4-(2′-Carboxyphenyl)-4-oxobutyryl Coenzyme A Ester, an Intermediate in Vitamin K_2 (Menaquinone) Biosynthesis

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4-(2′-Carboxyphenyl)-4-oxobutyryl coenzyme A ester, *o*-Succinylbenzoic Acid, 1,4-Dihydroxy-2-naphthoic Acid, Menaquinone, Vitamin K

Enzyme preparations from *Mycobacterium phlei*, *Escherichia coli* and *Galium mollugo* cell suspension cultures were incubated in the presence of 4-(2′-carboxyphenyl)-4-oxobutyrate (*i.e. o*-succinylbenzoic acid, OSB, **1**), ATP, coenzyme A and Mg^{2+}. The main product isolated from the incubation mixture was 4-(2′-carboxyphenyl)-4-oxobutyryl coenzyme A ester (**2**) as determined by comparison with synthetic coenzyme A esters. Synthetic and enzymically formed 4-(2′-carboxyphenyl)-4-oxobutyryl coenzyme A ester (**2**) was shown to be enzymically converted to an intermediate in vitamin K_2 biosynthesis *viz.* 1,4-dihydroxy-2-naphthoic acid (**5**). The enzymic formation of 2-(3′-Carboxypropionyl)benzoyl coenzyme A ester (**3**) and 4-(2′-carboxyphenyl)-4-oxobutyryl-di-coenzyme A ester (**4**) was also observed. They appeared in minor amounts, however. These esters were not convertible to 1,4-dihydroxy-2-naphthoic acid (**5**).

Introduction

In the biosynthesis of menaquinones (vitamin K_2) two aromatization processes are involved [1, 2]. In the first aromatization iso-chorismic acid and α-ketoglutaric acid are converted to 4-(2′-carboxyphenyl)-4-oxobutyrate (*i.e. o*-succinylbenzoate, OSB (**1**) [3–5]. In the second reaction **1** undergoes a ring closure reaction giving rise to 1,4-dihydroxy-2-naphthoate (**5**) DHNA [6–8]. The latter reaction proceeds through a mono coenzyme A ester [8]. Formation of this coenzyme A ester depends on the presence of coenzyme A and ATP which is hydrolyzed to AMP and pyrophosphate during this reaction [9].

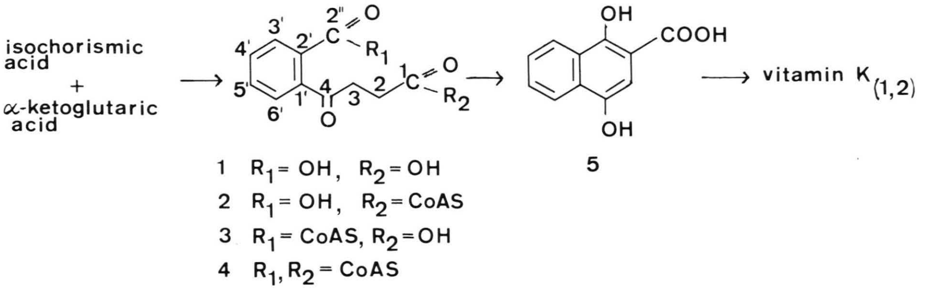

Fig. 1. Intermediates and suggested intermediates in vitamin K biosynthesis. The compounds shown are named in the text as follows: (**1**): 4-(2′-carboxyphenyl)-4-oxobutyrate or *o*-succinylbenzoic acid or OSB; (**2**): 4-(2′-carboxyphenyl)-4-oxobutyryl coenzyme A ester or "aliphatic" OSB CoA ester; (**3**): 2-(3′-carboxypropionyl)benzoyl coenzyme A ester or "aromatic" OSB CoA ester; (**4**): 4-(2′-carboxyphenyl)-4-oxobutyryl-dicoenzyme A ester or OSB di CoA ester; (**5**): 1,4-dihydroxy-2-naphthoic acid, DHNA.

