C_{55}-Dolichol: Occurrence in Pig Liver and Preparation by Hydrogenation of Plant Undecaprenol†

T. Mańkowski, W. Jankowski, T. Chojnacki,* and P. Franke

ABSTRACT: Apart from the long-chain dolichols (C_{80}-C_{110}), pig liver contains also a family of much shorter polyprenols with dominating C_{55}-polyprenol. This compound was identified as *cis/trans*-dihydroundecaprenol in which the OH-terminal isoprene residue was saturated. The number of internal *trans* isoprene residues in this compound was three in comparison with two such residues in long-chain C_{95}-dolichol. Chemical preparation of dihydroundecaprenol with a selectively saturated OH-terminal isoprene residue from fully unsaturated plant undecaprenol is presented.

The occurrence of partially hydrogenated long-chain cis/trans polyprenols was demonstrated in higher animals, yeast, and molds (Hemming, 1974). The long-chain "dolichols" ranging from C_{80} to C_{110} were found in mammalian tissues (Burgos et al., 1963; Dunphy et al., 1967), and C_{65-90}-dolichols in yeast (Dunphy et al., 1967). Similar dihydropolyprenols were found in marine invertebrates (Walton and Pennock, 1972). Hexahydropolyprenols were found in molds (Stone et al., 1967a; Barr and Hemming, 1972). On the other hand, in bacteria a fully unsaturated undecaprenol is rather typical (Gough et al., 1970). Similar undecaprenol is very abundant in several plants, in which it is usually accompanied by shorter and longer chain prenologues (Dunphy et al., 1967; Stone et al., 1967b; Sasak and Chojnacki, 1973).

Both types of polyprenols—the fully unsaturated and partially hydrogenated ones—were found to be obligatory intermediates in the form of prenyl phosphates in the biosynthesis of sugar polymers (Hemming, 1974). There were already indications that partial hydrogenation of a polyprenol resulted in a distinct change in the rate of formation of lipid-linked galactose in *Salmonella typhimurium* (Gajda et al., 1974).

The aim of the present paper is to describe the possibility of chemical conversion of fully unsaturated plant polyprenols into "dolichol-like" molecules in which the OH-terminal isoprene residue is hydrogenated. The product resembles thus the known dolichols isolated from pig liver and yeast, though it differs from the typical known dolichols in that it is composed of 11 isoprene residues only. It appeared on careful examination of dolichol from pig liver, which is believed to be a family of C_{80-110}-dolichols, that considerable proportions of much shorter polyprenols were also present in this preparation. One of them was isolated and characterized in this paper.

Materials and Methods

Hydroxy(C_{15})alkoxypropyl Sephadex (trade name: Lipidex-5000) was from Packard-Becker, B. V. Groningen, The Netherlands. Kieselgel G, Kieselgur G, and pre-coated TLC plates of silica gel 60 F-254 (250 μm) were from Merck, Darmstadt, Germany. PtO_2 was from Degussa Zweigniederlassung Hanau, Germany.

Analytical reversed phase partition thin-layer chromatography was done as described by Dunphy et al. (1967) using liquid-paraffin-saturated 98% aqueous acetone (solvent I) or 92% aqueous acetone (solvent II).

Analytical adsorption thin-layer chromatography was done on pre-coated silica gel plates using benzene–ethyl acetate (95:5, v/v; solvent III).

Preparative adsorption thin-layer chromatography was done on 1-mm layers of Kieselgel G with solvent III.

Spots of polyprenols were detected with anisaldehyde reagent (Dunphy et al., 1966) or with phosphomolybdic acid and iodine.

Column chromatography of polyprenols on Lipidex-5000 was performed with mixtures of acetone and water (Chojnacki et al., 1975).

Mass spectra were determined on an AEI MS 902 S instrument. NMR spectra were recorded in CCl_4 with a JEOL-JNM-MH-100 instrument by Mr. S. Maciaszek of Polytechnic School, Warsaw, Poland.

Fractionation of Dolichol Mixture from Pig Liver. Dolichol mixture (580 mg) was obtained from 5 kg of pig liver following the procedure of Burgos et al. (1963). The preparation of dolichol exhibited two groups of spots in reversed phase TLC (Figure 1, upper part, lane D): (A) corresponding to long-chain dolichols; and (B) polyprenols of shorter chain length. These two groups of compounds were separated by column chromatography on Lipidex-5000 (Figure 1, upper part). In this step the isolation of dolichol-19 (C_{95}) was achieved.

