Journal of Chromatography, 97 (1974) 293–296

CHROM. 7606

Note

Anomalous thin-layer chromatography of dolichol phosphate mannose in the presence of microsomal lipids

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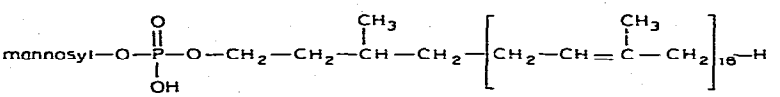
(Received April 8th, 1974)

When preparations of pig liver microsomes are incubated with guanosine-5'-diphosphate [^{14}C]mannose, of high specific activity, under appropriate conditions up to 70% of the radioactivity can be recovered as a lipid-soluble compound. It has been established that this compound is dolichol phosphate mannose (DPM, structure I)[1,2].



Structure I

It acts in several systems as an intermediate in the mannosylation of glycoproteins[1,3,4]. Although the level of incorporation of radioactivity is high the molar quantity of DPM formed is low and the product represents a quantitatively very minor component of the extractable lipid. Thin-layer chromatography of lipid extracts of microsomes has been an important aspect of the characterization of the radioactive product. In the course of many chromatographic studies of the product we have become aware of apparently anomalous R_F values and multiplicity of radioactive spots. We are reporting here the results of some of these studies partly because they have an intrinsic interest themselves, partly because they may help others avoid what can be a very confusing situation and partly because the discovery of other lipid-soluble products from guanosine-5'-diphosphate (GDP) mannose and which probably contain dolichol presents a greater need for caution in interpreting chromatographic data of compounds of this type.

EXPERIMENTAL

Preparation of DPM and of lipid

Pig liver microsomes were prepared and incubated with GDP [U-^{14}C]mannose (0.05 μCi, 0.35 nmoles) as described previously[1]. After 5 min the incubation was halted by the addition of 2 ml of chloroform–methanol (2:1) and 1 ml of water to 0.225 ml of the incubation mixture. After thorough mixing on a Vortex mixer the mixture was

separated by low-speed centrifugation into an upper aqueous layer and a lower chloroform layer with an interphase of insoluble protein. The chloroform layer was removed and the protein layer was extracted twice more in a similar way. The bulked chloroform extracts were washed twice with each time 4 ml of water. The washed chloroform extracts were then taken to dryness under nitrogen and were stored at −20°.

Thin-layer chromatography

Thin layers (0.25 mm) of Kieselgel G (Merck, Darmstadt, G.F.R.) were prepared on glass plates (20 × 20 cm). The adsorbent was activated by heating at 120° for 40 min. Lipid fractions were dissolved in chloroform–methanol (2:1) at a concentration such that application of 10 µl of solution as a single spot applied the appropriate amount of total lipid (see Table I). Chromatography proceeded until the solvent front had travelled approx. 15 cm. Solvent systems used were: (A) diisobutyl ketone–acetic acid–water (20:15:2); (B) chloroform–methanol–water (65:25:4); (C) *n*-propanol–water (7:3).

The position of ^{14}C on a chromatogram was determined by using a Panax RTLS-1A radioactivity scanner (Panax Equipment, Redhill, Surrey, Great Britain) and was confirmed by autoradiography.

Anisaldehyde–sulphuric acid spray reagent[5] was used to detect the presence of unlabelled lipids. Most lipids stained a pale pink-brown colour.

Mild alkali treatment

This treatment was the same as that used by Lahav *et al.*[6] except that ethyl formate rather than acetic acid was used to neutralise the alkali after treatment. The lipid was dissolved in 2 ml of chloroform–methanol (1:4) and to this was added 0.2 ml of 1 *M* NaOH. The mixture was incubated at 37° for 10 min and was then neutralised with ethyl formate (0.4 ml). Chloroform–methanol (9:1) (4 ml) and water (4 ml) were then added and the solution was mixed vigorously. Centrifugation separated the mixture into two layers and the lower layer was washed with 2 ml of water–methanol (2:1) before taking it to dryness under nitrogen.

TABLE I

TYPICAL R_F VALUES (±0.05) OF DOLICHOL PHOSPHATE MANNOSE IN THE PRESENCE OF TOTAL LIPID EXTRACT OF PIG LIVER MICROSOMES

*Quantity of total lipid chromatographed**	*System*		
	A	*B*	*C*
Low (up to 150 µg)	0.40	0.30	(0.07), 0.62 (trace)
Intermediate (150–450 µg)	0.12, 0.40	0.30–0.[illegible] (streak)	0.07, 0.62
High (450–600 µg)	0.12, 0.40	0.30–0.35 (streak) 0.55–0.60 (streak)	0.07, 0.62

* As a single spot.

