsilyl ether derivative and analyzed by gas chromatography-mass spectrometry, a peak was present with a C-value of 2144 that did not contain radioactivity when analyzed by gas chromatography with radioactivity monitoring and, therefore, was not a metabolite of Tbx<sub>2</sub>. However, the mass spectrum obtained for the peak was identical to that published for 2,3-dinor-PGF<sub>2</sub> recently reported by us to be the major urinary metabolite of PGD<sub>2</sub> in the monkey (16).