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CHIRAL 1,2-DIACYLGLYCEROLS IN THE HAEMOLYMPH OF THE LOCUST, *LOCUSTA MIGRATORIA* *

C.M. LOK [a] and D.J. VAN DER HORST [b]

[a] *Unilever Research, Vlaardingen, and* [b] *Laboratory of Chemical Animal Physiology, State University of Utrecht, Utrecht (The Netherlands)*

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Summary

A ^{1}H-NMR method using chiral shift reagents was applied in the stereochemical analysis of the haemolymph 1,2-diacylglycerols of *Locusta migratoria.* Conversion of the 1,2-diacylglycerols into 1,2-diacetyl-3-tritylglycerols allowed the accurate determination of the enantiomeric purity, whereas direct trimethylsilylation of the unmodified or hydrogenated haemolymph 1,2-diacylglycerols proved to be less suitable because of signal broadening.

In the haemolymph of *Locusta*, *sn*-1,2-diacylglycerols with a remarkably high optical purity were found to be present. In the resting locust, at least 96% of the haemolymph 1,2-diacylglycerols have the *sn*-1,2-configuration; in locusts in which the haemolymph diacylglycerol concentration was elevated by fat body triacylglycerol mobilization induced by flight activity or injection of adipokinetic hormone, over 97% of the 1,2-diacylglycerols is the *sn*-1,2-enantiomer. The few percent *sn*-2,3-enantiomer may not have been present initially.

Positional distribution of the fatty acids in the fat body triacylglycerols and in the haemolymph *sn*-1,2-diacylglycerols obtained from locusts after a 2 h flight revealed nearly identical occupation of the *sn*-2-positions in both acylglycerols. The distribution patterns in the *sn*-1-position of the 1,2-diacylglycerols and the combined *sn*-1 and *sn*-3 positions of the triacylglycerols are compatible with the possible existence of a stereospecific *sn*-3-triacylglycerol lipase.

Introduction

The major energy reserve for prolonged flight in the locust is fat, which is

composed mainly of triacylglycerols and located in the fat body. During flight, this reserve is mobilized and transported in the haemolymph as 1,2-diacylglycerols associated with lipoproteins [1—3]. Thus, the haemolymph 1,2-diacylglycerol level is elevated from 4.5 to 14.1 mg/ml, and simultaneously the turnover rate is increased by a factor seven [4]. Diacylglycerol release from the fat body is stimulated by an adipokinetic hormone secreted by the corpora cardiaca from the onset of flight activity [5,6].

In the resting locust the fatty acid spectrum of the haemolymph 1,2-diacylglycerols is more complex than during flight, indicating differentiation in metabolic function and biochemical pathways [4]. In fact, part of the haemolymph diacylglycerols in the resting locust has been suggested to originate from dietary fat hydrolysis and intestinal re-esterification [7]. These diacylglycerols may be transported to the fat body and subsequently be taken up to yield triacylglycerols. Besides, in the fat body a second pathway in triacylglycerol synthesis involving glycerol 3-phosphate originating from glucose degradation may be operative, in which part of the 1,2-diacylglycerols formed as intermediates is released into the haemolymph [8]. For the conversion of locust fat body triacylglycerols into 1,2-diacylglycerols during flight, either a stereospecific lipase may be present or diacylglycerols are formed by lipolysis of triacylglycerol to 2-monoacylglycerol followed by acylation with acyl-CoA [9]. Elucidation of the absolute configuration of the haemolymph 1,2-diacylglycerols and the positional distribution of fatty acids in both these diacylglycerols and the fat body triacylglycerols can be an aid in exploring biochemical pathways and can provide information about the stereospecificity of the processes involved. Tietz [9] already speculated on the presence of 1,2-diacyl-*sn*-glycerol in the haemolymph of *Locusta.*