RESULTS AND DISCUSSION

Typical R_F values of dolichol phosphate [^{14}C]mannose ([^{14}C]DPM) in the presence of crude lipid extracted from pig liver microsomes are shown in Table I. It can be seen that in systems A and C an increase in loading of the chromatogram readily resulted in the [^{14}C]DPM chromatographing as two distinct bands. The slower running of these two peaks which was absent at the lowest loading of lipid in system A and only present in trace amounts in system C, increased with loading and in many cases became the major radioactive area on the chromatogram.

System B was less susceptible to the increased loading phenomenon although excessive loading resulted in the appearance of a second band. Another characteristic feature of the loading effect in this system was that the increased loading caused the single band, and also the second band when it appeared, to streak markedly from the edges of the band. This gave an exaggerated horseshoe appearance to the darkened areas of the autoradiograms. Staining of the chromatograms showed no equivalent streaking of those lipids which were stained, and a regular distribution was observed. It was noticed, that [^{14}C]DPM, which was present in amounts too low to be detected by the stain (less than 50 ng/mg total lipid), was not associated with any of the stained lipid in the low-loaded chromatograms. Furthermore, as the loading increased and the chromatographic pattern of the DPM altered, the DPM became associated with stained unlabelled lipid, and there was some streaking of the latter, particularly in association with the second and faster running radioactive peak.

It is interesting that in system B the second band was of higher R_F than the normal position whereas in systems A and C the new band was of lower R_F. It appears that in the increased loading situation the [^{14}C]DPM tends to form a physical complex with other lipids in the mixture and that some of it travels as this complex during chromatography. In systems A and C the complex is more polar than [^{14}C]DPM itself but in system B the complex (not necessarily the same complex as in systems A and C) is less polar than [^{14}C]DPM itself. It may be relevant that both dolichol and [^{14}C]DPM are much less soluble in solvent systems A and C than in B. Possibly in systems A and C the non-polar dolichol side chain is innermost in the complex and the polar end of the molecule is exposed, whereas in system B the reverse arrangement occurs.

That the effect was due to a physical complexing and that no irreversible chemical change had occurred was shown by two-dimensional chromatography of intermediate quantities of lipid containing [^{14}C]DPM. Using system B for the first dimension, all of the radioactivity travelled as one compound (Table I) and was presumably not complexed to other lipids. On using systems A or C for the second dimension the [^{14}C]DPM chromatographed normally (see Table II).

Another method that was successful in removing the complexing lipid from the [^{14}C]DPM was mild treatment with alkali. The treatment is sufficient to deacylate most esters but is without effect on [^{14}C]DPM (ref. 1). Following this treatment the [^{14}C]DPM chromatographed normally (Table II).

It is common practice to use TLC of lipid extracts of microsomes previously incubated with GDP [^{14}C]mannose in order to establish the presence of [^{14}C]DPM by observation of ^{14}C at appropriate R_F values. It can be used further to distinguish between DPM and a second mannosylated lipid[7]. The R_F values of this second lipid are 0.05, 0.05 and 0.23 in systems A, B and C respectively[8]. Clearly to avoid confusion

TABLE II

TYPICAL R_F VALUES (± 0.05) OF DOLICHOL PHOSPHATE MANNOSE PRESENT IN INTERMEDIATE QUANTITIES (SEE TABLE I) OF THE TOTAL LIPID EXTRACT OF PIG LIVER ENDOPLASMIC RETICULUM AFTER TREATMENT OF THE LIPID EXTRACT

System	*After alkali treatment*	*After TLC (1st dimension) in system B*
A	0.40	0.40
B	0.30	0.30
C	(not determined)	0.62

it is essential either to use only low loadings of lipid or, if the specific radioactivity of the extract precludes this, to carry out a prior step of purification. Effective pretreatment as shown here, can be TLC in system B or mild alkali treatment. Pretreatment in the form of column chromatography on DEAE-cellulose acetate[6] appears also to be effective.

The incorporation of [^{14}C]mannose into lipid from GDP [^{14}C]mannose by mammalian microsomes is frequently sufficiently high (10–70%) to allow detection of ^{14}C on chromatograms with low loadings of lipid. The problem is more serious with other sugars such as glucose and N-acetyl glucosamine which in these systems are transferred from the appropriate nucleotide diphosphate sugar to lipid much less efficiently[3,9]. The corresponding glycosylated lipids appear to behave similarly to DPM in the TLC systems described.

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