Since it was known that the activated **1** is a mono-coenzyme A ester [8] it was desirable to determine the site of activation at either C-atom 1 or 2″ of **1** (Fig. 1). It had been suggested on mechanistic grounds that the "aromatic" carboxyl group (*i.e.* C-2″ in **1**) is activated [10, 11]. Experimental evidence seemed to support this assumption [2, 12]. However, recent results [1, 7] cast doubt on the validity of this conclusion. It was therefore essential to synthesize and characterize the three possible coenzyme A esters **2**, **3** and **4** [13]. Cell free synthesis of the acti-

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Abbreviations: CoA, CoASH, coenzyme A; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; LSC, liquid scintillation counting; MOPS, 3-(N-morpholino)propanesulfonic acid; OSB, *o*-succinylbenzoic acid; PC, paper chromatography; TLC, thinlayer chromatography.

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vated **1** and comparison of the resulting coenzyme A esters with synthetic samples of **2**, **3** and **4** and the enzymic conversion of the enzymically and chemically formed **2** to 1,4-dihydroxy-2-naphthoate (**5**) are reported in the present manuscript. A preliminary account of part of this work has been published [7].

Materials and Methods

Organisms

The source of *E. coli* K12 and *M. phlei* was reported previously [8]. A suspension culture of *G. mollugo* (anthraquinone producing line) has been described [14].

Enzyme extract from E. coli catalyzing the conversion of OSB (1) to OSB CoA esters (2) and (3) and the conversion of (2) to DHNA (5)

An enzyme extract prepared as described [8] was passed through Sephadex G 25 equilibrated in K_2HPO_4 buffer (0.1 M, pH 8.0) to remove low molecular weight compounds [15]. Protein (13.5 mg per ml) was determined according to Bradford [16].

Enzyme extract from M. phlei catalyzing the conversion of OSB (1) to OSB CoA esters (2), (3) and (4); (OSB: CoA ligase fraction)

Frozen cells (10 g) were thawed and suspended in MOPS buffer (10 ml, 0.02 M, pH 6.9) containing DTT (0.2 mM) and DMSO (20%). The cell suspension was cooled in an ice salt mixture and sonicated (Branson Sonifier) 10 times for 60 s at 60 Watts with intervals of 30 s. The supernatant of centrifugation (50000 × *g*, 10 min) contained 9 mg protein per ml. To this solution (10 ml) protamine sulfate (9.5 ml, 2% in 0.02 M MOPS buffer) containing DTT (0.2 mM) and DMSO (20%) was added with stirring during 15 min at 0 °C. The solution was stirred for further 20 min and centrifuged (10 min, 12000 × *g*). The supernatant containing the OSB CoA ligase was passed through Sephadex G 25 [15] equilibrated in KH_2PO_4 buffer (0.1 M, pH 7.5). The resulting solution contained 1 mg protein per ml and was free of any DHNA synthase activity.

Enzyme extract from M. phlei converting OSB CoA ester (2) to DHNA (5)

The pellet obtained during centrifugation at 12000 × *g* (see above) was suspended (ultrasonic bath, 5 min, 0 °C) in 20% saturated ammonium sulfate in MOPS buffer (5 ml, pH 6.9) containing DTT (0.2 mM) and DMSO (20%) [9]. After centrifugation (10 min, 12000 × *g*) the supernatant was passed through Sephadex G 25. This solution (8 mg protein per ml) contained the DHNA synthase of *M. phlei*. This fraction was devoid of OSB:CoA ligase activity.

Enzyme extract from G. mollugo catalyzing the conversion of OSB (1) to the "aliphatic" OSB CoA ester (2)

Frozen cells (5 g) were suspended in KH_2PO_4 buffer (10 ml, 0.1 M, pH 7.3) containing DTT (0.2 mM) and protamine sulfate (0.1%) and sonicated (60 Watts, 0 °C) 10 × 10 s with intervals of 50 s. After centrifugation (4 °C, 10 min, 50000 × *g*) the clear red supernatant was treated dropwise with protamine sulfate solution (500 µl, 2% in 0.1 M KH_2PO_4 buffer) and stirred for 15 min at 0 °C. The suspension was centrifuged (5 min, 12000 × *g*).

