The Application of Countercurrent Methods to the Fractionation of Lipid Material from Human Placenta

P. G. COLE, G. H. LATHE AND C. R. J. RUTHVEN

Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W. 6

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The method of countercurrent fractionation has been applied to the separation of a wide variety of substances. Among animal lipids, Lovern (1952) has studied ox-brain phospholipids, Lovern & Olley (1952) fractionated haddock-muscle lipids, while the lipids of human brain have been investigated by Cole, Lathe & Ruthven (1953). The latter study has now been extended to the lipid components of human placenta.

The composition of placental lipids has been estimated previously by Pratt, Kaucher, Richards, Williams & Macy (1946) using ordinary chemical procedures. This work involves the assumption that the types of lipid found in placenta do not differ widely from those of brain, for which most of the analytical methods have been perfected. In the work now reported it has been found that in using the countercurrent method, lipid fractions obtained from placenta differ from those of brain. In particular, it seems impossible to account for the nitrogen and phosphorus contents of the placenta lipids on the basis of the types of phospholipid predominating in brain.

METHODS

The methods of countercurrent distribution of lipid, together with analytical and paper-chromatographic procedures, have already been published (Cole *et al.* 1953), and the apparatus used was that of Lathe & Ruthven (1951). Unless otherwise stated, distributions were made through 49 transfers. Figures of the distributions are drawn with the tubes containing the polar material to the left.

Extraction. Placental lipids were extracted from whole, full-term, organs by the method of Murray, Lathe, Clark,

Ruthven & Levine (1950). The membranes and cord were detached, and the placentae treated within 2–10 hr. of delivery.

Solvent systems. The solvent systems are given in Table 1. Systems used previously (Cole *et al.* 1953) are referred to by the same letters.

Partition coefficient of neutral fat. About 55 mg. of neutral fat (prepared from beef suet) was equilibrated between 10 ml. of each of the phases of system A. The two layers were separated, and equal volumes taken to dryness *in vacuo*. The partition coefficient was 0.092.

EXPERIMENTAL

Distribution of crude placental lipid

Two schemes of fractionation were used. The first (Fig. 1) was similar to that used for brain lipid. After each distribution the solvent was evaporated from every tube, and the residue was dried and weighed. As this probably involved some damage to the lipid through oxidation or decomposition, scheme 2 (Fig. 2) was devised. Here two samples of lipid were distributed in a solvent system suitable for polar materials. One of the sets of fractions was taken to dryness and weighed, and on the basis of these weights the second set was divided into three groups of tubes, each of which was combined, and redistributed in an appropriate new solvent system. In this way repeated drying of individual fractions was avoided.

Scheme 1. Fig. 1a shows the countercurrent distribution, through 49 transfers, of 1.25 g. of crude placental lipid in solvent system A. Three main groups of fractions were evident: tubes 0–16, 17–38, and 39–49. These were of polar, intermediate, and non-polar character, and weighed 20, 63 and 17% of the whole, respectively. In order to obtain larger amounts of these materials for redistribution, 8.7 g. of crude lipid were distributed, in flasks, through eight transfers in system A, using 100 ml. of each phase. After

Table 1. *Solvent systems for countercurrent distributions*

(Figures in the body of the table are volumes of individual components required to prepare system; LP, light petroleum, b.p. 40–60°.)

System	...	...	A	B	C	D	E	F	G	H
Volume ratio of upper to lower phase			1/1	1/4	1/1	1/1	1/2	1/8	2/1	4/1
Components	...		CCl ₄ 62 CH ₃ OH 35 Water 3.15	As system A	LP 31 CCl ₄ 31 CH ₃ OH 35 Water 3	CCl ₄ 25 CHCl ₃ 15 CH ₂ Cl ₂ 10 CH ₃ OH 40 Water 10	As system D	As system D	As system A	As system A

Fig. 1. (Scheme 1.) Distributions of placental lipid. (a) Distribution of crude placental lipid in system A by weight (—), showing the position of cholesterol (....) and the iodine number of the non-cholesterol material (---). (Bases are shown as follows: ethanolamine (E), choline (C), serine (S).) (b) Distribution of half of the combined polar fractions of crude placental lipid (tubes 0-16 of a) in system D. (c) Distributions (c_1) (—) and (c_2) (---) in system C of tubes (0-4) and (5-9) respectively of a 9-transfer distribution in system A of the intermediate fractions of tubes 17-38 of a. (d) Distribution of half of the combined polar fractions of crude placental lipid (tubes 0-16 of a) in system B.

removing the solvent *in vacuo* and reconstituting each tube to 20 ml. of each phase, the distribution was continued in the countercurrent apparatus for an additional 23 transfers. The fractions were then divided into three groups, namely, tubes 0-10, 11-24 and 25-31, based on the divisions of the 49-tube distribution. As the fractions were combined a graph of the 31-transfer distribution is not shown. In Fig. 1a

the division of this distribution is referred directly to the 49-tube distribution, which is more suitable for comparisons.