Usually enzymatic methods are used to analyse enantiomeric 1,2- and 2,3-diacyl-*sn*-glycerols [10,11]. These methods rely on the ability of stereospecific enzymes to differentiate between enantiomeric diacylglycerols or their phosphorylated derivatives. Recently a ^{1}H-NMR method using chiral shift reagents has been used to determine the enantiomers of 1,2-diacylglycerols [12]. After chemical modification of the 1,2-diacylglycerols, sharp and isolated ^{1}H-NMR signals were produced that allowed an accurate determination of the enantiomeric purity [13,14]. We investigated whether this technique could be used for the identification of the locust haemolymph 1,2-diacylglycerols. As different 1,2-diacylglycerol pools seem to exist at rest and during periods of increased lipid mobilization, we studied the absolute configuration of the haemolymph 1,2-diacylglycerols originating from locusts at rest, after flight, and after injection with extracts of corpora cardiaca containing adipokinetic activity. In addition, positional distributions of fatty acids were determined in the fat body triacylglycerols and in haemolymph 1,2-diacylglycerol samples obtained from locusts after flight. For stereochemical analysis, we compared the conversion of the 1,2-diacylglycerols into 1,2-diacyl-3-tritylglycerols, applied in the identification of chiral 1,2-diacylglycerols in milkfat [14], with the trimethylsilylation method [13].

Materials and Methods

Insect experiments

Locusta migratoria were reared under crowded conditions and fed on reed (*Glyceria maxima*) supplemented with rolled oats [4]. Adult males (12 days after imaginal ecdysis) were used in all experiments. Corpora cardiaca were dissected from anaesthetized animals and extracts prepared as described by Holwerda et al. [15]. Locusts were injected into the haemocoelom with a 10 μl volume of hormone extract containing 0.05 pair corpus cardiacum equivalents. 90 min after injection, haemolymph was sampled. In experiments involving flight, locusts were flown for 2 h on motordriven roundabouts rotating at constant speed [4].

Isolation of haemolymph 1,2-diacylglycerols

From groups of locusts at rest, after flight and after hormone injection, samples of 25 μl haemolymph per animal were taken from a puncture in the ventral membrane between head and thorax using a Hamilton micro-syringe and pooled for each experimental group. Samples of 1 to 2 ml were centrifuged for 2 min at 12 000 $\times g$ and the supernatants were immediately mixed with excess $CHCl_3/CH_3OH$ (1/1, v/v). Lipids were extracted by the chloroform/methanol/water system according to Bligh and Dyer [16]. The evaporated total lipids were chromatographed over a 20 cm column (0.4 cm inner diameter) filled with silicic acid containing 10% boric acid [17] to prevent isomerisation. A gradient of diethyl ether in light petroleum was used as eluent. The 1,2-diacylglycerols isolated were purified by preparative thin-layer chromatography.

Isolation of fat body triacylglycerols

From groups of ten locusts, fat bodies were removed and rinsed with saline at 0°C. Pooled tissue samples of 1—1.2 g were homogenised in $CHCl_3/CH_3OH$ (1/1, v/v) using a Branson sonifier, and lipids extracted [16]. Total lipids were separated by column chromatography on silicic acid and triacylglycerols isolated.

^{1}H-NMR analysis

Spectra were measured on a Bruker WP 200 spectrometer. Tris(3-(heptafluoropropylhydroxymethylene)-*d*-camphorato)europium (III), $Eu(hfc)_3$ and tris(3-heptafluoropropylhydroxymethylene)-*d*-camphorato)praseodymium (III), $Pr(hfc)_3$ were obtained from Aldrich-Europe (Belgium), and Willow Brook Laboratories (U.S.A.), respectively. Proton chemical shifts (δ) are given in p.p.m. downfield from internal tetramethylsilane.

Silylation procedure

Unmodified or hydrogenated haemolymph 1,2-diacylglycerols were silylated with hexamethyl disilazane/trimethyl chlorosilane/pyridine (1 : 4 : 4, v/v/v) for 15 min, followed by evaporation under vacuum. Residues were dissolved in light petroleum, filtered to remove pyridinium hydrochloride and evaporated once more. The products were used for the NMR experiments without further

purification. Hydrogenation of the samples before silylation was carried out in hexane solutions, using 10% Pd on carbon (Merck) as catalyst.