Servachrom XAD 2 (Serva, Heidelberg, FRG) was added to the supernatant (2 g, equilibrated in 0.1 M phosphate buffer pH 7.3 containing 0.2 mM DTT) and stirred for further 10 min at 0 °C. The resin was removed by filtration and the pH of the solution adjusted to 7.3 [14].

Protein: 2 mg per ml as determined by Lowry [17].

Influence of pH on the formation of OSB CoA esters

Enzyme extracts of *G. mollugo* (50 µl) or *M. phlei* (50 µl of the OSB:CoA ligase fraction) were incubated (10 min, 30 °C, various pH values) with ATP (500 nmol), CoASH (125 nmol), $MgCl_2$ (1 µmol) and [1-^{14}C]OSB (3.58 nmol, 925 Bq) in a final volume of 70 µl. The reaction was stopped by formic acid (10 µl). 30 µl of this solution were chromatographed immediately on cellulose TLC sheets (Cel 300 UV_{254}, MN, Düren) in *n*-butanol-acetic acid-water 5:2:3. CoA esters formed were detected with a radio scanner, the radioactive material eluted (H_2O) and analyzed by LSC.

Influence of time on the yield of OSB CoA esters from M. phlei

The OSB CoA ligase fraction from *M. phlei* (100 µl) was incubated (30 °C, pH 7.8) with ATP (1 µmol), CoASH (250 nmol), $MgCl_2$ (2 µmol) and [1-^{14}C]OSB (7 nmol, 1850 Bq) in a final volume of

140 μl. 20 μl fractions were taken at different times and mixed subsequently with formic acid (5 μl). Chromatography and quantitative determination as in the preceeding paragraph.

Enzymic synthesis of OSB CoA esters in extracts from G. mollugo, E. coli, and M. phlei

Enzyme extracts (2 ml) were incubated (10 min, 30 °C) with ATP (10 μmol), CoASH (3 μmol), $MgCl_2$ (20 μmol) and inactive OSB (1 μmol) or [1-^{14}C]OSB (1 μmol, 0.26 MBq). The pH was adjusted to 7.2 (*G. mollugo* and *E. coli*) or to 6.5 or 7.9 (*M. phlei*). The reaction was stopped (conc. HCl, 150 μl) and the mixture cooled. Denaturated protein was removed by centrifugation. The clear supernatant was passed through a Centricon 10 membrane filter (Amicon, Witten, FRG) for complete deproteinization (5000 × *g*, 10 °C, 2 h, rotor angle 60°).

The clear filtrate was injected onto a HPLC column (RP-8, 250 × 8 mm), and gradient elution was carried out with water (solvent A) and methanol (solvent B) increasing from 10 to 35%.

Both water and methanol contained H_3PO_4 (0.15 mM). The CoA ester fractions were concentrated in high vacuum and rechromatographed on an analytical column (250 × 4 mm) in the same solvent system with a gradient from 12 to 25% B [13] and stored at −20 °C in the HPLC solvent.

Formation of DHNA (5) from OSB CoA esters

Enzymically formed OSB CoA esters as well as chemically synthesized OSB CoA esters [13] were incubated. The purified CoA esters (dissolved in HPLC solvent) were concentrated to 100 μl (high vacuum, 30 °C).

3580 pMol (925 Bq) of the [1-^{14}C]OSB CoA ester (**2**), (**3**) or (**4**) were incubated with enzyme extract from *E. coli* or with the DHNA synthase fraction of *M. phlei* (2 ml, 30 min, 34 °C, pH 7.8). Incubation was terminated by cooling and addition of concentrated HCl (80 μl). Non-radioactive OSB (**1**), OSB-spirodilactone and DHNA (**5**) were added as carriers. The mixture was extracted with peroxide free ether (2 × 5 ml) and the concentrated ether extract chromatographed (TLC; silica gel; $CHCl_3$/EtoAc/HCOOH 45:6.6:0.5) [18]. Radioactive spots were detected by a radioactivity scanner, scraped off and determined quantitatively by LSC.

