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MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE DURING THE DEVELOPMENT OF THE DIPTEROUS *CERATITIS CAPITATA*

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Abstract—1. Developmental changes in phosphatidylcholine (PC) molecular species composition from larval to pharate adult stages of *Ceratitis capitata* are mainly related to a decrease in the relative level of tetraenoic molecules, dropping to trace amounts in pharate adults, and to an increased appearance of trienoic species as development proceeds.

2. Most disaturated, mono-, and dienoic molecules show compositional patterns comparable to those exhibited by PCs from higher animals. Nevertheless, some "unusual" components have also been detected, primarily, tri and tetraenoic molecules containing linoleic acid.

3. Characteristic features of the insect PCs are (i) the high relative abundance of di-monounsaturated species throughout development; (ii) larval stages—containing 1-palmitoyl,2-palmitoleyl and 1,2-dilinoleoyl PCs, and (iii) pharate adult containing 1-linoleoyl, 2-oleoyl PC.

INTRODUCTION

Observations from a number of publications on the molecular composition of phosphoglycerides from many tissues of higher animals (Renkonen, 1966; Kuksis & Marai, 1967; Privett & Nutter, 1967; Montfoort *et al.*, 1971; Hunter *et al.*, 1973; Abad *et al.*, 1976; Gonzalez-Ros & Ribera, 1980) allowed for the establishment of certain general features either in the positional distribution of acyl chains or in their respective combinations to render a given molecular type. These general patterns seem to be dependent on the precise type of tissue (Montfoort *et al.*, 1971) and on its developmental stage (Abad *et al.*, 1976; Gonzalez-Ros & Ribera, 1980a) while independent from differences in animal species (Montfoort *et al.*, 1971; Gonzalez-Ros & Ribera, 1980a).

The known influence of phospholipids on the physical properties of biological membranes and on the activity of membrane proteins (Van Deenen, 1965; Fourcans & Jain, 1974; Sandermann, 1978; Holub & Kuksis, 1978) encourages studies on the precise structure of the major phospholipid classes present in order to correlate certain membrane functions with specific lipid compositions. In this regard, systematic studies on developing living systems, which undergo drastic changes in their functional capacities, could be of interest in determining the possible associated lipid compositional changes (Wells & Dittmer, 1967; Wood, 1974; Abad *et al.*, 1976; Gonzalez-Ros & Ribera, 1980a,b). No attempts have yet been made to describe these kinds of biochemical events in the vast group of Insecta. Developmental changes in the holometabolous dipterous *Ceratitis capitata* have already been described from the point of view of quantitative levels, fatty acid composition, and positional distribution of acyl chains in the two major phospholipid classes, phosphatidylcholine (PC) and phosphatidylethanolamine (Castillon *et al.*, 1971; Fernandez-Sousa *et al.*, 1971a,b). With this background the present paper describes the molecular species composition of PC throughout different stages of development of the same insect in an attempt to compare these with the better known patterns from higher animal tissues.

Abbreviations used: PC—phosphatidylcholine; DAG—1,2-diacyl-3-acetyl-*sn*-glycerol; TLC—thin-layer chromatography; GLC—gas-liquid chromatography.

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MATERIALS AND METHODS

Culturing of *Ceratitis capitata* (Wiedemann) was carried out as previously described (Madariaga *et al.*, 1972). Larvae were collected at 5–6 days and carefully washed before being used. Pharate adults were 5–6 days old (Hinton, 1968).

Insect homogenization, lipid extraction and fractionation of total lipids into neutral and polar lipid fractions by silicic acid column chromatography were performed as described (Fernandez-Sousa *et al.*, 1971a).

PC was obtained from the polar lipid fractions by thin layer chromatography (TLC) (1.0 mm thick) on silica gel G (E. Merck A.G., Darmstad, G.F.R.) using chloroform–methanol–water (65:25:4, v/v/v) as the solvent system. Aliquots containing up to 80 mg of polar lipids were applied as narrow bands to each 20 × 20 cm plate. Bands of PC were detected by spraying the margins of the plates with the Dittmer reagent (Dittmer & Lester, 1964), then scraped off the plates and extracted by successive treatments with 10 vol of chloroform–methanol (2:1, 1:1, 1:2 and 1:9, v/v) and finally methanol. PC isolated in this way was further purified by W-200 neutral Al_2O_3 (Woelm M., Eschwege, G.F.R.) column chromatography (Singleton *et al.*, 1965) and finally appeared as a single spot on two-dimensional TLC using chloroform–methanol–water (65:25:4, v/v/v) and *n*-butanol–acetic acid–water (3:1:1, v/v/v).

