ANNA RADOMIŃSKA-PYREK, TADEUSZ CHOJNACKI and WIESŁAW ZULCZYK

ACYL ESTERS OF POLYPRENOLS: SPECIFICITY OF MICROSOMAL TRANSACYLASE FOR POLYPRENOLS OF DIFFERENT CHAIN LENGTH AND SATURATION*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences,
Rakowiecka 36; 02-532 Warszawa, Poland
and Institute of Nuclear Research, Warszawa, Poland

Transfer of fatty acids from phospholipids to polyprenols, catalysed by the transacylase from rat liver microsomes, was investigated. The specificity of the enzyme for polyprenols of different chain length and different degree of saturation was studied using individual isoprenologues, the preparation of which in highly tritiated form is described. It was found that short-chain polyprenols are better substrates for the enzyme than long-chain polyprenols, and α-saturated better than unsaturated or multiply saturated polyprenols. Short-chain, α-saturated single isoprenologues were several-fold more active as acyl acceptors than natural dolichol.

Polyprenols occur in living cells in the free or in the esterified form. Free polyprenols make up about 50% of the total pool in pig liver cells (Butterworth & Hemming, 1968), Aspergillus fumigatus (Stone & Hemming, 1968), and Neurospora crassa (Radomińska-Pyrek, unpublished). The remaining 50% consist mainly of acyl esters. Phosphoryl esters and possible other forms of bound polyprenols amount to less than 1% of the total.

There are two possible biosynthetic pathways leading to acylated polyprenols: transfer of fatty acids from phospholipids to polyprenols and direct acylation via acyl-coenzyme A (Radomińska-Pyrek, unpublished). The former transacylation reaction was first demonstrated by Keenan & Kruczek (1976) in the system in which natural dolichol, a mixture of isoprenologues, served as the acceptor molecule.

The aim of the present work was to obtain additional information on the transacylase, especially on its specificity towards different polyprenol species. The problem seems to be of considerable interest because of the central role polyprenols play in glycosylation reactions. The function of polyprenol coenzymes in the synthesis

* This work was supported by the Polish Academy of Sciences within the project 09.7.1. and by the Agriculture Research Service of the U.S. Department of Agriculture.

[125]
of bacterial cell walls is known (e.g., Hemming, 1974), and there is growing interest in their involvement in the synthesis of glycoproteins in eukaryotic cells (Hemming, 1974; Waechter & Lennarz, 1976; Palamarczyk, 1978). This crucial role of poly
prenols in such an important biosynthetic pathway makes the investigation of their metabolism especially attractive. It is possible that acylation and deacylation reactions are responsible for the size of the pool of available poly
prenols and represent a regulatory mechanism of glycosylation.

Study of the specificity of the transacylation reaction requires poly
prenol substrates differing in chain length and degree of saturation. The preparation of such compounds, in tritiated form, is also described in the present paper.

MATERIALS AND METHODS

Materials. [3H]NaBH₄ (8 Ci/m mole) was purchased from the Radiochemical Centre (Amersham, Bucks., England). NaBH₄, Triton X-100 and Nonidet P-40 were from B.D.H. Laboratory, Chemical Division (Poole, England). Lecithin from eggs, extra pure, was purchased from E. Merck (Darmstadt, F.R.G.). Palmitic acid was obtained from Koch-Light Laboratories Ltd (Colnbrook, England). PtO₂ was from Degussa Zweigniederlassung (Hanau, F.R.G.). Hydroxy(C₁₅)alkoxy-
propyl Sephadex (trade name: Lipidex-5000) was from Packard-Becker, B.V. (Groningen, The Netherlands). Tricium gas was obtained from the Ośrodek Pro-
dukci i Dystybcyj Isotopów (Świerk near Warsaw, Poland).

Single isoprenologues: C₅₅-OH (from leaves of Magnolia kobus), C₈₅-OH (from needles of Pinus sylvestris) and farnesol C₁₅-OH (obtained from Fluka AG, Buchs S.G., Switzerland) were isolated and hydrogenated as described previously (Sasak et al., 1976; Mańkowski et al., 1975, 1976, 1977). Bovine pituitary dolichols, a mixture of isoprenologues of C₈₅ to C₁₁₅, were obtained as described by Rado-
ńska-Pyrek et al. (1979).