DHNA (**5**) formed from the aliphatic OSB CoA ester (**2**) was also analyzed as follows: The ether was evaporated and the residue dissolved in MeOH (200 μl). This solution was chromatographed (HPLC, RP-8, 250 × 4 mm, MeOH/H_2O gradient from 55 to 75% MeOH containing 0.15 M H_3PO_4).

Methylation and subsequent hydrolysis of OSB CoA esters (2) and (3) isolated from M. phlei extracts

Either (**2**) or (**3**) (2.5 nmol; 650 Bq) were dissolved in methanol−water 1:1 (800 μl) and diazomethane in ether added (1 min; 0 °C): The solution was freed from excessive diazomethane with a stream of N_2 and concentrated to 300 μl (high vacuum; 25 °C). After mild alkaline hydrolysis (20 °C; pH 11.5; 30 s for (**2**) and 12 min for (**3**)) the solution was acidified to pH 1.5 with HCl and extracted with ether (2 × 3 ml). The concentrated ether extract was chromatographed (TLC; silica gel; $CHCl_3$/EtOAc/HCOOH 45:6.6:0.5) [12].

Results and Discussion

Cell free synthesis of the 4-(2′-carboxyphenyl)-4-oxobutyryl coenzyme A ester had been observed in protein extracts from *E. coli, M. phlei* [8] and cell suspension cultures of *Galium mollugo* [19]. In the experiments now described extracts from all three sources were used. The coenzyme A ester isolated from incubations with protein from *Galium mollugo* is converted by enzyme preparations from *M. phlei* or *E. coli* to DHNA [19]. This and the following data show that the coenzyme A esters from *Galium mollugo* and from bacteria *(E. coli, M. phlei)* are identical.

The incubations were carried out under conditions which gave 30−40% *(M. phlei)* or 10% *(E. coli)* yield of the "aliphatic" OSB CoA ester (**2**) with reference to the OSB employed. Incubation mixtures containing enzymically formed coenzyme A esters were worked up in different ways (see Materials and Methods): Radioactive coenzyme A ester obtained from incubations with ^{14}C-labelled **1** and enzyme from *Galium mollugo* cells [19] was either purified by PC and the radioactive ester eluted (H_2O) and subsequently analyzed by HPLC (Fig. 2) or analyzed directly by HPLC after complete deproteinization (see later).

Fractions were collected and their radioactivity determined. Two of the fractions turned out to be radioactive (Fig. 2). The material corresponding to

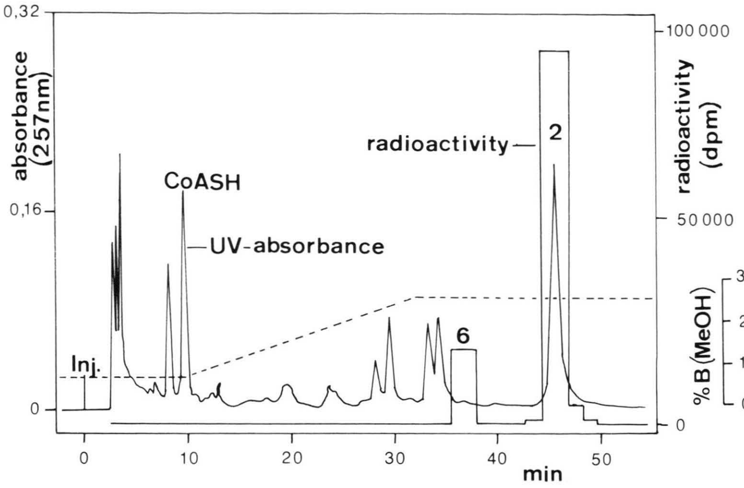
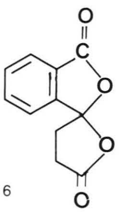