Before distributing the polar group of fractions (tubes 0-16 of Fig. 1a) in new solvents they were distributed again in solvent A through 24 transfers. The result is not shown, as little further separation took place, 87.5% of the material

remaining in tubes 0-8. This was combined (the other 12.5% being added to the lipid of intermediate polarity in tubes 17-38 of Fig. 1a), and halved. One half was distributed in solvent system *D* (Fig. 1b) and the other in solvent system *B* (Fig. 1d). These two solvent systems represent alternative methods of moving the polar fractions toward the centre of the distribution. In each case the lipid was split into two groups (tubes 0-10 and 11-25). As they were not yet centrally placed later studies (Scheme 2) utilized the combined effect of changing the solvents and altering the ratio of phases.

The intermediate fractions (tubes 17-38 of Fig. 1a), after being combined, were given a preliminary distribution in the same system *A* for 9 transfers. Tubes 0-4 and 5-9 were then combined, and the solvent removed from each. The two materials were then distributed separately in system *C* (Fig. 1c₁ and 1c₂). This resulted in greater differentiation, both distributions showing the following groups of fractions: tubes 0-5, 6-26, 27-42 and 43-49. As would be expected from the origin of the materials, there was a preponderance of non-polar lipid in distribution c₂, and of the polar lipid in c₁.

The non-polar fractions (tubes 39-49 of Fig. 1a) from the distribution in system *A* were redistributed in system *H*. The graph, which is not included, showed that the components were not separated but remained as one group in tubes 30 to 45.

system (Fig. 1c), tubes 0-24 of this distribution were taken to dryness and weighed individually, while tubes 25-49 were combined. Most of the weighed material was found in tubes 10-24 with a peak at 16 (Fig. 2d). The combined, non-polar part was redistributed in system *G*, giving two groups of fractions in tubes 12-30 and 31-49 (Fig. 2e).

Characterization of countercurrent fractions from placental lipid

The nature of the various fractions produced by countercurrent distribution has been investigated by chemical analyses. In addition, the contents of each tube were hydrolysed with acid, and paper chromatography applied to the identification of bases and phosphates.

Cholesterol. The crude placental lipid contained approximately 16% of cholesterol, according to the Liebermann-Burchard reaction. Estimation of cholesterol in the individual tubes of a distribution in solvent *A* (Fig. 1a) showed that cholesterol occurred in tubes 24-49, with peaks at tubes 34 and 45, which contained 70 and 20% cholesterol, respectively. The partition coefficient of cholesterol in system *A* (Cole *et al.* 1953) was calculated to give a peak at tube 35. The less polar of the two peaks, at tube 45, may be attributed to cholesterol esterified with fatty acids.

Cholesterol was estimated in the two distributions in solvent *C* (Fig. 1c). The cholesterol curve, which is not

Scheme 2. About 3 g. of crude placental lipid were distributed in solvent system *B*, and taken to dryness. On the basis of the weight curve (Fig. 2a) division into the following groups seemed appropriate:

Character of lipid	Tube nos.	% of whole
Very polar	0- 9	6
Polar	10-23	12
Intermediate and non-polar	24-49	82

Accordingly, a larger scale distribution of 6 g. of crude lipid in solvent system *B*, using 100 ml. of solvent per tube, was run through 49 transfers, after which the tubes were combined according to the above table, and then each of the three solutions was reduced in volume for redistribution in other systems.

The redistribution of the very polar fractions (tubes 0-9 of Fig. 2a) in system *F* spread the bulk of the material widely (Fig. 2b). The slightly less polar lipid (tubes 10-23 of Fig. 2a) when distributed in system *E* was found mainly in tubes 18-32, with a peak at tube 25 (Fig. 2c). The remaining fraction of Fig. 2a (tubes 24-49) was distributed in system *C* (Fig. 2d). In view of the previous experience with this

shown, had a peak at tube 35, and traces were present in tubes 6-30. There was no peak at tube 45, nor was this to be expected since the non-polar fractions had been separated before these distributions were made.