Preparation of 1-³H-labelled poly
prenols. Single isoprenologues: 9-dihydro-
-C₅₅-OH, C₅₅-OH, and bovine pituitary dolichols were subjected to oxidation with chromium trioxide-pyridine complex followed by reduction with [³H]NaBH₄, as described by Keenan & Kruczek (1975).

Preparation of microosomal fraction. Rat livers (white Wistar rats, 200 - 220 g) were homogenized in 0.25 M-sucrose containing 1 mM-disodium EDTA. The homogenate was centrifuged for 20 min at 18 000 g and the resulting supernatant was reacentrifuged for 60 min at 105 000 g. The pellet was suspended in 0.25 M-sucrose in 1 mM-Tris/HCl, pH 7.0 (1 ml per 1 g of liver), the suspension divided into small portions and stored at −20°C. Under such conditions, the preparation was stable for several weeks.

Standard acylation assay conditions. If not stated otherwise, the assay conditions were as follows: poly
prenols (10 - 30 nmoles) were dried under a stream of nitrogen, suspended in 20 µl of 3% Triton X-100 using a Vortex mixer, and added to the assay mixture, which contained finally, in a total volume of 0.11 ml, 0.55% Triton X-100 and 127 mM-Tris/HCl, pH 6.0. The reaction was initiated by the addition
of microsomes (300 μg protein) and was carried out for 60 min at 37°C. It was terminated by the addition of 5 ml of a CHCl$_3$/CH$_3$OH mixture (2:1, v/v). The extract was shaken with 1.0 ml of 0.72% NaCl and the resulting upper phase discarded. The lower phase was washed with chloroform/methanol/water, 3:48:47, by vol. (Folch et al., 1957) and was dried under a stream of nitrogen. The residue was applied to a Merck precoated silica gel plate and developed in solvent A or B (see below). The radioactivity was located using the Berthold II scanner. The area corresponding to the esterified polypropenols was scraped off and counted in dioxane scintillation fluid.

**Chemical synthesis of palmityl esters of polypropenols.** The palmityl esters of natural dolichol and z-dihydro-C$_{55}$-OH were synthesized from the free acid and polypropenol alcohols using triphenylphosphine and diethyl azodicarboxylate as dehydratation agent according to the method described by Mitsunobu & Eguchi (1971). To a mixture of polypropenol (0.27 mmole), palmitic acid (0.4 mmole) and triphenylphosphine (0.5 mmole) in 5 ml of tetrahydrofuran (freshly distilled from lithium aluminium hydride), an excess of azodicarboxylate was added until the solution became yellow. Isoprenyl alcohols were transformed into their palmityl acid esters quantitatively, as shown by t.l.c. Tetrahydrofuran was evaporated in vacuo, the residue dissolved in hexane and, after removal of undissolved triphenylphosphine oxide and diethyl azodicarboxylate by filtration, the product was purified on a silica gel column in hexane. The purity of the product was checked by t.l.c. and proton n.m.r.

**Chromatography.** Thin-layer chromatography was carried out on precoated silica gel plates (0.25 mm thick; Merck, Darmstadt, F.R.G.) in, alternatively, solvent A (hexane/benzene/ethyl acetate, 60:30:1, by vol), solvent B (light petroleum ether/ethyl ether/acetic acid, 80:20:1, by vol), and solvent C (benzene/ethyl acetate, 95:5, v/v). The radioactivity was located using the Berthold II scanner.

Thin-layer reversed phase chromatography was performed on cellulose plates (Cellulose-TLC, Serva, Heidelberg, F.R.G.) impregnated with paraffin (Stone et al., 1967) in solvent D (acetone) and solvent G (acetone/water, 92:8, v/v) saturated with paraffin.

For chromatography on alumina columns (Brockman grade III, neutral, 6 x 0.7 cm), labelled lipids from ten samples (incubated for 2 h) were used. The columns were eluted stepwise (8 ml) with increasing concentrations of ethyl ether in petroleum ether (Stone et al., 1967). Each 8 ml eluate was collected as one fraction.

General methods. Proton n.m.r. spectra were recorded in CCl$_4$ or benzene with a Jeol-JNM-100 instrument. Protein was determined by the method of Lowry et al. (1951).