Conversion of purified PC in 1,2-diacyl,3-acetyl-*sn*-glycerol (DAG) was performed as follows: Twenty-five units of phospholipase C (EC 3.1.4.3) from *Clostridium welchii* (Sigma Chemical Co., St Louis, MO, U.S.A.) were dissolved into 8.0 ml of 0.05 M Tris buffer, pH 7.2, and 10.0 ml of 5 mM $CaCl_2$ and washed twice with ethyl–ether to remove lipid contaminants. To this solution were added 35 mg of PC dissolved in 5.0 ml of freshly-distilled ethyl–ether. The mixture was shaken under N_2 at 29°C for 2 hr and then extracted five times with ethyl–ether. Combined extracts were evaporated to dryness and weighed. An aliquot containing up to 1 mg of dry extract was analyzed by TLC (0.5 mm thick) using *n*-hexane/ethyl-ether/acetic acid (50:50:1, v/v/v) as the solvent system. The 1,2-diacyl-*sn*-glycerol spot was visualized by spraying the plate with a 0.1% Rhodamine-6-G methanol solution or by exposing it to iodine vapor. The remaining dry extract was acetylated with a 24-fold molar excess of acetic anhydride and 1 vol of anhydrous pyridine at 40°C for 4 minutes. The reaction was stopped by adding 3.0 ml of anhydrous methanol. Diacylglycerol acetates were extracted five times with ethyl–ether and the combined extracts were washed with 2 vol 2 N HCl, 2 vol 2% $NaHCO_3$ and 3 vol distilled water. The organic phase was dried over anhydrous sodium sulfate, concentrated in a rotary evaporator and purified by TLC on 0.5 mm layers of silica gel G, using hexane–ethyl-ether–acetic acid (50:50:1, v/v/v) as the solvent system. Bands of diacyl–glycerol acetates were detected with Rhodamine-6-G solution and the 1,2-diacyl,3-acetyl-*sn*-glycerols (DAG) were scraped off the plate and eluted from the adsorbent with several volumes of chloroform. Initial PC and DAG derivatives did not show significant differences in their fatty acid compositions (Table 1).

The DAG mixture was resolved by argentation TLC ($AgNO_3$–silica gel G) on the basis of total number of double bonds per molecule. Two plates (200 × 200 × 0.5 mm) were prepared as described by Renkonen (1966), except that the plates were dried at 120°C for 35 min. The plates were developed using benzene–chloroform–methanol (90:10:1, v/v/v) as solvent system. After development the plates were sprayed with a 0.05% methanol–water (1:1, v/v) solution of 2′,7′-dichlorofluorescein and the DAG subfractions were located under u.v. light. Those subfractions corresponding to the various degrees of unsaturation were recovered separately by repeated extraction of the gel scrapings with ethyl–ether. Silver nitrate was eliminated by washing the extracts with a 0.1% NaCl solution. The organic phase was evaporated to dryness after addition of small amounts of methanol to remove last traces of water. The ratio of the subfractions obtained was determined by gas–liquid chromatography (GLC) by adding methyl–pentadecanoate (as an internal standard) to the methyl ester derivatives. The positional distribution of the fatty acids in DAG subfractions was determined by hydrolysis with pancreatic lipase (EC 3.1.1.3) (Calbiochem, Los Angeles, CA) as previously reported (Gonzalez–Ros & Ribera, 1980a). Preparation of the methyl esters and analysis of the fatty acids by GLC was previously described (Gonzalez–Ros & Ribera, 1980b). Analytical data represent an average of two–four different samples and are expressed as molar percentages of total fatty acids. Standard deviation for data in Tables 1, 2 and 3 was no more than 2.1% and 1.3% respectively, for molar relative abundances higher and lower than 10%.