Preparation of 1,2-diacetyl-3-tritylglycerols

Samples of crude haemolymph extracts containing 12 mg of 1,2-diacylglycerols were dissolved in 0.15 ml toluene/pyridine (3 : 1, v/v) and reacted with 30 mg tritylchloride at 60°C for 50 h. About 15 mg 1,2-diacyl-3-tritylglycerols were obtained by chromatography of the mixtures over a 20 cm column of silicic acid (inner diameter 0.4 cm) which was deactivated with 8% water to avoid detritylation [18]. These samples were dissolved in a few drops of toluene and mixed with 0.1 ml of a solution of sodium ethoxide in ethanol (0.1 mol/l) and allowed to react at room temperature for 1.5 h. After evaporation, the residues were reacted with acetic anhydride in pyridine overnight, evaporated and treated with a few drops of a solution of diazomethane in diethyl ether. The resulting 1,2-diacetyl-3-tritylglycerols were purified by column chromatography over 20 cm silicic acid (inner diameter 0.4 cm) which was deactivated with 8% water, using toluene as the eluent. The NMR spectra of these compounds were identical to those of authentic 1,2-diacetyl-3-trityl-*rac*-glycerol. The yields were about 7 mg.

Fatty acid analysis

Samples of the haemolymph 1,2-diacylglycerols and the fat body triacylglycerols were interesterified with methanolic HCl (1 mol/l) in sealed ampoules at 100°C for 1.5 h. The methyl esters were analysed by GLC on a 1.8 m PEGA column using a Hewlett Packard 5700A chromatograph. Peaks were identified by comparison with reference fatty acid methyl esters and by graphical analysis.

Lipase assays

Lipolysis of haemolymph 1,2-diacylglycerols and fat body triacylglycerols was carried out essentially according to the procedure of Luddy et al. [19]. The pancreatic lipase, grade B, was obtained from Calbiochem and was defatted with diethyl ether before use. Fatty acid analysis of the resulting 2-monoacylglycerols was performed after interesterification.

Results

To test the feasibility of analysing mixtures of trimethylsilylated 1,2-diacylglycerols by the ^{1}H-NMR method, a mixture of 1,2-dimyristoyl-3-trimethylsilyl-*rac*-glycerol and 1,2-distearoyl-3-trimethylsilyl-*rac*-glycerol was prepared. The complete overlap of the corresponding ^{1}H-NMR signals (200 MHz) of the trimethylsilyl groups was maintained after addition of the chiral shift reagent, indicating that simple mixtures of 1,2-diacylglycerols can be analysed. As a chiral shift reagent $Pr(hfc)_3$, which shifts the absorptions to higher fields free from other signals [13], was used. Purified haemolymph 1,2-diacylglycerol samples were subjected to trimethylsilylation. The resulting mixture of 1,2-diacyl-3-trimethylsilylglycerols, however, could not be analysed sufficiently because of considerable broadening of the trimethylsilyl signals, probably due

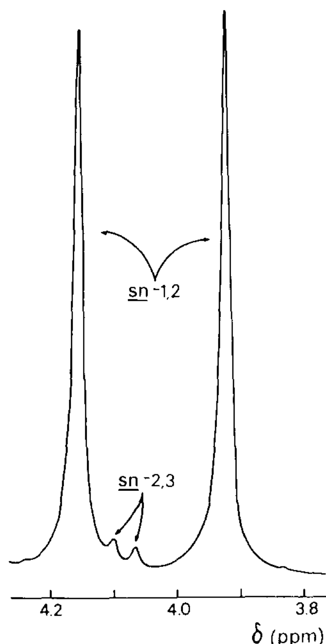

Fig. 1. ^{1}H-NMR spectrum (200 MHz, C^2HCl_3 solution) of the acetyl methyl protons of 1,2-diacetyl-3-tritylglycerol originating from the haemolymph 1,2-diacylglycerols of *Locusta* after a 2 h flight.