**RESULTS AND DISCUSSION**

**Preparation of selectively tritiated polypropenols.** Selectively tritiated polypropenols were prepared by the slightly modified method of Manikowski et al. (1976), originally developed for selective hydrogenation of polypropenols. Approximately 150 mg of pure polypropenol in 20 ml of freshly distilled absolute ethanol was mixed with 15 mg of PtO$_2$ and 5 mg NaBH$_4$. The suspension was placed in a 25 ml Erlenmeyer flask
and saturated with tritium gas (up to 100 mCi) under atmospheric pressure for 2 h with constant stirring. The partially hydrogenated products were filtered and dissolved in 50 ml of chloroform. Figure 1 shows the reversed phase thin-layer chromatogram of the partially hydrogenated product of C₈₅ polypropenol taken as an example. Fractions 2T, 4T, 6T represent labelled polypropenols with one, two, and three double bonds hydrogenated, respectively. More highly hydrogenated molecules (fraction \( m \)) did not separate well on the plate. The chloroform phase was washed with water, evaporated, and the residue was applied to the Lipidex-5000 column. The column was developed according to Mańkowski et al. (1976). Fractions 2T, 4T and 6T were completely separated on the column; fraction \( m \) was collected and subjected to second hydrogenation as above, except that hydrogen gas was used in this step instead of tritium, and NaBH₄ was omitted. The labelled perhydro-polypropenol was the product of the reaction.

Specific radioactivities of the individual polypropenols obtained by the described method are shown in Table 1; products labelled according to Keenan & Kruczek (1975) are included for comparison. Both methods yield polypropenols of similar specific radioactivity. However, Keenan & Kruczek (1975) used liver dolichols as starting material and introduced the label without otherwise changing the molecule. In the present work, we use as substrates plant polypropenols of high purity, which can be obtained in large quantities. The conversion of plant polypropenols into dolichol-like molecules requires hydrogenation of the \( \alpha \)-double bond. Labelling can be achieved in the same step, thus greatly simplifying the procedure. Moreover, a series of highly labelled modified polypropenols of different degree of saturation can be obtained in the same reaction. It has been observed that the remaining not reduced, fully unsaturated polypropenol has also acquired the tritium label due to the
Table 1

Specific radioactivities of tritiated single isoprenologues differing in the degree of saturation and chain length

Structures of polyrenols are expressed according to Hemming (1974): o, o-isoprene residue; T, trans-isoprene residue; C, cis-isoprene residue; S, saturated isoprene residue.

<table>
<thead>
<tr>
<th>[3H]Polyrenol</th>
<th>Structure</th>
<th>Specific radioactivity (μCi/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Dihydro-C15s-OH</td>
<td>o-T5S-OH</td>
<td>39.5</td>
</tr>
<tr>
<td>α-Dihydro-C25s-OH</td>
<td>o-T3C9S-OH</td>
<td>72</td>
</tr>
<tr>
<td>Tetrathydro-C25s-OH</td>
<td>o-T&lt;sub&gt;6&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S-OH</td>
<td>144</td>
</tr>
<tr>
<td>Perhydro-C35s-OH</td>
<td>(S)&lt;sub&gt;10&lt;/sub&gt;S-OH</td>
<td>64</td>
</tr>
<tr>
<td>α-Dihydro-C8s-OH</td>
<td>o-T1&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;13&lt;/sub&gt;S-OH</td>
<td>13.6</td>
</tr>
<tr>
<td>Tetrathydro-C8s-OH</td>
<td>o-T&lt;sub&gt;6&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S-OH</td>
<td>27.2</td>
</tr>
<tr>
<td>Hexahydro-C8s-OH</td>
<td>o-T&lt;sub&gt;6&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S&lt;sub&gt;8&lt;/sub&gt;S-OH</td>
<td>54.2</td>
</tr>
<tr>
<td>Perhydro-C18s-OH</td>
<td>(S)&lt;sub&gt;10&lt;/sub&gt;S-OH</td>
<td>126.1</td>
</tr>
<tr>
<td>α-Dihydro-C25s-OH*</td>
<td>o-T&lt;sub&gt;7&lt;/sub&gt;C&lt;sub&gt;9&lt;/sub&gt;S-OH</td>
<td>61.3</td>
</tr>
<tr>
<td>C15s-OH*</td>
<td>o-T&lt;sub&gt;5&lt;/sub&gt;C&lt;sub&gt;9&lt;/sub&gt;-C-OH</td>
<td>55.8</td>
</tr>
<tr>
<td>Dolicohol*</td>
<td>o-T&lt;sub&gt;7&lt;/sub&gt;C&lt;sub&gt;11&lt;/sub&gt;14s-OH</td>
<td>58.1</td>
</tr>
</tbody>
</table>

* Tritiated according to Keenan & Kruczek (1975).

expected catalytic exchange mechanism. Thus, our method makes possible the preparation of large quantities of well defined tritiated isoprenologues differing in chain length and degree of saturation.