RESULTS AND DISCUSSION

The fatty acid composition of PC and DAG derivatives from larval and pharate adult stages of *Ceratitis capitata* is shown in Table 1. Palmitic, palmitoleic, oleic and linoleic acids account for more than 90% of PC fatty acid contents in both developmental stages. Nevertheless, the higher relative abundance of dienoic fatty acids (linoleic acid) in larval stages provide a decrease in the overall degree of PC unsaturation as development proceeds to pharate adult.

On the other hand, the resemblance between the fatty acid composition of the starting PC and DAG derivatives (Table 1) indicates that no significant alteration of the acyl moieties occurs as a consequence of the analytical procedure utilized.

Fractionation by argentation–TLC of DAG derived from *Ceratitis capitata* PC provides: (a) the relative abundance and fatty acid composition of each fraction and (b) the positional distribution of acyl groups (GLC data is available from the authors upon request). From these complementary results, the experimental molecular species composition was estimated (Table 3).

Five different groups of DAG derivatives (Table 2) with different degrees of unsaturation were obtained from the two developmental stages considered in *Ceratitis capitata*, i.e. disaturated, mono-, di-, tri- and

Table 1. Fatty acid composition of PC and DAG derivatives from developing *Ceratitis capitata**

	Larva			Pharate adult		
Fatty acids	PC	DAG†	DAG‡	PC	DAG†	DAG‡
12:0	2.0	1.7	2.4	Tr.§	Tr.	0.7
14:0	0.8	1.0	1.8	0.9	0.8	2.4
16:0	24.0	23.3	25.3	30.7	29.5	32.6
16:1	26.4	26.3	24.1	20.3	20.9	18.5
18:0	3.4	3.9	4.5	3.7	3.4	5.7
18:1	17.4	18.2	16.8	27.0	27.5	24.1
18:2	23.7	24.5	23.4	16.1	16.9	16.0
18:3	2.1	2.0	1.7	1.1	1.1	Tr.

* A comprehensive study on the changes in PC fatty acid composition during the development of *Ceratitis capitata* is available from Fernandez-Sousa *et al.* (1971a).

† Experimental fatty acid composition of DAG derivatives (see Methods).

‡ Fatty acid composition of DAG as calculated from molar relative abundances and fatty acid compositions of recovered DAG subfractions on argentation TLC.

§ Traces; fatty acid molar relative abundance less than 0.5%

Table 2. Molar relative abundance of DAG subfractions derived from *Ceratitis capitata* PC

Molecular classes*	Larva	Pharate adult
Disaturated	13.1†	17.5
Monoenoic	22.5	28.4
Dienoic	36.6	27.6
Trienoic	12.8	26.5
Tetraenoic	14.9	Tr.

* Subfractionation of DAG derivatives in achieved by argentation-TLC on the basis of unsaturation degree (see Methods section).

† Molar relative abundance of different DAG subfractions within the whole DAG population from each developmental stage.

tetraenoic molecules. These DAG subfractions undergo distinctive changes in their relative abundances as the development proceeds. Dienoic and mainly tetraenoic molecules are mostly characteristic of larval stages whereas a higher relative abundance of trienoic molecules appears to be associated with pharate adults. Nonetheless, the previously reported decrease in the absolute amount of PC from larval to pharate adult stages (Castillon *et al.*, 1971) provides a steady developmental decrease which can be estimated as, at least, a reduction by half of all the different molecular groups.

The percentage distribution of individual PC molecular species is shown in Table 3. Eighteen to twenty major ($\geqslant 2\%$ of total PC population) molecular species are found which undergo significant and distinctive variations in relative abundance between the two stages considered in the development of the insect.

PC populations are composed of molecules with a fairly selective fatty acid distribution, i.e. unsaturated fatty acids are primarily attached to the *sn*-2 position, whereas saturated acyl groups bind mainly to the *sn*-1 glycerol hydroxyl group. Nevertheless, this positional selectivity appears to be somewhat less conspicuous than the one described elsewhere for phosphoglycerides from higher animals (Holub & Kuksis, 1978), as illustrated, for instance, by the linoleic acid containing-tetraenoic molecules.