In system *G*, scheme 2, the position of cholesterol is shown in Fig. 2e. Peaks were found at tubes 27 and 39, containing 70 and 24% of cholesterol, respectively. This was in agreement with the position of the cholesterol peaks in system *G* when calculated from the maxima found in system *A*. The shape of the less polar of these peaks indicated that more than one compound of cholesterol was present.

Neutral fat. The partition coefficient of a specimen of neutral fat (not of human placental origin) was found to be 0.09 in system *A*. From this it was calculated that neutral fat would form a peak at tube 45 in a distribution of 49 transfers. Such a peak, representing 17% of the original lipid, was present in Fig. 1a. However, it contained some cholesterol and P as well. In system *G* the theoretical peak of neutral fat would be at tube 42, which coincides with the non-polar peak of Fig. 2e.

Degree of unsaturation. Since oxidation could not be prevented completely, the values found for unsaturation may not be of great significance, but they should reveal the

Fig. 2. (Scheme 2.) Distributions of placental lipid by weight (—) showing cholesterol (....) and the iodine number of the non-cholesterol material (---), glutamic acid (G), ethanolamine (E), choline (C), serine (S), glycerophosphate (G.P.), orthophosphate (O.P.), phosphoryl choline (P.C.) and phosphoryl ethanolamine (P.E.). (a) Distribution of crude placental lipid in system B. (b) Distribution of the most polar fractions of crude placental lipid (tubes 0-9 of a) in system F. (c) Distribution of the polar fractions of crude placental lipid (tubes 10-23 of a) in system E. (d) Distribution of fractions of intermediate polarity and non-polar fractions of crude placental lipid (tubes 24-49 of a) in system C. (e) Distribution of the non-polar fractions of crude placental lipid (tubes 25-49 of d) in system G.

relative degree of unsaturation. In all calculations allowance was made for cholesterol, and the values given refer to the iodine number of the non-cholesterol material.

Inspection of the iodine numbers showed that the very polar material (Figs. 1*a*, 2*b*) was relatively unsaturated. Among the materials of intermediate polarity in Fig. 1*a* a steep gradient appeared between tubes 15 and 36. However, in the distributions of comparable materials in scheme 2 (Fig. 2*d*, *e*) this was absent.

Nitrogen and phosphorus analyses. The distribution of lipid in system *A* (Fig. 1*a*) was examined for P, while N analyses were made on a second similar distribution. For the purpose of comparing amounts of N and P with weights of non-cholesterol lipid it has previously been found convenient to chart P in terms of the amount of phosphatide to which it was equivalent, using the factor $P \times 100/3.85$. Similarly, a figure of $N \times 100/1.8$, based on monoaminophosphatides, has been used for charting N. Fig. 3 is a composite graph of these data, together with a curve of the mean of the non-cholesterol material in the two distributions.

Fig. 3. Nitrogen and phosphorus content of the non-cholesterol material (—) of crude placental lipid distributed in solvent *A* (compare Fig. 1*a*). Nitrogen (....) and phosphorus (---) are expressed as equivalent amounts of monoaminophosphatides ($N \times 100/1.8$ and $P \times 100/3.85$).

The most striking feature of the results was the disparity between the phosphatide curve based on the P content, and that calculated from N. The more polar components (tubes 0–15 of Fig. 3) had a N content comparable with monoaminophosphatides, while the P content was too low in this region. In the area of intermediate polarity (tubes 15–30 of Fig. 3) N declined and the P content became much greater than could be attributed to phosphatides of the usual type. Moreover, considerable amounts of P occurred in the non-polar regions, with peaks in tubes 35 and 46, which contained no N.

Identification of bases. The contents of the individual tubes from the distribution of placental lipid in system *A* (Fig. 1*a*), as well as the distributions from scheme 2, were hydrolysed with acid, desalted, and the bases were identified by paper chromatography using two solvent systems. The findings are shown in Figs. 1*a* and 2*b*–*e*.

The small amounts of material in the most polar tubes (0–4) of the distribution in system *A* (Fig. 1*a*) made chromatographic identification difficult. The only amino acid

found here was serine. However, in comparable fractions from scheme 2 (Fig. 2*b*) glutamic acid was found. Glutamic acid also appeared in the polar tubes of Fig. 2*c*, and also in the less polar materials (Fig. 2*e*).

The three phosphatide bases, choline, serine and ethanolamine, were observed widely spread throughout the polar tubes and those of intermediate polarity (Fig. 2*b*–*d*), giving the impression of many different fractions. The non-polar material (Fig. 2*e*) was free of bases except for a little ethanolamine about tube 20.