Fig. 2. Time-course of acylation of [3H]α-dihydro-C15s-OH by microsomal transacylase. The reaction conditions were as described in Methods; the final concentration of polyrenol was 0.3 mM.
Characterization of the transacylation reaction. Optimal conditions for the transacylation reaction were checked using $[^3]$H$\alpha$-dihydro-C$_{35}$-OH. Under standard assay conditions, the rate of esterification of $\alpha$-dihydro-C$_{35}$ polypropenol was proportional to the concentration of microsomal protein up to 5.5 mg per ml. The formation of polypropenol esters was linearly dependent on time up to 60 min in the presence of a sub-saturating concentration of $[^3]$H$\alpha$-dihydro-C$_{35}$-OH (0.27 mm) (Fig. 2). The apparent $K_m$ for $\alpha$-dihydro-C$_{35}$-OH polypropenol was 0.2 mm (Fig. 3), in good agreement with the value of 0.3 mm reported by Keenan & Kruczek (1976). However, our enzymic preparation differed in some respects from theirs. Triton X-100 could be omitted from the assay mixture without a significant decrease of the activity (not shown), and Nonidet P-40 stimulated this activity by about 40%. Interestingly, there was no stimulation of the transacylase by egg yolk lecithin (added as a sonicated suspension in 3% Triton X-100), whereas Keenan & Kruczek (1976) observed a more than seven-fold stimulation of the enzyme activity by lecithin liposomes. This discrepancy might be due to the use of different microsome preparations (we used crude microsomes instead of the density gradient-purified material). A more attractive explanation would be a possible preference for lecithin membranes rather than for phospholipid-detergent micelles.

Identification of the reaction products. Incubation of $[^3]$H$\alpha$-dihydro-C$_{35}$-OH with rat liver microsomes yielded a single radioactive product, as revealed by t.l.c. in three different solvents (A, B, and G, see Methods). The $R_f$ values were 0.35 - 0.38, 0.85, and 0.32, respectively. The material co-chromatographed with synthetic $\alpha$-dihydro-C$_{35}$-palmitate in all three systems did not reveal any additional spots. The reaction mixture could be also fractionated on alumina columns. The elution profile is shown in Fig. 4B. The acylated $[^3]$H$\alpha$-dihydro-C$_{35}$ was eluted with 5%
Fig. 4. Alumina column chromatography of the transacylation products. For conditions of chromatography see Methods. I, Transacylation products of tritiated natural pituitary dolichol; II, transacylation products of $[^3]$H$\alpha$-dihydro-C$_{27}$-OH. A, Acylated polyprenol; B, unreacted polyprenol; C, unidentified compound.

Fig. 5. Scans of thin-layer chromatograms of the radioactive products obtained following incubation of $[^3]$H$\alpha$-dihydro-C$_{27}$-OH (A), $[^3]$H$\alpha$-dihydro-C$_{27}$-OH (B), $[^3]$H$\alpha$-dihydro-C$_{15}$-OH (C), $\alpha$-unsaturated C$_{27}$-OH (D), and natural dolichol (E). Conditions of incubation and chromatography in solvent A (hexane/benzene/ethyl acetate, 60:30:1, by vol.) were as described in Methods.
ethyl ether in petroleum ether. Besides the ester and the unreacted free polyprenol, an unidentified, more polar substance was eluted from the column (Fig. 4). The esterified polyprenol, isolated either by t.l.c. or by alumina column chromatography could be completely converted to the free alcohol by treatment with alkali under mild conditions (Stone et al., 1967).

Similarly, other [³H]isoprenologues of different chain length and saturation (Table 1), after acylation by rat liver microsomes showed on t.l.c. with solvent A a single peak of radioactivity (Fig. 5). In the case of [³H]C₁₅₅ α-unsaturated single polyprenol, at least two separate peaks of radioactivity were obtained. The reaction product of natural pituitary dolichol (a mixture of seven isoprenologues, average C₁₁₀₀) migrated as a broad peak (Rf 0.37 to 0.46). From the alumina column, dolichol was eluted at a higher concentration of ethyl ether (10%) than the acylated α-dihydro-C₁₅₅ polyprenol (5%).