Fig. 2. HPLC analysis of a radioactive fraction obtained after incubation of protein from *Galium mollugo* L. cells with radioactive OSB (**1**), ATP, CoASH and Mg^{2+}. Before HPLC the incubation mixture was purified by paper chromatography. Radioactivity was associated with the "aliphatic" OSB CoA ester (**2**) and the spirodilactone (**6**) of OSB.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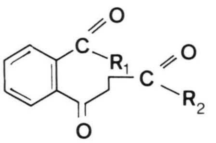

7 R_1 = OH , R_2 = OCH_3
8 R_1 = OCH_3 , R_2 = OH

Fig. 3. Derivatives of *o*-succinylbenzoic acid (**1**). The compounds shown are named in the text as follows: (**6**): Spirodilactone of (**1**); (**7**): "aliphatic" methyl ester of (**1**); (**8**): "aromatic" methyl ester of (**1**); (**9**): dimethyl ester of (**1**).

one of the radioactive fractions coincided with the spirodilactone (**6**) of **1**, a compound known to be an artifact of isolation, derived from the enzymically formed mono coenzyme A ester of **1** [8]. The retention time of the other radioactive material corresponded to that of an authentic sample [13] of the "aliphatic" coenzyme A ester (**2**). Its retention time was clearly different from that of synthetic samples of **3** and **4**.

Low molecular weight compounds contained in incubation mixtures were also separated from protein using Centricon 10 membrane filters. HPLC analysis of this low molecular weight fraction showed that in addition to the "aliphatic" coenzyme A ester (**2**) compounds appeared which in the case of *E. coli* coincided with **3** and in the case of *M. phlei* coincided with both **3** and **4** provided the incubation was carried out at pH 7.9 (Fig. 4). At pH 6.5 only **2** but no **3** and **4** were detectable (Fig. 4). In each case, however, **2** was the main component. Relative amounts of coenzyme A esters are given in Table I.

Identification of the enzymically formed coenzyme A esters was based on comparison (HPLC, UV-spectra, FAB mass-spectra) with synthetic samples

Table I. Relative amounts of coenzyme A ester **2**, **3** and **4** detected in enzyme extracts from different sources.

Source of OSB coenzyme A ligase preparation	pH of incubation mixture	Relative amounts [%] of coenzyme A esters **2**	**3**	**4**
E. coli	7.2	>95	<5	n.d.
G. mollugo	7.2	100	n.d.	n.d.
M. phlei	6.5	100	n.d.	n.d.
M. phlei	7.9	80–85	10–15	<10

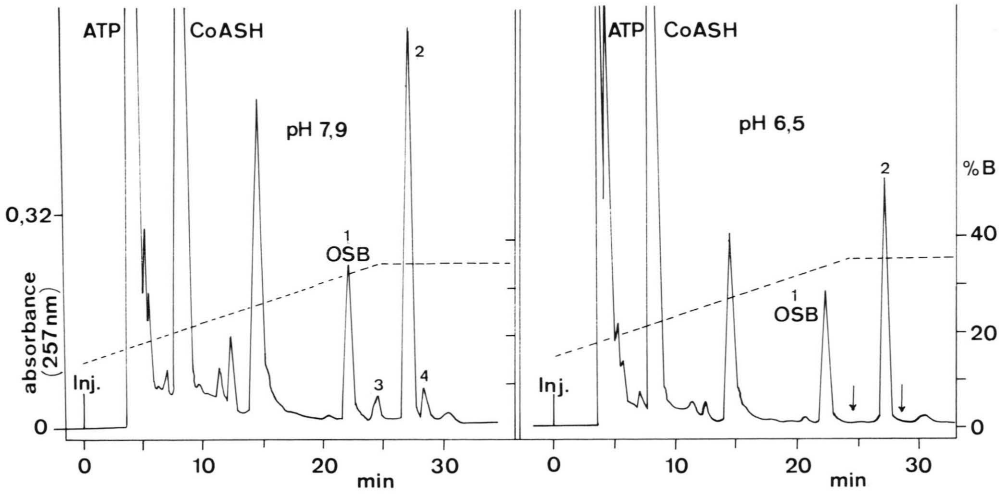

Fig. 4. Influence of the pH of the incubation mixture on the formation of different coenzyme A esters of OSB (**1**) in cell free extracts from *Mycobacterium phlei*. Arrows indicate retention times of coenzyme A esters **3** and **4**.