Chromatograms stained for phosphate. The distributions of scheme 2 were examined for P compounds by paper chromatography, after acid hydrolysis of the fractions. Inorganic P and glycerophosphate were found throughout the fractions. The amount of inorganic P decreased towards the non-polar end of all the distributions except that of the most non-polar material in system *G* (Fig. 2*e*). Here there was a relative increase of orthophosphate and glycerophosphate toward the non-polar end of the distribution, but very little was present in the region of the free cholesterol peak (tubes 20–30). Phosphatidylcholine or phosphatidylethanolamine (which were not distinguished easily in the systems used) also appeared in tubes 3–17 and 26–43 of the distribution in system *F* (Fig. 2*b*), in tube 29 of system *E* (Fig. 2*c*) and tubes 0–24 of system *C* (Fig. 2*d*).

DISCUSSION

Human placenta is an organ of rather diverse structures, which includes many blood vessels and much loose connective tissue, in addition to the trophoblast. It is not surprising then that placental lipids appear to be at least as complex as those of brain (Cole *et al.* 1953).

The lipid was prepared from placentae contaminated with maternal and foetal blood and thus contained a certain amount of fat-soluble material from these sources. However, a comparison of the lipid composition of washed placenta, as determined by Pratt *et al.* (1946), with the lipid composition of blood, as estimated by Boyd (1933), shows that the main effect of contamination with blood is to increase the placental content of free and ester cholesterol.

In scheme 2 manipulation, particularly warming and drying, was reduced to a minimum in order to avoid oxidation and decomposition of the lipid. Apart from the known lability of lipid materials, the need for these precautions was suggested by a number of observations, including the presence of a large polar peak (approximately 35% of the distributions) in Fig. 1*c* of scheme 1. This was difficult to explain other than by decomposition. The absence of this peak in the corresponding distribution (Fig. 2*d*) of scheme 2 strengthens this impression. Apart from this difference between the two schemes, the general pattern of the lipid distributions is very similar.

The composition of the lipids of placenta and of brain can be compared, since the initial distributions were made under the same conditions (Fig. 1*a*, this

paper, and Fig. 4*a*, Cole *et al.* 1953). A similarity in composition is shown by peaks in both distributions at about tube 10, and in the central region. However, there were three main differences. First, placenta contained a large fraction (equivalent to 17% of the crude lipid) in the non-polar region (tubes 39–49, Fig. 1*a*) in the position of neutral fat. While neutral fat is almost absent from brain, human placental lipid contains 30%, according to Pratt *et al.* (1946). The fact that the non-polar peak of Fig. 1*a* also contained cholesterol and phosphorus accentuated the quantitative difference between our findings and those of Pratt. A possible explanation of this discrepancy may lie in the fact that Pratt estimated neutral fats from the amount of acetone-soluble glycerol, since recently Lovern & Olley (1952) demonstrated that in at least one tissue, haddock muscle, an important proportion of the phosphatide is acetone-soluble.

A second difference between placenta and brain was found in the centre of the distributions (tubes 15–35). Here the greater prominence of the placental curve on the polar side may be attributed to its greater content of lecithin, while the smaller amount of material in the region of tube 29 of the placental curve may be due to the larger amount of cerebroside in brain. On the non-polar side of the central peak cholesterol was present in equal amounts (16%) in brain and placenta. The fact that this was higher than reported for placenta by Pratt *et al.* (1946), was probably due to contamination with blood in our preparations. As regards sphingomyelin, which in brain is thought to have a peak at tube 21, we have no data for placenta.

Thirdly, in placenta there was no peak corresponding to the prominent polar fractions found in the first five tubes of the brain distribution, and characterized by the presence of much reducing material and many amino acids.

The distribution of bases in placenta and brain can be compared (Fig. 1*a*, this paper, and Fig. 5, Cole *et al.* 1953). In addition to the absence in the placental distribution of the polar, amino-acid-containing fraction (tubes 0–4), it was noted that while choline was present only in the central fractions of brain, in placenta it occurred not only there but also in the more polar tubes (5–11). This latter position probably corresponds with the main concentration of choline which Lovern (1952) found in distributions of ox-brain phosphatide. Serine was detected in placenta over a much smaller area of the distribution in system *A* than it was in brain, although the more detailed study of bases made in scheme 2 showed that here it was much more widely distributed. The distributions of bases, scheme 2, indicated the presence of many compounds, and suggested that the placental lipids were very complex. There were three or four areas of

high ethanolamine concentration, three of choline and two or three of serine. Glutamic acid occurred in all the distributions except that of the central fractions (Fig. 2). This amino acid was noted in the most polar brain fractions by Cole *et al.* (1953), and it has also been found in kephalin prepared from brain (Hecht & Mink, 1952). Wynn & Williams (1950), who found glutamic acid, together with other amino acids, in light petroleum extracts of plasma, considered that it occurred as part of a peptide combined with plasma lipid. The widespread occurrence of glutamic acid in the placental lipid distributions reported here suggests that there is some general relationship between this amino acid and the placental lipids, rather than the existence of a definite glutamic acid-containing lipid.