Disaturated PC molecular species account for approximately 10% of the total PC population. The most abundant disaturate is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine which exhibits increased relative levels as development proceeds to pharate adult stages. Relative abundance of dipalmitoyl-PC is somewhat comparable to previously described biological systems (Montfoort *et al.*, 1971; Abad *et al.*, 1976), except for brain or lung where it is found in a much greater amount (Montfoort *et al.*, 1971; Holub & Kuksis, 1978; Gonzalez-Ros & Ribera, 1980a). The monoenoic PC molecular species are present in the developing insect with twice as much the relative levels of the disaturated ones, the main components being 1-palmitoyl, 2-palmitoleoyl and 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphocoline. The latter is the main type of monounsaturated molecules in pharate adult stages, whereas the 1-palmitoyl, 2-palmitoleoyl combination appears to be preferred in larva. Interestingly, 1-palmitoyl, 2-palmitoleoyl-*sn*-glycero-3-phosphocholine has not been found in significant amounts in avian or mammalian tissues, with the exception of rat lung (Montfoort *et al.*, 1971) whereas in the insect larvae it is the major component (12.4%) in the monoenoic group.

Other possible combinations of monounsaturated (oleic and palmitoleic) and saturated (palmitic and stearic) fatty acids have also been detected by forming monoenoic molecular species, except that stearic acid was never found attached to the *sn*-2 position.

Dienoic PC molecular species represent about 37 and 28%, respectively, of the total PC population from larval and pharate adult stages. They are formed either by having two double bonds in the same apolar chain (1-palmitoyl, 2-linoleoyl-PC being the main representative of this type), or by concomitance of two monounsaturated apolar chains within the same PC molecule. The latter molecules are the most common type of dienoic PCs in both developmental stages of the insect in contrast with higher animal tissues where they have rarely been found (Montfoort *et al.*, 1971; Hunter *et al.*, 1973; Abad *et al.*, 1978) with the exceptions of mammalian milk (Privett & Nutter, 1967) and avian embryonic brain (Gonzalez-Ros & Ribera 1980a). Di-monounsaturated molecules are formed indistinctively by combination of oleic and palmitoleic acids in either *sn*-1 or *sn*-2 positions, even though preferential combination of two palmitoleoyl residues within the same molecule as well as 1-oleoyl, 2-palmitoleoyl-PC appear to be predominant in larval stages.

Table 3. Molecular species composition of PC from developing *Ceratitis capitata**

Molecular classes†	Larva		Pharate adult	
	Found	Random‖	Found	Random
16:0/16:0	9.0	5.8	11.9	9.3
16:0/16:1 (+18:0/16:1)‡	12.4	7.2	8.7	6.9
16:0/18:1 (+18:0/18:1)	6.7	4.8	11.7	9.2
16:1/16:0 (+18:1/16:0)	2.2	10.5	5.2	14.4
16:1/16:1 (+18:1/16:1)	13.3	11.6	7.1	9.6
16:1/18:1 (+18:1/18:1)	7.6	7.6	7.3	12.8
16:0/18:2	10.0	5.7	7.8	4.9
18:0/18:2	3.7	0.8	2.7	0.6
18:2/16:0	Tr.	5.7	2.3	4.9
16:1/18:2	2.8	6.3	5.0	3.3
18:1/18:2	3.8	4.1	9.1	4.4
18:2/16:1	2.7	6.3	2.6	3.3
18:2/18:1	Tr.	4.1	5.5	4.7
18:2/18:2	8.1	5.6	Tr.	2.6
Total accounted§	82.2	86.1	87.0	90.6

* Molecular species with molar relative abundances higher than 2% have been represented.

† *sn*-1 position/*sn*-2 position.

‡ Molecular species in brackets indicate minor constituents in not fully-resolved mixtures.

§ Molar percentage of total PC population accounted by the analytical procedure.

‖ Calculations assuming random distribution of acyl groups were done according to Kuksis *et al.* (1963).

