The Phospholipid Composition of Embryonic Chick Liver Microsomes

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(Received 9 January 1967)

1. The phospholipid composition of hepatic microsomal fractions from different developmental stages of embryonic chick was established. The major components were phosphatidylcholine (approx. 66%), phosphatidylethanolamine plus phosphatidylserine (approx. 21%) and sphingomyelin (approx. 9%). 2. There were no significant changes in the phospholipid composition during embryonic development from 9 to 20 days. 3. When microsomal subfractions were prepared it was found that the smooth-microsomal fractions (Ia and Ib) had a significantly greater sphingomyelin content than the rough-microsomal fraction (II). This was compensated by a lower phosphatidylcholine content in fractions Ia and Ib and an increase of phosphatidylcholine in fraction II. 4. The significance of the differences in the phospholipid composition of smooth and rough microsomes is discussed with particular reference to the origin and interrelation of smooth and rough endoplasmic reticulum.

The most significant change in the chemical composition of the microsomal fraction of embryonic chick liver during morphogenesis is the increase in phospholipid (Pollak & Ward, 1967). Along with the increase in the phospholipid content between the embryonic ages of 6 and 16 days there is a marked increase in the extent of the membranous components of the endoplasmic reticulum (Karrer, 1960; Pollak & Ward, 1967). Since phospholipids make up the major lipid component of the lipoprotein complexes that constitute the membranous structures of the endoplasmic reticulum (Dallner, 1963), it was decided to investigate any changes occurring in the phospholipid composition of the microsomal fraction of embryonic chick liver during development.

The phospholipid composition of hepatic microsomes of newly born rats and of young adult rats has been investigated by Dallner, Siekevitz & Palade (1966). The results of that investigation showed that there were little or no significant changes between 2 hr. *post partum* and the young adult stage in the distribution of the constituent phospholipids of the microsomal fraction (Dallner *et al.* 1966).

The microsomal fraction contains several different membrane systems, and thus the overall phospholipid composition may not provide significant information about the phospholipid distribution

within individual membrane systems during development. Thus the fractionation of the microsomal fraction into three subfractions, consisting of membranes with different physicochemical properties (Dallner, 1963), provides a much more informative basis for the determination of changes in the phospholipid composition during development. The significance of this point cannot be overemphasized, particularly since the contribution of each of the membrane fractions varies at different developmental stages. Further, two enzymes, glucose 6-phosphatase and adenosine triphosphatase, have a differential distribution pattern between the smooth-microsomal and the rough-microsomal fractions (Pollak & Ward, 1967). In the present investigation the contributions from the individual phospholipids were determined within each of the three microsomal subfractions obtained from several developmental stages. It was found that the phospholipid composition of the smooth microsomes differed from that of the rough microsomes, but that each microsomal subfraction had a constant phospholipid composition in all the developmental stages investigated.

MATERIALS AND METHODS

The fertile eggs were obtained from the same source as in previous investigations (Pollak & Shorey, 1964; Pollak & Ward, 1967), and incubated under the same conditions, though they were derived from a different strain of white Leghorn hens, and in some instances cross-bred chick

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embryos were used, as in the later stages of a previous investigation (Pollak & Ward, 1967).

All reagents were of analytical grade. Chloroform, methanol and ethanol were purified and redistilled before use.

The microsomal fractions and the microsomal sub-fractions Ia, Ib and II were prepared as described by Pollak & Ward (1967). These fractions were extracted with chloroform-methanol (2:1, v/v), 2 ml./g. of tissue equivalent. This extract was kept at room temperature in the dark for 30–60 min. and was then centrifuged. The supernatant was transferred to a separating funnel, and the pellet was re-extracted as before. After centrifugation, the two extracts were combined and 0.2 vol. of water was added. This mixture was shaken and the chloroform layer collected; when necessary the mixture was centrifuged to ensure good separation of the two layers. The solvent was driven off with a stream of O₂-free N₂ in the dark and cold. The residue was then redissolved at room temperature in a small volume (70–200 μ l.) of chloroform-methanol (2:1, v/v), and a portion of the solution (20 μ l. or less) containing 5–50 μ g. of phosphorus was applied on to a thin-layer chromatogram. When necessary the phospholipid extracts were stored under N₂ at –20°.