Dolichol mixture (500 mg) was dissolved in 8.5 ml of acetone to which 1 g of Lipidex-5000 was added, followed by the addition (dropwise with shaking) of 0.7 ml of water. The resulting slurry containing suspension of hydrophobic gel with most of dolichol material adsorbed to gel particles was applied to the column (1 × 80 cm) of Lipidex-5000 that was previously equilibrated with 93% aqueous acetone. The column was eluted with acetone–water mixture containing linearly increasing concentrations of acetone (600 ml of 93% acetone in the mixing vessel and 600 ml of 97% acetone in the reservoir). Five-milliliter fractions were collected at the flow rate 40 ml/h. The elution of polyprenols was followed by reversed phase TLC in solvent I of 10-μl samples of the eluate. The spots of polyprenols of shorter chain length (spots of group B; Figure 1, upper part) were detected in the early fractions of the eluate (it is noteworthy that partial separations of polyprenols of group B from

† From the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warszawa, Poland, and the Central Institute of Molecular Biology, Academy of Sciences of German Democratic Republic, 1136 Berlin, German Democratic Republic. *Received April 25, 1975.* This work was supported by the Polish Academy of Sciences within the project 09.3.1. and by the Agricultural Research Service, U.S. Department of Agriculture, and the Petroleum Research Fund, administered by the American Chemical Society.

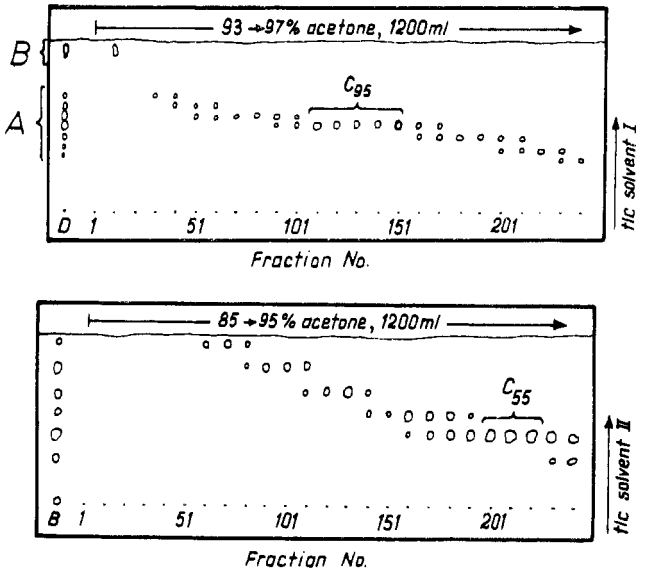


FIGURE 1: Fractionation of dolichols from pig liver on the column (1 × 80 cm) of Lipidex-5000. Tracings of reversed phase thin-layer chromatograms: (D) dolichol mixture; (A) C_{80-110}-dolichols; (B) polyprenols of shorter chain length; (1–241) fractions of effluents from the column eluted with indicated acetone–water system. Upper part of the figure shows the chromatographic run in which fraction of pure dolichol-19 (C_{95}) was obtained (tubes 111–151) and fraction of polyprenols B of shorter chain length (tubes 11–15). The latter was further fractionated on the same column as shown in the lower part of the figure, and one of the main components of the mixture was collected in tubes 201–221 (C_{55}). Spots stained with iodine.

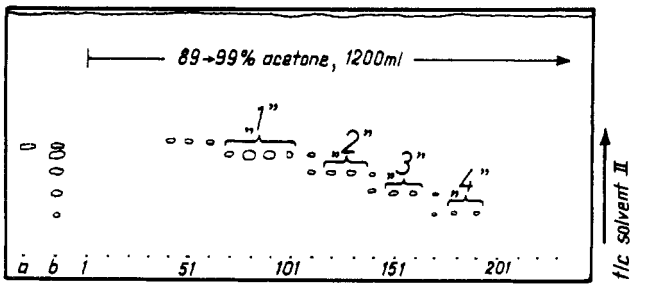


FIGURE 2: Fractionation of hydrogenation products of plant undecaprenol on the column (1 × 80 cm) of Lipidex-5000. Tracing of reversed phase thin-layer chromatogram: (a) plant undecaprenol; (b) undecaprenol after treating with hydrogen gas for 3 h; (1–241) fractions of effluent from the column eluted with indicated acetone–water system. Fractions containing individual components of the applied mixture (products "1", "2", "3", and "4") were pooled as indicated. Spots stained with phosphomolybdic acid.

the bulk of longer chain dolichols are also observed in the course of purification of dolichols on alumina on applying the procedure of Burgos et al. (1963); polyprenols B are eluted earlier than long-chain dolichols (A) from alumina with 10% ethyl ether in petroleum ether). The appropriate fractions of the eluate from the column of Lipidex-5000, containing polyprenols of group B (tubes 11–15), were pooled (80 mg of dry mass). Upon further elution of the gel, all dolichols of group A were recovered as incompletely separated fractions of seven individual dolichols. A portion of the eluate exhibiting single spot (solvent I) of the main prenologue (tubes 111–151) marked C_{95} (Figure 1, upper part) was analyzed by NMR and mass spectrometry (Figures 5D and 6D).