[13]. Hydrolysis of the esters and quantitative determination of the resulting *o*-succinylbenzoic acid (**1**) and coenzyme A [13] gave a 1:1 molar ratio for **2** and **3** and a 1:2 molar ratio for **4**.

The stability of the enzymically formed esters were also investigated [13], using Ellman's reagent. It was found that coenzyme A esters **2** and **4** were much more labile (halflife 7.5 min at 30° and pH 7.5) than **3** (halflife 8.5 h).

The observation that all three coenzyme A esters (**2**, **3** and **4**) were formed during the incubation was unexpected and made it necessary to test the enzymic conversion of all three esters to 1,4-dihydroxy-2-naphthoic acid (**5**). Two different enzyme preparations were used: Firstly a crude enzyme preparation from either *M. phlei* (Table II, experiments 1–6) or *E. coli* (experiments 7–18) and secondly an enzyme preparation which contained an enriched naphthoate-synthase (experiments 4–6). The latter preparation was devoid of *o*-succinylbenzoic acid coenzyme A ligase.

Experiments 1 to 3 show that only the "aliphatic" ester (**2**) but not the aromatic ester (**3**) or the dicoenzyme A ester (**4**) is converted to 1,4-dihydroxy-2-naphthoic acid (**5**). This conclusion is confirmed by the results of experiments 4 to 6 in which no coenzyme A ligase was present. This shows that synthesis of **5** did not occur after hydrolysis of either **2**, **3** or **4** and reactivation of OSB (**1**). Again, the "aliphatic" coenzyme A ester (**2**) is the only isomer which is converted to DHNA (**5**). These results are in agreement with those obtained from incubations with protein from *E. coli*. Only the "aliphatic" coenzyme A ester (**2**) was converted to **5**. Addition to the incubation mixture of either **3** or **4** or **3** and **4** (experiments 10 to 13) did not give an increased yield of **5**. Experiments 15 and 16 showed that conversion of OSB (**1**) and coenzyme A to DHNA (**5**) was not observed in the presence of "diamide" (*i.e.* azodicarboxylic acid bis-(dimethylamide)). This compound oxidizes coenzyme A [20]. Nevertheless the "aliphatic" coenzyme A ester is converted to **5** even in the presence of diamide (experiment 17) whereas **4** is not (experiment 18). Thus it can again be concluded that formation of **5** does not occur after hydrolysis and recombination, and shows that **5** is directly formed from **2** without release of the coenzyme A residue. DHNA (**5**) was identified as previously described [8]. Moreover, the identity of **5** was secured by HPLC analysis and a UV-spectrum of the enzymically formed DHNA (**5**) which was identical with the spectrum of an authentic sample.

We conclude that the "aliphatic" coenzyme A ester (**2**) is the true natural intermediate in vitamin K and anthraquinone biosynthesis. This is at variance with previous assumptions and interpretations [2, 12], and made it necessary to check the results of previous experiments [2, 12] in order to detect the sources of possible errors. In these experiments [2, 12] the activated OSB (**2** or **3**) was isolated by TLC

Table II. Results of incubations of naphthoate synthase containing protein fractions with different coenzyme A esters (**2**), (**3**), (**4**) of OSB (**1**). In each case 3580 pmol (925 Bq) of the respective ester was incubated for 30 min at 34 °C at pH 7.8 in a final volume of 2 ml.