The phospholipids were separated by thin-layer chromatography on silicic acid (Kieselgel G nach Stahl; E. Merck A.-G., Darmstadt, Germany; 500 μ thickness). The solvent used was chloroform-methanol-water (16:7:1, by vol.) (Wood, Imaichi, Knowles, Michaels & Kinsell, 1964). After development and drying, the chromatograms were sprayed with an aqueous solution of Rhodamine 6G (0.01%) and examined while still damp under ultraviolet light (either 340 m μ or 253 m μ). For the identification of choline-containing components the chromatograms were sprayed with Dragendorff reagent (Bregoff, Roberts & Delwiche, 1953), and ninhydrin was used to identify phosphatidylserine and phosphatidylethanolamine (Marinetti, Erbland & Stotz, 1958).

The phosphatides were also chromatographed in several other solvent systems so that their R_F values could be compared with those quoted in the literature. The three major phospholipid-containing spots were first isolated in the solvent system of Wood *et al.* (1964) and subsequently rechromatographed in one of the following solvent systems: 1, di-isobutyl ketone-acetic acid-water (40:20:3, by vol.); 2, chloroform-methanol-acetic acid-water (25:15:4:2, by vol.) (Skipski, Peterson & Barclay, 1964); 3, phenol-aq. 28–30% (w/w) ammonia (99:1, v/v) (Redman & Keenan, 1964).

Spots identified as phosphatidylcholine and sphingomyelin were also shown to contain choline after they had been hydrolysed and the hydrolysate rechromatographed. Complete alkaline hydrolysis was carried out according to the method of Axelrod, Reichenthal & Brodie (1953). The samples were then dried by rotary evaporation, redissolved in 0.1 ml. of water, and 10–20 μ l. portions of the solutions chromatographed together with a standard of choline chloride on Whatman 3MM paper by developing in butan-1-ol-acetic acid-water (4:1:1, by vol.) overnight.

The phospholipid-containing areas of the thin-layer chromatograms were removed and eluted by the method of Skipski *et al.* (1964) for total phosphate determination. The lipid extracts were evaporated and ashed, and total phosphate was determined either by the method of Allen

(1940) or by that of Chen, Toribara & Warner (1956). The recovery of phospholipid phosphorus after chromatography was 80–110%.

RESULTS

On chromatography of the lipid extracts, prepared either from the total microsomal fraction of embryonic chick liver or from any of the sub-fractions investigated, six components were detected when chloroform-methanol-water (16:7:1, by vol.) was used as a solvent. The spots were viewed under ultraviolet light after spraying with Rhodamine 6G. The R_F values varied somewhat from one experiment to another, but this did not affect the order of separation of these components. All but 2.4% of the lipid phosphorus was located in the three spots that were analysed to consist of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine plus phosphatidylserine (Table 1).

The identification of sphingomyelin was based on a comparison of R_F values of a Dragendorff-positive spot in three different solvent systems (Wood *et al.* 1964; Skipski *et al.* 1964; Marinetti *et al.* 1958).

The component identified as phosphatidylcholine possessed R_F values comparable with those quoted for this compound in the above-mentioned solvent systems. This spot was also Dragendorff-positive, indicating the presence of choline.

After alkaline hydrolysis, choline was obtained as a product. In each of the three solvent systems only one spot of the lipid extract gave a positive reaction with ninhydrin, and the R_F value of this spot was consistent with it being phosphatidylethanolamine or phosphatidylserine. We could effect only partial separation of phosphatidylserine and phosphatidylethanolamine with chloroform-methanol-water (16:7:1, by vol.) as solvent. After alkaline hydrolysis of this component, two ninhydrin-positive products were obtained, which were identified as ethanolamine and serine. Hence the single spot on the original chromatogram consisted of both phosphatidylethanolamine and phosphatidylserine, with the former in larger amounts, though the exact proportions between these two compounds were not determined.