Polyunsaturated PC, comprised mainly of trienoic and tetraenoic molecular species, have been found with the same overall relative abundance ($\sim 26\%$) in both of the two developmental stages considered. Nevertheless, tetraenoic molecules appear to be exclusively associated with larval stages, whereas trienoic species are mainly related to pharate adults.

Trienoic molecular species are formed by preferential combinations of monounsaturated residues (palmitoleic and mainly oleic acids) at *sn*-1 position with linoleic acid at *sn*-2. Additionally, 1-linoleoyl, 2-oleoyl-*sn*-glycero-3-phosphorylcholine has also been found exclusively associated with pharate adult stages. Trienoic PC molecules have shown a very irregular distribution in other known biological systems, i.e. they have considerably high relative abundance in some vegetable oils (Privett & Nutter, 1967), mammalian milk (Privett & Nutter, 1967), several rabbit and pig tissues (Montfoort *et al.*, 1971) and in the hen egg yolk (Kuksis & Marai, 1967), whereas they are virtually absent from pig and chicken liver (Hunter *et al.*, 1973; Abad *et al.*, 1976) and from chick embryo brain (Gonzalez-Ros & Ribera, 1980a).

Tetraenoic molecules ($\sim 14\%$) are exclusively associated with larval stages, the main representative being 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine. Nevertheless, dipolyunsaturated molecular species such as the latter were predicted as not likely to be found in biological systems because of the observed instability of membrane model systems containing high amounts of these phosphoglyceride molecules (DeGier *et al.*, 1968). Actually, such a prediction has been confirmed in most known higher animal tissues (see for instance Montfoort *et al.*, 1971; Abad *et al.*, 1976; Gonzalez-Ros & Ribera, 1980a).

Also shown in Table 3 is the molecular composition of PC that would be expected on the basis of a completely random distribution of the fatty acids present in the initial PC (Table 1). As can be seen by comparison, experimental values from mono- and dienoic molecules containing *sn*-1 saturated and *sn*-2 unsaturated acyl residues are higher than expected from the statistical assumption, whereas the opposite occurs for *sn*-1 unsaturated, *sn*-2 saturated molecules (see for instance 1-oleoyl, 2-palmitoyl and 1-linoleoyl, 2-palmitoyl-PC). This observation is in agreement with the generally observed positional selectivity of acyl groups in animal phosphoglycerides. Conversely, trienoic species in pharate adult and tetraenoic species in larve are also favored over expected random distribution values, even though it implies favoring the appearance of "unusual" molecular species such as 1,2-dilinoleoyl and 1-linoleoyl, 2-oleoyl-PC in larve and pharate adults respectively.

The observed non-randomness would mean that certain acyl groups of PC from the developing insect tend to associate with each other on some basis other than molar concentration, perhaps fulfilling a certain requirement in the assembly of the developing membranes. Another possibility is that insect PC represents pooled contributions of molecules from several PC populations, each of which may possess a random distribution for its fatty acids. Whether or not these results are a reflection of the specificity of endogenous PC synthesis is not known yet.

In conclusion, PC from developing *Ceratitis capitata* shows a molecular composition whose features basically agree with the trend shown by phosphoglycerides from higher animals. A non-random distribution and a positional selectivity of acyl groups have been observed in most saturated, mono- and dienoic molecules where saturated and unsaturated fatty acids, respectively, are primarily attached to the *sn*-1 and *sn*-2 glycerol hydroxyl group. On the other hand, linoleic acid containing tri- and tetraenoic molecular species in pharate adult and larva, respectively, do not exhibit such a positional distribution in their acyl groups. Indeed, appearance of "unusual" (as compared with higher animal tissues) di-linoleoyl (larval stages) and 1-linoleoyl, 2-oleoyl (pharate adults) molecular species is favored. Other characteristic features of the insect PC are the high relative abundance of dienoic molecules containing two monounsaturated acyl chains throughout development, and 1-palmitoyl, 2-palmitoleoyl-*sn*-glycero-3-phosphocholine being the major monoenoic component in larval stages.

Developmental changes are mainly related to a decrease in the relative levels of tetraenoic molecules, dropping to trace amounts in pharate adults, and to an increased appearance of trienoic species as development proceeds.

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