Exp. No.		Source of enzyme	OSB-CoA-ester	DHNA formed	
				[pmol]	[%]*
A	1	*M. phlei*	(**2**)	802	22
	2	*M. phlei*	(**3**)	< 15	< 0.4
	3	*M. phlei*	(**4**)	< 15	< 0.4
B	4	*M. phlei*	(**2**)	1081	30
	5	*M. phlei*	(**3**)	< 15	< 0.4
	6	*M. phlei*	(**4**)	< 15	< 0.4
C	7	*E. coli*	(**2**)	2055	57
	8	*E. coli*	(**3**)	< 15	< 0.4
	9	*E. coli*	(**4**)	100	< 3
	10	*E. coli*	(**3**) + (**4**)	90	< 2
	11	*E. coli*	(**2**) + (**3**)	1518	42
	12	*E. coli*	(**2**) + (**4**)	1947	54
	13	*E. coli*	(**2**) + (**3**) + (**4**)	1575	44
	14	*E. coli*[a]	(**2**)	< 15	< 0.4
	15	*E. coli*	OSB + CoASH	120	3
	16	*E. coli*	OSB + CoASH + diamide	< 15	< 0.4
	17	*E. coli*	(**2**) + diamide	537	15
	18	*E. coli*	(**4**) + diamide	< 15	< 0.4

* The amount of CoA ester employed is set to 100%.

[a] Enzyme heat denatured.

[2, 12]. This method does not distinguish between compounds **2** and **3**. It was assumed that treatment of the esters **2** or **3** with diazomethane [2, 12] or diazoethane [2] would give a mixed ester in which one carboxyl group would be linked to coenzyme A whereas the other carboxyl group would be methylated [2, 12] or ethylated [2]. Mild alkaline hydrolysis was expected to give the methyl ester or ethyl ester of OSB (**1**) with the methyl or ethyl group attached to either the "aliphatic" (compare **7**) or "aromatic" (compare **8**) carboxyl group.

When these experimental steps were applied to a synthetic sample of [1-^{14}C]OSB-2″-CoA ester (*i.e.* "aromatic" OSB CoA ester) (**3**) [13] the "aliphatic" methyl ester (**7**) (Fig. 3) of OSB was indeed detected as had been expected (Fig. 5). However, when the "aliphatic" coenzyme A ester (**2**) was treated sequentially with diazomethane and alkali, an unidentified product was formed which was neither the "aliphatic" nor the "aromatic" methylester of OSB (Fig. 6). This compound was named compound X. The methods which had previously been used [2, 12] to determine the site of activation in the mono coenzyme A ester of OSB (**1**) are evidently unreliable and their application led to erroneous conclusions because the possibility was overlooked that enzyme preparations from bacteria may contain both of the mono coenzyme A esters of OSB (Fig. 4). The two esters are not separable either by paper chromato-

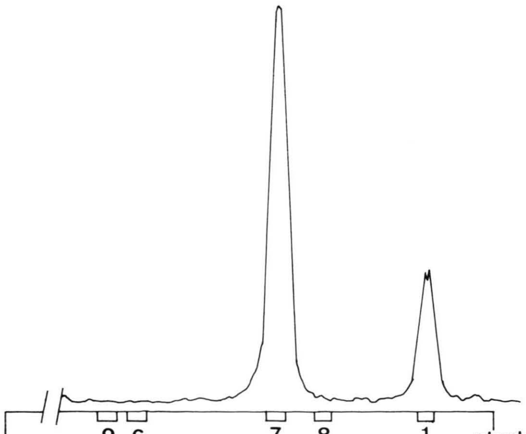

Fig. 5. Radioactivity scan of a chromatogram obtained after sequential treatment of a synthetic sample of "aromatic" OSB coenzyme A ester (**3**) with diazomethane and base. R_f values of the following compounds are indicated: **1** OSB; **7** "aliphatic" OSB methyl ester; **8** "aromatic" OSB methyl ester; **6** spirodilactone of OSB; **9** OSB dimethylester.