One spot with blue fluorescence under ultraviolet light and with an R_F value closely corresponding to that of phosphatidylinositol was present in most of the chromatograms, but the phosphate values from this spot were so low that quantitative determination was not possible.

There did not appear to be any marked change in the phospholipid composition of the chick microsomes during the period of embryonic development studied (Table 1).

Table 1. *Percentage composition of the major phospholipids of the microsomal fraction of embryonic chick liver*

Details of methods are given in the Materials and Methods section. The percentage values of sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine plus phosphatidylserine (PE + PS) are given as means $\pm$ S.D., taking the total phospholipid content/g. of tissue as 100%. Total phospholipid content is expressed in terms of mg. of phospholipid/g. of tissue.

Embryo age (days)	No. of experiments	Total phospholipid (mg./g. of tissue)	Composition (%)			Recovery (%)
			SM	PC	PE + PS	
9	2	3.8 $\pm$ 0.3	7 $\pm$ 1	69 $\pm$ 5	17 $\pm$ 1	93
12	3	3.3 $\pm$ 0.1	8 $\pm$ 2	66 $\pm$ 8	26 $\pm$ 6	100
14	2	5.0 $\pm$ 0.6	11 $\pm$ 2	59 $\pm$ 4	23 $\pm$ 3	93
16	5	5.6 $\pm$ 0.5	11 $\pm$ 3	71 $\pm$ 3	19 $\pm$ 2	101
20	3	4.6 $\pm$ 0.4	8 $\pm$ 3	64 $\pm$ 3	20 $\pm$ 2	92

Table 2. *Percentage distribution of phospholipid within microsomal subfractions II, Ib and Ia of developing chick liver*

Details of experimental procedures are presented in the Materials and Methods section. The values shown were obtained by determining the phospholipid content of fractions II, Ib and Ia. The sum of the phospholipid content of these three fractions was taken as 100% and the phospholipid content of each fraction is expressed as a percentage of the total phospholipid content. The results represent mean values $\pm$ S.D.

Embryo age (days)	No. of experiments	Distribution of phospholipid in fractions (%)		
		Fraction II (rough microsomes)	Fraction Ib (Mg ²⁺ -binding smooth microsomes)	Fraction Ia (Mg ²⁺ -free smooth microsomes)
9	4	68 $\pm$ 5	22 $\pm$ 7	10 $\pm$ 3
12	3	68 $\pm$ 2	21 $\pm$ 6	11 $\pm$ 5
16	6	69 $\pm$ 6	0	31 $\pm$ 6
20	3	58 $\pm$ 2	0	42 $\pm$ 2

When the microsomes were subfractionated by the method of Dallner (1963), three membrane fractions were obtained, one rough-membrane fraction and two smooth-membrane fractions, one of which binds Mg²⁺. The phospholipid content of these three membrane fractions was determined with liver of 9-, 12-, 14-, 16- and 20-day chick embryos. During this time there is an increase in the membrane content within the embryonic chick liver cell (Pollak & Shorey, 1967). (It was not feasible to carry out these determinations on livers of chicks of earlier embryonic age because of the difficulty in obtaining sufficient tissue for a phospholipid fractionation). These results are presented in Table 2. The phospholipid values are expressed as a percentage of the sum of the three fractions. This in turn represents 80–100% of the total microsomal phospholipid phosphorus. The phospholipid content of the microsomal fraction more than doubles between the embryonic ages of 6 and 16 days (Pollak & Ward, 1967). On the other hand, the distribution of the phospholipids between the three microsomal subfractions stays relatively constant as development progresses, with the

exception of the Mg²⁺-free smooth-microsomal fraction at 16-day or older developmental stages. This is associated with the disappearance of the Mg²⁺-binding microsomal fraction at these late developmental stages (Table 2).

When the three microsomal subfractions were analysed with respect to their composition of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine plus phosphatidylserine, the results indicated that the phospholipid composition of a given membrane fraction did not alter as embryonic development proceeded (Table 3). There were, however, very definite differences between the phospholipid composition of the rough and smooth membranes, and only slight differences between the two types of smooth membranes (Table 3).