To achieve the resolution of the material eluted first from the column (polyprenols B), it was applied to the same column of Lipidex-5000 equilibrated with 85% aqueous acetone, and eluted with linearly increasing concentrations of acetone (600 ml of 85% acetone in the mixing vessel and 600 ml of 95% acetone in the reservoir). Five-milliliter fractions were collected; 20-μl samples of the eluate were applied to reversed phase TLC in solvent II. At least five components were observed in the eluate (Figure 1, lower part), though the separation was not complete. Part of the eluate in which the main component of the original mixture (Figure 1, lower part, lane B) was seen as a single spot in reversed phase TLC (tubes 201–221) was analyzed by NMR and mass spectrometry (Figures 5C and 6C).

Preparation of Undecaprenol from Plant Polyprenol Mixture. A mixture of C_{45-70}-polyprenols, similar to that isolated from *Ficus elastica* (Stone et al., 1967b), was prepared from leaves of *Trichosantes palmata* (cf. Sasak and Chojnacki, 1973) by Dr. J. Pyrek from the Institute of Organic Chemistry, Warsaw, Poland. Pure undecaprenol was isolated from this mixture by column chromatography on Lipidex-5000 as described previously (Chojnacki et al., 1975). It was identified by NMR, mass spectrometry, and thin-layer chromatography (Figures 3, 5A, and 6A).

Reaction of Undecaprenol with Hydrogen. Undecaprenol (150 mg) was dissolved in 20 ml of freshly distilled absolute ethanol, and 15 mg of PtO_2 and 5 mg of $NaBH_4$ were added. The suspension in a two-necked 25-ml Erlenmeyer flask was connected to the system filled with hydrogen gas under atmospheric pressure. The contents of the flask was mixed with a magnetic stirrer. After the appropriate time (usually 2–4 h for obtaining partially hydrogenated products, and 10–16 h for obtaining fully hydrogenated product) when about 1.5 mol of hydrogen was consumed per mol of undecaprenol, the contents of the flask was filtered, chloroform was added to 50 ml, and the solution was triturated with water. Aqueous phase was washed twice with chloroform, and chloroform extracts were evaporated to dryness. The residue was dissolved in 7.5 ml of acetone for preparative separation of partially hydrogenated products on the column of Lipidex-5000 similarly as described above for dolichols, except the gradient system was 89–99% aqueous acetone (Figure 2). Reversed phase TLC in solvent II has revealed the presence of several products of hydrogenation of undecaprenol (Figure 2, lane b), and their incomplete separation (Figure 2, 1–241). Fractions containing individual products (single spots in reversed phase TLC) were pooled as indicated in Figure 2 (products: "1", "2", "3", and "4"), evaporated to dryness, and studied by mass spectrometry. Product "1" was analysed by thin-layer chromatography, NMR, and mass spectrometry (Figures 3, 5B, and 6B).

Results

Selective Hydrogenation of the OH-Terminal Isoprene Residue in Plant Undecaprenol. Hydrogenation of pure undecaprenol from *Trichosantes palmata* for 3–4 h under conditions described in Materials and Methods leaves a little unchanged material, giving rise to the formation of a number of products. They exhibit the same R_f in adsorption TLC on silica gel in solvent III, slightly lower than original undecaprenol, but differ in thin-layer and column reversed phase chromatographic systems (Figures 2 and 3). The individual products of hydrogenation of undecaprenol were isolated by column chromatography on Lipidex-5000 and examined by mass spectrometry. Each of these products ("1", "2", "3", and "4"; see Figures 2 and 3) differs from the previous one by two hydrogen atoms and can be described as: "1", dihydroundecaprenol ($M^+ = 768$); "2", tetrahydroundecaprenol ($M^+ = 770$); "3", hexahydroundecaprenol ($M^+ = 772$); and "4", octahydroundecaprenol ($M^+ = 774$). Mass spectra of these products