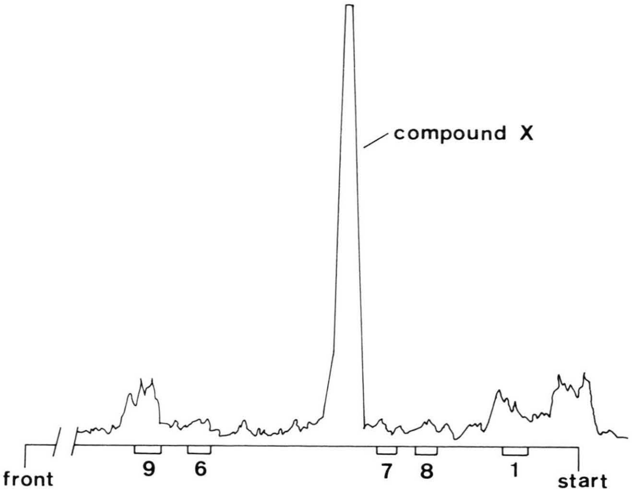

Fig. 6. Radioactivity scan of a chromatogram obtained after sequential treatment of a synthetic sample of "aliphatic" OSB coenzyme A ester (**2**) with diazomethane and base. R_f values of compounds are indicated as in Fig. 5.

graphy or thin layer chromatography on cellulose [2, 12]. The fraction which had been obtained after diazomethane [2, 12] and alkali treatment therefore contains mainly the unknown compound X (Fig. 6) together with a small amount of the "aliphatic" methyl ester (**7**) of OSB. Treatment of compound X (Fig. 6) with alkali did not give OSB (**1**).

Because the "aliphatic" methyl ester (**7**) was isolated after sequential diazomethane and alkali treatment the conclusion was drawn [2, 12] that activation of OSB occurs at the aromatic carboxyl group during vitamin K biosynthesis in agreement with earlier postulates [11, 12].

It is now evident that previous conclusions [2, 12] are in error and that the "aliphatic" OSB CoA ester (**2**) is the true intermediate in vitamin K biosynthesis.

It had previously been pointed out [8] that activation of OSB (**1**) at the "aliphatic" carboxylgroup is likely to occur also in the biosynthesis of an OSB-derived alkaloid *viz.* shinunine [21].

Another question to be raised is why enzyme preparations contain both (**2**) and (**3**) (*E. coli* and *M. phlei*) as well as (**4**) (*M. phlei* only). At present we are unable to answer this question. It might be that chemical transfer of a coenzyme A residue from one carboxyl group to the other carboxyl group of **1** occurs inter- or intramolecularly. We are inclined to assume, however, that this is not the case because we have never observed that synthetic coenzyme A esters of **1** [13] were accompanied by their isomers when kept in solution. It might be, however, that the coenzyme A ligase lacks specificity or regioselectivity to a certain extent and is also able to activate the aromatic carboxyl group of OSB (**1**) albeit with a higher K_M. A lack of substrate specificity has also been observed with acetyl coenzyme A synthetase [22]. Another possibility would be that the enzyme preparations employed contained contaminating non-specific coenzyme A ligases. Thus this question can only be solved after purification of the ligase.

It is now demonstrated that the aliphatic coenzyme A ester (**2**) is the naturally occurring intermediate. It follows that ring closure leading to DHNA (**5**) takes place at the nonactivated "aromatic" carboxyl group. The question arises what provides the driving force for the ring closure.

If the "aliphatic" ester **2** undergoes a ring closure reaction, the primary product of the reaction would be an activated naphthoic acid coenzyme A ester in which the energy of the thioester bond is conserved (**10**, Fig. 7). The energy may be "recycled" and used for an intermediate activation of either the enzyme or the next "aliphatic" coenzyme A ester (**2**) entering the ring closure reaction (Fig. 7). Thus the immediate product of the ring closure reaction would be a cofactor of the overall reaction.

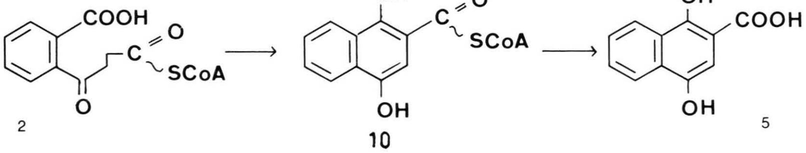

Fig. 7. Hypothesis in which the participation of the coenzyme A ester of 1,4-dihydroxy-2-naphthoic acid (DHNA) (**10**) is postulated.

Acknowledgements

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