to the presence of too great a variety of molecular species. Hydrogenation of the 1,2-diacylglycerols before trimethylsilylation did not improve the quality of the signal. To simplify the sample, all acyl radicals were replaced by acetyl groups. As both acetyl groups at the primary and secondary positions of glycerol have excellent properties for stereochemical analysis, contaminations causing signals to coincide with only one of the acetyl signals are easily detected. Prior to removing the fatty acids by ethoxide catalysed ethanolysis, the hydroxyl group was blocked by the trityl group which is stable to base and unlike the tetrahydrophyranyl group does not introduce an additional chiral centre.

The ^{1}H-NMR signals of the acetyl methyl groups of the converted haemolymph 1,2-diacylglycerols obtained after flight are shown in Fig. 1. $Eu(hfc)_3$ was added to the sample. The assignment of the acetyl signals was confirmed by adding 1,2-diacetyl-3-trityl-*rac*-glycerol [14] to the sample. The outer set of signals originates from the *sn*-1,2-diacetyl compound, the inner set from its enantiomer [14,20]. At least 97% of the initial 1,2-diacylglycerols had the *sn*-1,2-configuration. The 2 to 3% *sn*-2,3-enantiomer may not have been present in the haemolymph initially. The presence of small amounts of 1,3-diacylglycerols in the haemolymph lipids indicates that some isomerisation may have taken place. The corresponding spectra of the converted 1,2-diacylglycerols isolated from haemolymph samples obtained from locusts at rest or after hormone injection were almost identical to Fig. 1, showing the presence of acylglycerols of high optical purity as well. In resting locusts, 96% of the converted 1,2-diacylglycerol is the *sn*-1,2-diacetyl enantiomer, in hormone injected

TABLE I

FATTY ACID DISTRIBUTION IN *LOCUSTA* FAT BODY TRIACYLGLYCEROLS AND HAEMOLYMPH *sn*-1,2-DIACYLGLYCEROLS AFTER A 2 h FLIGHT

	Position	Fatty acids (mole percent)							
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Haemolymph *sn*-1,2-diacylglycerols	*sn*-1	—	4.1	54.6	2.8	6.9	21.2	8.3	3.1
	sn-2	—	0.4	3.2	2.7	0.4	64.5	10.4	18.5
Fat body triacylglycerols	$\frac{sn\text{-}1 + sn\text{-}3}{2}$	0.1	4.5	46.3	3.2	6.6	26.5	7.0	5.9
	sn-2	—	0.1	2.5	1.4	0.5	61.0	9.0	25.4

locusts a similar *sn*-1,2-content was measured: 97%. Thus, in *Locusta* haemolymph *sn*-1,2-diacylglycerols with a remarkably high optical purity are present both during storage of the fat body triacylglycerols and during fat mobilization induced either by flight activity or by injection of corpus cardiacum extracts.

The positional distribution of the fatty acids in the haemolymph *sn*-1,2-diacylglycerols after a 2 h flight and the fatty acid distribution in position 2 and in the average of positions 1 and 3 of the fat body triacylglycerols are given in Table I. The proportional fatty acid distribution of the *sn*-1,2-diacylglycerols has been corrected for the presence of approx. 3% *sn*-2,3-diacylglycerols in the various samples analysed. Both in the triacylglycerols and the diacylglycerols the distribution of fatty acids is non-random. In the triacylglycerols 96.8% and in the diacylglycerols 96.1% of the fatty acids in the 2-position is unsaturated, oleic acid (18 : 1) and linolenic acid (18 : 3) are the most pronounced components. In both the *sn*-1-position of the diacylglycerols and the combined positions 1 and 3 of the triacylglycerols, saturated acids, mainly palmitic acid (16 : 0) and stearic acid (18 : 0), are predominant.