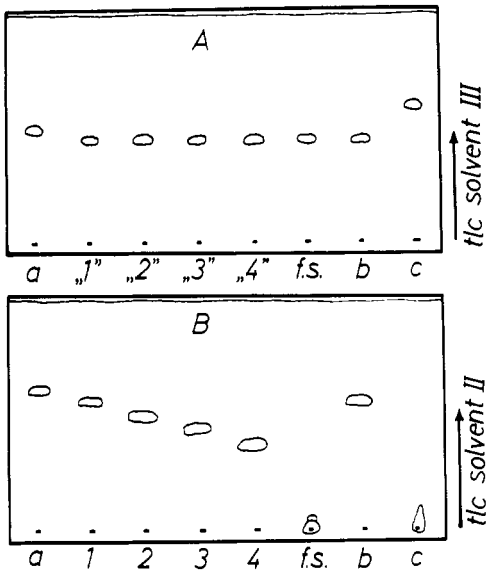


FIGURE 3: Tracings of thin-layer chromatograms of individual natural and hydrogenated polyprenols. (a) Plant undecaprenol; ("1", "2", "3", and "4") individual hydrogenated products (cf. Figure 2); (f.s.) fully saturated undecaprenol formed from undecaprenol after 12 h of hydrogenation; (b) dolichol-11 from pig liver; (c) dolichol-19 from pig liver. (A) Thin-layer chromatography on silica gel in solvent III; (B) reversed phase thin-layer chromatography in solvent II. Spots stained with anisaldehyde reagent.

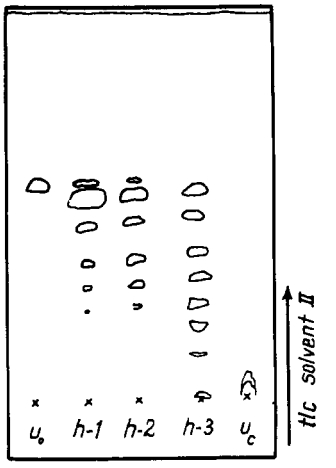


FIGURE 4: Tracing of reversed phase thin-layer chromatogram of hydrogenated products of plant undecaprenol. (U_o) Plant undecaprenol; (h-1, h-2, and h-3) reaction mixtures after 3, 4, and 5 h of hydrogenation, respectively (the measured uptake of hydrogen gas in h-1, h-2, and h-3 was 1.5, 3.0, and 5.0 mol per mol of undecaprenol, respectively); (U_c) fully saturated undecaprenol (12 h of hydrogenation). Spots stained with phosphomolybdic acid.

showed the pattern of fragmentation typical for polyprenols of dolichol type (saturated OH-terminal isoprene residue) with a strong signal of molecular ion and a weak one $(M - 18)^+$ corresponding to the loss of water from molecular ion (cf. Gough and Hemming, 1970). NMR spectra of the tetra- and hexahydroundecaprenol showed that both these compounds contained saturated OH-terminal isoprene unit, but there was no evidence concerning the selectivity of hydrogenation of other isoprene residues with respect to their cis/trans configuration.

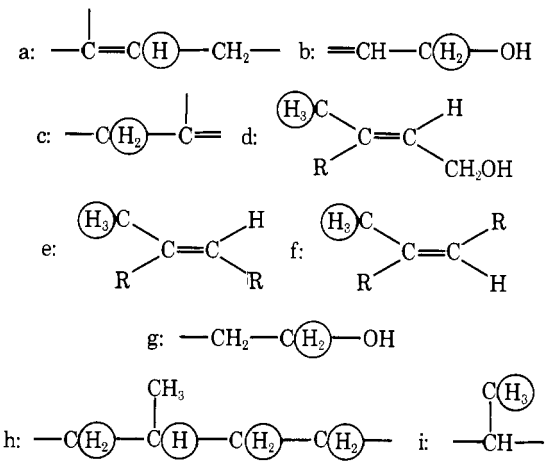
The mass spectrum of product "1" is presented in Figure 6B. In the described conditions of hydrogenation, product "1" was the main component of reaction mixture. With the increase of the volume of consumed hydrogen, the number and the amounts of more saturated products increased, while there was a relative decrease of the amount of product "1" (Figure 4). We could choose experimentally the volume of consumed hydrogen in order to obtain the highest yield of product "1". In our experiments we obtained up to 60% of product "1".

After 10–16 h of hydrogenation of undecaprenol no partially hydrogenated products were observed. In this case the product had no mobility in reversed phase TLC (Figures 3B and 4), though in adsorption TLC its R_f was the same as that of partially hydrogenated undecaprenols (Figure 3A).