The phospholipids of fraction II (rough-microsomal fraction) were composed of 10% sphingomyelin, 70% phosphatidylcholine and 20% aminophospholipid. Fraction Ia differed from fraction II by having a higher sphingomyelin and aminophospholipid content and a lower phosphatidylcholine content (Table 3).

Table 3. *Percentage composition of the major phospholipid components within the hepatic microsomal subfractions of the developing chick embryo*

Details of experimental procedures are presented in the Materials and Methods section. The values shown represent the distribution of sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine plus phosphatidylserine (PE+PS) within the microsomal subfractions II, Ib and Ia. The only other phosphatide detected was phosphatidylinositol, which was present either in trace amounts or less than 3%. The mean values $\pm$ S.D. of the phosphatides are expressed as percentages of the total, with the numbers of determinations in parentheses.

Embryo age (days)	Fraction	Composition (%)		
		SM	PC	PE+PS
9	II	10 $\pm$ 3 (2)	67 $\pm$ 1 (2)	22 $\pm$ 1 (2)
	Ib	13 $\pm$ 1 (2)	58 $\pm$ 2 (2)	27 $\pm$ 1 (2)
	Ia	18 $\pm$ 5 (2)	54 $\pm$ 4 (2)	27 $\pm$ 1 (2)
12	II	10 $\pm$ 2 (7)	70 $\pm$ 3 (7)	20 $\pm$ 2 (7)
	Ib	18 $\pm$ 2 (3)	58 $\pm$ 3 (3)	24 $\pm$ 2 (3)
	Ia	19 $\pm$ 3 (4)	59 $\pm$ 7 (4)	22 $\pm$ 4 (4)
14	II	9 $\pm$ 1 (5)	71 $\pm$ 2 (5)	20 $\pm$ 2 (5)
	Ib	16 $\pm$ 2 (4)	62 $\pm$ 4 (4)	22 $\pm$ 4 (4)
	Ia	19 $\pm$ 1 (4)	57 $\pm$ 1 (4)	25 $\pm$ 1 (4)
16	II	10 $\pm$ 3 (4)	67 $\pm$ 5 (4)	20 $\pm$ 1 (4)
	Ib	0 (3)	0 (3)	0 (3)
	Ia	18 $\pm$ 2 (3)	59 $\pm$ 3 (3)	23 $\pm$ 2 (3)
20	II	8 $\pm$ 3 (3)	72 $\pm$ 4 (3)	20 $\pm$ 3 (3)
	Ib	0 (3)	0 (3)	0 (3)
	Ia	14 $\pm$ 3 (3)	63 $\pm$ 1 (3)	23 $\pm$ 3 (3)

Fraction Ib resembled fraction Ia in phospholipid composition, but for any given experiment the sphingomyelin content was slightly lower than that of fraction Ia and the phosphatidylcholine content was slightly higher (Table 3). These latter differences are not regarded as being highly significant, but are pointed out because of their consistency within each experiment.

The differences of the sphingomyelin content between smooth-microsomal fractions (Ia and Ib) and rough-microsomal fractions (II) were statistically highly significant for the 12-, 14-, 16- and 20-day chick embryos.

In the 9-day chick embryo the difference between fractions II and Ia may be regarded to have statistical significance, whereas the difference of the sphingomyelin content between fractions II and Ib was not statistically significant. The method used to measure the statistical significance of the differences in sphingomyelin content consisted of a comparison of the differences in concentration with the standard error of the differences.

DISCUSSION

The results reported here indicate that the hepatic microsomal fractions from embryonic chicks are similar in phospholipid composition to microsomes obtained from rat liver (Dallner *et al.* 1966; Getz, Bartley, Stirpe, Notton & Renshaw, 1962; Macfarlane, Gray & Wheeldon, 1960; Manganiello &

Phillips, 1965) in that they contain as the major phospholipid components phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. Moreover, the percentage composition of the major phospholipid classes in the chick microsomes agrees reasonably well with the results obtained by these workers for rat liver microsomes (Table 1).