Some rather unexpected features were presented by the nitrogen and phosphorus analyses of placenta. In brain, these two elements were present only in the polar and intermediate tubes, and at concentrations which were in reasonable agreement with the predominance of phosphatides. In placenta, however, as shown in Fig. 3, the concentration of phosphorus was too low in the polar material, and much too high in the intermediate and non-polar lipid, to be explained on this basis. The nitrogen content, on the other hand, was comparable with that of phosphatides in the more polar half of the distribution, but was much lower in the non-polar region. It was also noticed that in the areas where the phosphorus was high, a qualitative Feulgen reaction for aldehyde appeared to be particularly strong.

The identity of these high-phosphorus compounds has not been established. Acetal phosphatides (Thannhauser, Boncoddio & Schmidt, 1951) may be present, as the reaction for aldehyde suggests, but since the areas with high phosphorus content are low in nitrogen, plasmalogens can account for only a small proportion of the phosphorus. On the other hand, compounds like cardiolipin (Pangborn, 1947), phosphatidylinositol (Folch, 1942), and phosphatidic acids (Chibnall & Channon, 1929), although devoid of nitrogen, have approximately the same phosphorus content as the monoaminophosphatides. Diphosphoinositide (Folch, 1949), contains more phosphorus (7.3%) and negligible nitrogen. However, from its properties, a much more polar position in the distribution would be expected. It seems more probable that phosphate-containing lipids of a new type occur in the placenta, although the possibility that they have been produced by hydrolysis of phospholipids between delivery and extraction of the lipids, might be considered.

Recent studies have tended to emphasize the dynamic role played by the placenta in the nutrition of the foetus. This has been demonstrated in relation to both fat (Popják & Beeckmans, 1950) and carbo-

hydrate metabolism (Huggett, Warren & Winterton, 1949) as well as by the high overall rate of oxygen consumption of the tissue (Wang & Hellman, 1943). The pattern of placental lipids, and in particular the small proportion of neutral fats, and the abundance of compounds which are high in phosphate, is in agreement with the conception of the placenta as an actively metabolizing tissue. Together with the complexity of the phospholipids, as indicated by the distribution of bases, these are the most striking features of the lipid components of placenta.

SUMMARY

1. The lipids of mature human placentae have been fractionated by countercurrent distribution in

a solvent system of carbon tetrachloride, methanol and water, which was further modified for use with polar and non-polar lipids.

2. The placental lipids have been compared with those of brain, distributed under the same conditions.

3. Ethanolamine, choline and serine each occur in several fractions of placental lipid.

4. Glutamic acid is distributed so widely as to indicate some general relation to the placental lipids.

5. The nitrogen and phosphorus contents are not consistent with the predominance of the usual phospholipids, but indicate that compounds of high phosphorus and low nitrogen content are present.

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

1. THE ISOLATION AND PROPERTIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE

By DWIGHT B. MCNAIR SCOTT AND S. S. COHEN

The Children's Hospital of Philadelphia (Department of Pediatrics) and the Department of Physiological Chemistry of the University of Pennsylvania School of Medicine, Philadelphia

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It has been shown that some bacterial viruses change the metabolism of the host cell in such a way that virus deoxyribosenucleic acid is synthesized instead of host deoxyribosenucleic acid and ribosenucleic acid. The hypothesis has been advanced that the routes of carbohydrate metabolism leading to the production of pentose and deoxypentose were different and that the relative amounts of glucose used by the two pathways were changed during elaboration of virus by the host (Cohen, 1947, 1951a). The glycolytic, or Embden-Meyerhof,

breakdown of one molecule of glucose 6-phosphate gives rise to two molecules of triosephosphate. It has been shown by Racker (1951) that condensation of glyceraldehyde phosphate with acetaldehyde by enzymes from *Escherichia coli* and other cells yields deoxyribose 5-phosphate which may be used in the synthesis of deoxyribosenucleic acid.

By the oxidative, or Warburg-Lipmann-Dickens pathway (Warburg, Christian & Griese, 1935; Lipmann, 1936; Dickens, 1938), oxidation of glucose 6-phosphate yields 6-phosphogluconate. It