The NMR spectrum of product "1" is shown in Figure 5B. As judged from the absence of signal from methyl group of OH-terminal isoprene residue at 1.72 ppm and the presence of triplet at 3.55 ppm, the substance was dihydroundecaprenol with saturated OH-terminal isoprene residue (Feeney and Hemming, 1967; cf. also the NMR spectra of untreated undecaprenol and of dolichol-19 in Figures 5A and 5D). The number of trans internal isoprene residues calculated from the NMR spectra according to Feeney and Hemming (1967) was close to three both in the starting plant undecaprenol (2.82) and in product "1" (2.74).

The Presence of Dolichol-11 in Pig Liver. As shown in Figure 1, apart from the C_{80-110}-dolichols, pig liver contains also a group of polyprenols of shorter chain length. The main

Chart I



polyprenol of the latter group was isolated. Its mass spectrum exhibited high peak of molecular ion ($M^+ = 768$), a lower one of the molecular ion less water, and the splitting pattern characteristic for dolichols (Figure 6C). In this respect its mass spectrum was similar to that of dolichol-19 (Figure 6D) estimated in our experiments and also similar to that published by Gough and Hemming (1970).

The NMR spectrum of the isolated shorter chain length polyprenol (Figure 5C) confirmed the results of mass spectrometry, showing characteristic signals, a triplet at 3.55 ppm and other signals common to long-chain polyprenols (Feeney and Hemming, 1967). The signal from protons of methyl group of OH-terminal isoprene residue at 1.72 ppm was absent. The NMR spectrum of this substance shows, however, a peak at 1.35 ppm which is not characteristic for polyprenols; it seems to be derived from the presence of impurities in the preparation (cf. also Stone et al., 1967a), as its relative intensity was decreasing upon purification by preparative TLC on Kieselgel G in solvent III. There was no evidence that this peak could represent an internal isoprene residue as shown by both mass and NMR spectra (cf. also Feeney and Hemming, 1967). Unfortunately the amount of this substance was insufficient for further purification to obtain more distinct NMR spectrum.