Discussion

The successful identification of chiral 1,2-diacylglycerols in locust haemolymph provides another example of the versatility of the ^{1}H-NMR method in the stereochemical analysis of chemically modified natural 1,2-diacylglycerols. The conversion into 1,2-diacetyl-3-tritylglycerols gave better results than the direct trimethylsilylation of unmodified or hydrogenated 1,2-diacylglycerols. If pure enantiomeric 1,2-diacylglycerols are to be characterised, the combination of the ^{1}H-NMR method and the lipase analysis as described in this paper in principle yields the same information as the methods based on the stereospecificity of phospholipases for phosphorylated 1,2-diacylglycerols. If small amounts of enantiomers formed by isomerisation are present, the initial fatty acid composition can be derived by correction. However, for samples in which initially both *sn*-1,2- and *sn*-2,3-diacylglycerols are present, only the determination of the absolute configuration is useful as lipase analysis cannot distinguish between the *sn*-1 and *sn*-3 positions. In the latter case the methods relying on phospholipase assays provide more complete information about the diacylglycerols present.

Now that the *sn*-1,2-configuration of the locust haemolymph 1,2-diacylglycerols has been established, two alternatives for their biochemical formation by triacylglycerol mobilization during flight become plausible. Tietz [9] suggested that triacylglycerols are degraded to 2-acylglycerols by a non-stereospecific lipase. These 2-acylglycerols would then act as substrates in the resynthesis of 1,2-diacylglycerols [8,21]. Though at that time the stereospecificity of the haemolymph diacylglycerols was only suspected, the requirement of a stereospecific reacylation does not seem unreasonable, since Johnston et al. [22] showed that *sn*-1,2-enantiomers of diacylglycerols are almost exclusively formed in hamster mucosal microsomes using 2-palmitoylglycerol as substrate. Other studies, however, indicate that significant amounts of 2,3-diacyl-*sn*-glycerols are also formed by rat intestinal monoacylglycerol transferase [23—25]. The direct degradation of triacylglycerols into 1,2-diacyl-*sn*-glycerols by an enzyme specific for the *sn*-3-position of glycerol might be a second possibility. Apart from its intrinsic interest, the discovery of a stereospecific lipase would give a further impulse to the search for stereospecific triacylglycerol lipases. These should be the ideal tools for stereochemical analysis of triacylglycerols. At present, apart from lipoprotein lipases [26], only lingual lipase [27] and hepatic lipase [28] have been shown to have some preference for one of the two primary positions of glycerol.

Concerning the positional distribution patterns of the locust fat body triacylglycerols and haemolymph *sn*-1,2-diacylglycerols, the occupation of the *sn*-2-position in both glycerides is in close agreement. Some minor differences exist between the *sn*-1-position of the diacylglycerols and the average of positions 1 and 3 of the triacylglycerols which, however, in view of the accuracy of the analyses are considered to be significant. This would be in favour of the 2-monoacylglycerol pathway. On the other hand, since our lipase assay could not differentiate between the two primary positions in the triacylglycerol, the possibility of an asymmetrical fatty acid distribution should be considered. In the depot fat of the mealworm *Tenebrio molitor*, Brockerhoff et al. [29] observed an asymmetrical triacylglycerol fatty acid distribution, in which for instance 14 : 0 and 16 : 0 tend to prefer positions 1 to 3, whereas 16 : 1 and 18 : 1 tend toward position 3. Assuming that similar differences exist in *Locusta*, a specific *sn*-3 lipase would account completely for the observed differences in the average of the positions 1 and 3 of the triacylglycerols and the *sn*-1 position of the diacylglycerols. Though fairly speculative, this finding is compatible with the possibility of a stereospecific triacylglycerol lipase. Incubation experiments with the fat body lipase system of the locust and optically active triacylglycerols labelled at the primary positions of glycerol, which are in progress in the authors laboratories, could possibly distinguish more conclusively between the alternatives.

In resting locusts, pathways for the production of 1,2-diacylglycerols in the haemolymph other than triacylglycerol mobilization are probably relatively more important than those during flight or after hormone injection. However, the evidence is not conclusive since the 1,2-diacylglycerols having the same configuration as those mobilized during flight or after injection of the adipokinetic hormone.

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