In the results stated for the whole microsomal fraction, the values refer to what in itself is a heterogeneous membrane fraction. When this is fractionated by the method of Dallner (1963) into rough membranes and two smooth-membrane fractions, the smooth-membrane and rough-membrane fractions are found to differ in enzyme activity (Dallner, 1963; Pollak & Ward, 1967). What appears to be a constant composition with regard to the phospholipids in whole liver microsomes prepared from chicks differing in embryonic age from 9 to 20 days (Table 1) is really a reflection of the predominance of one type of membrane, which obscures the difference in the phospholipid composition between it and the other two membranes (Tables 1 and 3).

When the magnitude of the three fractions, as determined from their phospholipid content, is determined for the developmental period of 9 to 20 days, it is found that the rough-microsomal subfraction constitutes a fairly constant proportion of about 60% of the total microsomal phospholipid (Table 2). Since the phospholipid composition of

the rough-microsomal subfraction does not alter as a function of age (Table 3), its composition of 10% sphingomyelin, 70% lecithin and 20% aminophospholipid is to a large extent responsible for the values obtained for the whole microsomal fraction (Tables 1 and 3).

The absence of the Mg^{2+} -binding smooth-microsomal fraction (Ib) from the 16- and 20-day embryonic liver was always associated with a 10% increase of phospholipid in the non- Mg^{2+} -binding smooth-microsomal fraction (Ia) (Tables 2 and 3). The higher phospholipid content of fraction Ia at 16 and 20 days seems all the more significant because it forms such a constant proportion of the total microsomal phospholipid content at the other stages of development studied here (Table 2). Further, the fact that the sum of the phospholipid content of fractions Ia and Ib at 9 and 12 days approximates to the phospholipid content of fraction Ia at 16 and 20 days, when subfraction Ib cannot be isolated, suggests that between 14 and 16 days some change occurs that alters the nature of fraction Ib so that it cannot bind Mg^{2+} .

The smooth-microsomal fractions (Ia and Ib) have a sphingomyelin content of approx. 17% of the total phospholipid content, and in the rough-microsomal fraction (II) this amounts to about 10% (Table 3). There is an inverse relationship to this with respect to the phosphatidylcholine content, which amounts to approx. 60% in the smooth-microsomal fraction and 70% in the rough-microsomal fraction (Table 3). Preliminary experiments in our Laboratory have shown that in rat liver fractions Ia and Ib have also a higher sphingomyelin content than fraction II (J. K. Pollak & M. Woog, unpublished work). Skipski *et al.* (1965) showed an increased sphingomyelin content in liver cell membranes as compared with total liver; conversely the percentage phosphatidylcholine content in liver cell membranes was less than half that of total liver. Since the smooth-microsomal fraction (Ia) is derived at least in part from the plasma or cell membranes of the liver the results obtained by Skipski *et al.* (1965) corroborate the results obtained in the present investigation.

The differences of aminophospholipids between the smooth-microsomal and rough-microsomal fractions are also consistently present throughout the developmental period investigated, but these differences are only of a minor nature (Table 3).

Palade & Siekevitz (1956) have shown that the regions of smooth and rough endoplasmic reticulum may be continuous with one another. In the present context this would mean that the endoplasmic reticulum is made up of a mosaic of rough and smooth areas. On disruption of the cell the membranes of the endoplasmic reticulum as well as the membranes of some other organelles roll up into

vesicles, each of which is homogeneous with respect to its structural lipid and protein components. Differential or gradient centrifugation then sort out and separate the different kinds of membranous vesicles. Thus the evidence from this investigation does not support the concept that the rough membranes are the precursors of smooth membranes, as suggested by Dallner *et al.* (1966) and Manganiello & Phillips (1965). Instead it is envisaged that the microsomal fraction is made up of several types of morphologically, chemically and functionally different membranous vesicles, as pointed out by Sjöstrand (1964).

The authors are indebted to Miss Maria Woog for her excellent technical assistance. This work was supported by grants of the New South Wales State Cancer Council and the Australian Research Grants Committee. The authors are also indebted to the National Health and Medical Research Council and the University of Sydney Cancer Research Fund for a grant to purchase a Beckman Spinco L2 centrifuge.

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