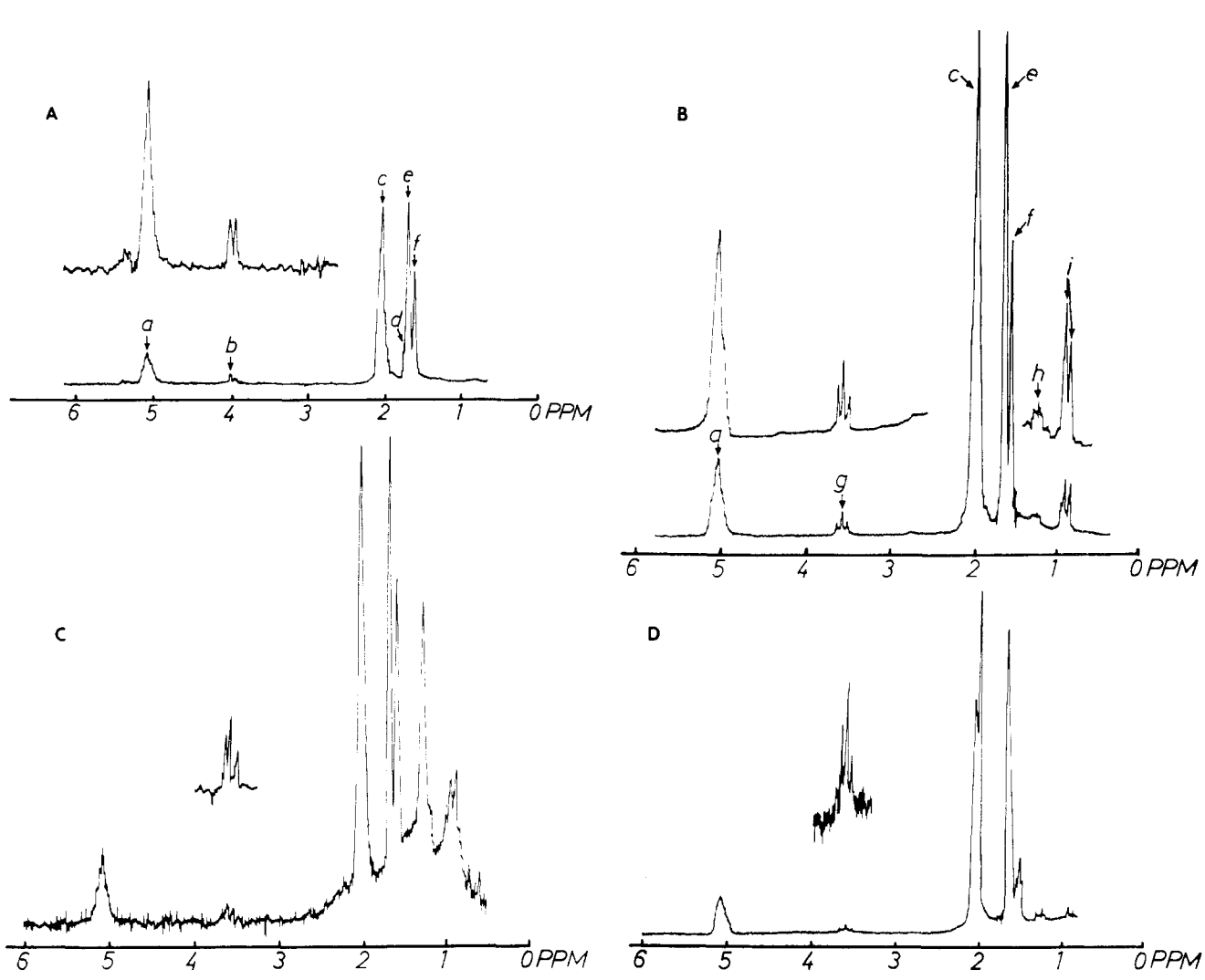


FIGURE 5: Tracings of NMR spectra at 100 MHz in CCl_4 of (A) plant undecaprenol; (B) dihydroundecaprenol formed by selective hydrogenation (product "1"); (C) dolichol-11 from pig liver; (D) dolichol-19 from pig liver. Assignment of characteristic signals is as shown in Chart I.

The comparison of mass and NMR spectra of this polyprenol (Figures 6C and 5C) with those of dihydroundecaprenol prepared by chemical hydrogenation (Figures 6B and 5B) and identical mobility of both compounds in thin-layer chromatography (Figure 3) suggest that they are identical. The name dolichol-11 can thus be ascribed to the isolated short-chain polyprenol from pig liver. The number of internal trans isoprene residues in dolichol-11 estimated from its NMR spectrum in CCl_4 according to Feeney and Hemming (1967) was close to 3 (2.76 and 3.2 in two separate analyses), in comparison with 2 internal trans residues found for dolichol-19 (1.78 and 2.33 in two separate analyses).

Dolichol-11 isolated from pig liver is one of the members of a group of compounds exhibiting similar R_f in thin-layer chromatography on silica gel in solvent III, and staining similarly with anisaldehyde reagent. These compounds can only partially be separated by column chromatography on Lipidex-5000. The total content of this material was ca. 16 mg per kg of tissue. Dolichol-11 accounted for ca. ¼ of this material. The content of C_{80-110}-dolichols in pig liver obtained in these studies was 100 mg per kg of tissue, i.e., the same as found by Burgos et al. (1963).

Discussion

It was shown in this paper that, during catalytic hydrogenation of undecaprenol in the presence of $NaBH_4$, the OH-terminal isoprene residue was more susceptible to hydrogenation than the others. The dihydroundecaprenol with saturated OH-terminal isoprene residue was dominating in the partially hydrogenated mixture. In the tetrahydroundecaprenol and in more saturated undecaprenols, one of the saturated residues was always the OH-terminal one as shown by NMR and mass spectrometry. With increase in the volume of consumed hydrogen, there was an increase in the number and the amount of more saturated products and a relative decrease in the amount of dihydroundecaprenol (product "1"). It seems, therefore, that hydrogen attacks first the OH-terminal isoprene residue, and, the tetra-, hexa-, and octahydroundecaprenols are not formed directly from fully unsaturated undecaprenol, but from product "1". The reason for adding $NaBH_4$ in our experiments on hydrogenation of undecaprenol with hydrogen gas was only empirical. Neither hydrogen gas nor $NaBH_4$ alone in the presence of PtO_2 gave any selective hydrogenation of OH-terminal isoprene residue. So far the nature of action of $NaBH_4$ is unknown.