A tendency to tail formation and broadening of the peak was often observed, even when single isoprenologues were used as substrates (e.g., Fig. 5). This can be best explained by heterogeneity of the acyl part of the polyprenol ester, possibly due to participation of endogenous microsomal lipids as acyl donors. However no closer characterization of these endogenous fatty acids was yet attempted.

![Diagram](image)

**Fig. 6.** Substrate specificity of rat liver microsomal transacylase. The transacylation reaction was carried out as described in Methods, with 0.1 mM-polyprenol (open bars) and 0.3 mM-polyprenol (shaded bars). The individual polyprenols are described by their ratio of saturating hydrogens to the total number of isoprene units. Activities are given as means of 3 - 6 determinations ± S.D.
Specificity of the transacylase. Specificity of the reaction was investigated using polypropenol substrates differing in chain length and number of double bonds (Fig. 6). Both these features were found to influence the rate of transacylation. Generally, short-chain polypropenols were better acceptors than the long-chain ones, although the difference between C₅₅ and C₈₅ was rather small. Hydrogenation of the α-double bond caused an increase in the reaction rate. However, hydrogenation of further double bonds led to a decrease in the activity.

The activity with the natural dolichol mixture was only half that with the corresponding α-saturated C₅₅ and C₈₅ compounds obtained by chemical hydrogenation.

The fact that short-chain polypropenols are better substrates than the long-chain polypropenols is difficult to explain. However, several possibilities can be considered. In our system, the transacylase is associated with the microsomal membrane. Therefore short-chain polypropenols might be more easily incorporated into a membrane and, consequently, might be more mobile in a lipid bilayer, thus facilitating the reaction. On the other hand, it is possible that the size of the polypropenol molecule influences properties of the mixed polypropenol-detergent micelles, the most likely form of the lipid dispersed in Triton X-100. In such a case, the differences in the reaction rate would reflect availability of the substrate rather than its higher intrinsic reactivity. In any case, for studies in vitro short-chain polypropenols are clearly superior to long-chain ones.

In the present work, no other endogenous microsomal acceptors except polypropenols were investigated. It was previously shown by Keenan & Kruczek (1976) that retinol cannot be a substrate for transacylase; these authors also assumed that cholesterol is acetylated by a separate system. The problem of transacylase specificity is complicated by a possible presence of a variety of acyl donors in the microsomes used as the enzyme source. Therefore, specificity of the transacylase can be unequivocally established only after solubilization of the enzyme, which is presently pursued in our laboratory. In contrast to the low specificity of transacylase with respect to the type of polypropenyl radical, the polypropenyl phosphate-dependent transglycosylases were found to be highly specific (Mańkowski et al., 1977; Pless & Palamarczyk, 1978).

We thank Dr. Tadeusz Mańkowski for help in tritiation of some of the polypropenols, and Dr. Grażyna Palamarczyk for stimulating discussions and critical reading of the manuscript.

REFERENCES


ESTRY ACYLOWE POLIPRENOLOI: SPECYFIKCyNOŚĆ TRANSACYLASY
MIKROSOMALNEJ W STOSUNKU DO POLIPRENOLOI RóżNIAČNYCH SIĘ DŁUGOŚCIĄ ŁAŃCUCHA I STOPNIEM NASYCENIA

Streszczenie

Badano reakcję przenoszenia kwasów tłuszczowych z fosfolipidów na poliprenol, katalizowaną przez mikrosomowy wątroby szczura. Specyficzność transacylasy w stosunku do poliprenolii określono, stosując znakowane trytem indywidualne poliprenole o znanej długości łańcucha i różnej ilości wiązań podwójnych. Opisano procedurę preparacji znakowanych trytem poliprenoli.
Stwierdzono, że poliprenole o krótkim łańcuchu są lepszymi substratami w reakcji katalizowanej przez transacylazę niż poliprenole długoląńcuchowe, oraz że poliprenole o nasyconej reszcie są lepszymi substratami niż całkowicie nasycone i wyżej nasycone poliprenole. α-Nasycone pojedyncze poliprenole o krótkim łańcuchu wykazały wielokrotnie wyższą aktywność jako akceptory reszty acylowej niż naturalny dolichol.

Received 6 October, 1978.