The occurrence of dolichol-11 in pig liver extends the list of mammalian polyprenols. This polyprenol may be active in glycosylation reactions similar to the typical longer chain dolichols in the reactions so far described (Hemming, 1974). The preparations of dolichol commonly used for chemical phosphorylation and subsequently employed as dolichyl phosphates in studies on the formation of prenyl phosphate

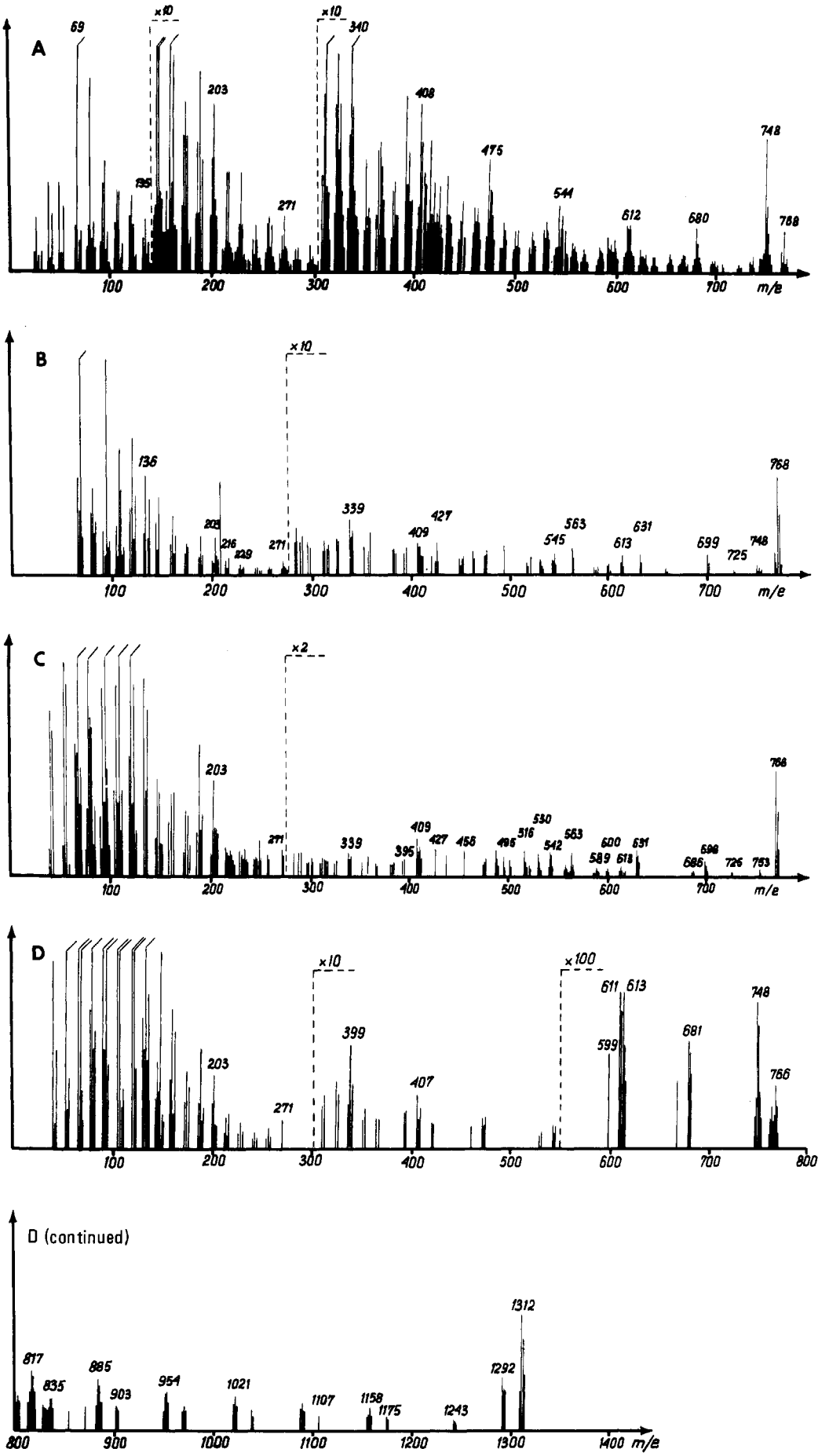


FIGURE 6: Histograms of more prominent peaks of mass spectra of (A) plant undecaprenol; (B) dihydroundecaprenol formed by selective hydrogenation (product "1"); (C) dolichol-11 from pig liver; (D) dolichol-19 from pig liver.

sugars may have also contained dolichol-11.

The question concerning the origin of dolichol-11 in pig liver arises in view of the fact that the proportion of three internal trans isoprene residues in this molecule is rather typical for plant undecaprenol (Feeney and Hemming, 1967). As revealed by reversed phase thin-layer chromatography, the polyprenols of pig liver form two different families (group A and group B, Figure 1). The more abundant are the longer chain dolichols ranging from C_{80} to C_{110} and possessing two internal trans isoprene residues (cf. also Feeney and Hemming, 1967). The components of group B, in which dolichol-11 is present, occur in lower amounts. One can assume that dolichol-11 may arise from plant polyprenols present in the diet, though direct evidence is lacking on the possibility of its adsorption from the intestine and specific enzymic reduction of its OH-terminal isoprene residue.

Using the procedure of selective hydrogenation of undecaprenol, we recently performed the synthesis of dihydrosolanesol with saturated OH-terminal isoprene residue from *all-trans*-(C_{45})solanesol. This shows that the method presented here for selective hydrogenation is convenient with respect to both cis/trans- and all-trans polyprenols and also is irrespective of the configuration of the OH-terminal isoprene unit.

Acknowledgments

We thank Professor Gustav Dallner of the University of Stockholm for a sample of Lipidex-5000 and Mrs. Josephine Hertel for her skilled technical assistance.

References

Barr, R. M., and Hemming, F. W. (1972), *Biochem. J. 126,* 1193-1202.

Burgos, J., Hemming, F. W., Pennock, J. F., and Morton, R. A. (1963), *Biochem. J. 88,* 470-482

Chojnacki, T., Jankowski, W., Mańkowski, T., and Sasak, W. (1975), *Anal. Biochem. 69,* 114-119.

Dunphy, P. J., Kerr, J. D., Whittle, K. J., and Pennock, J. F. (1966), *Chem. Ind. (London)* 1549-1550.

Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J., and Feeney, J. (1967), *Biochim. Biophys. Acta 136,* 136-147.

Feeney, J., and Hemming, F. W. (1967), *Anal. Biochem. 20,* 1-15.

Gajda, A., Jankowski, W., Mańkowski, T., and Chojnacki, T. (1974), Abstracts, 7th International Symposium on Carbohydrate Chemistry, Bratislava, Czechoslovakia, p 233.

Gough, D. P., and Hemming, F. W. (1970), *Biochem. J. 118,* 163-166.

Gough, D. P., Kirby, A. L., Richards, J. B., and Hemming, F. W. (1970), *Biochem. J. 118,* 167-170.

Hemming, F. W. (1974), *MTP Int. Rev. Sci. Biochem., Ser. One 4,* 39-98.

Sasak, W., and Chojnacki, T. (1973), *Acta Biochim. Pol. 20,* 343-350.

Stone, K. J., Butterworth, P. W. H., and Hemming, F. W. (1967a), *Biochem. J. 102,* 443-455.

Stone, K. J., Wellburn, A. R., Hemming, F. W., and Pennock, J. F. (1967b), *Biochem. J. 102,* 325-330.

Walton, M. J., and Pennock, J. F. (1972), *Biochem. J. 127,* 471-479.

Isolation of an Inhibitor of 25-Hydroxyvitamin D_3-1-Hydroxylase from Rat Serum†

Kathleen M. Botham, Jacob G. Ghazarian,‡ Barbara E. Kream, and Hector F. DeLuca*

ABSTRACT: An inhibitor of chick kidney mitochondrial 25-hydroxyvitamin D_3-1-hydroxylase has been isolated from rat serum by ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and preparative polyacrylamide disc gel electrophoresis. The purified protein was shown to contain iron and has a mol wt of 52 000. The protein is indistinguishable on gel electrophoresis from a similar inhibitor found in rat kidney tissue. The physiological significance of the inhibitor is not known; however, it seems possible that it is responsible for the failure to demonstrate in vitro 25-hydroxyvitamin D_3-1-hydroxylation with rat and other mammalian tissues.

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] the most active metabolite of vitamin D_3 known in both induction of intestinal calcium transport and bone mobilization (Holick et al., 1971a; Haussler et al., 1971; Omdahl et al., 1971), is synthesized from 25-hydroxyvitamin D_3 (25-OH-D_3) in vivo in the kidney of vitamin D-deficient chicks and rats (Holick et al., 1971a, b; Fraser and Kodicek, 1970; Gray et al., 1971).

Hydroxylation of 25-OH-D_3 in the 1 position has been demonstrated in vitro using kidney tubules, homogenates, and isolated mitochondria from vitamin D-deficient chicks (Shain, 1972; Fraser and Kodicek, 1970; Gray et al., 1972; Norman et al., 1971). However, although experiments with nephrectomized animals have shown that the kidney is the site of 1,25-$(OH)_2D_3$ formation in the rat in vivo (Gray et al., 1971), it has not yet been possible to demonstrate this reaction with rat kidney tubules, homogenates, or mitochondria in vitro.

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received July 28, 1975.* Supported by grants from the National Institutes of Health (AM-14881 and AM-15512).

‡ Present address: Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wis. 53233.

[1] Abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; EDTA, ethylenediaminetetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene; NADPH, reduced nicotinamide adenine dinucleotide phosphate.