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THE PHOSPHOLIPIDS OF LIVER-CELL FRACTIONS

I. THE PHOSPHOLIPID COMPOSITION OF THE LIVER-CELL NUCLEUS

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SUMMARY

1. Nuclei have been isolated from rat liver by the citric acid method of MIRSKY AND POLLISTER and their purity investigated by electron microscopy.

2. Nuclear phospholipid, cholesterol, DNA and protein have been determined; the composition and appearance of the nuclei have been compared with those of nuclei prepared in sucrose by the method of CHAUVEAU.

3. The phospholipid composition of the nuclei has been investigated by hydrolysis and two-dimensional paper chromatography and compared with the phospholipid composition of whole liver.

4. The amount of lipid in the nucleus is small, but the pattern of lipids is similar to that in the whole cell with the exception that cardiolipin is absent from the nucleus.

5. Evidence is presented that citric acid removes the outer membrane from the nucleus whilst in sucrose the double-membrane structure remains intact. By means of Langmuir trough experiments, the amount of lipid found in the nuclear fraction has been related to the amount required to cover the surface of a single nucleus. This has been found sufficient to constitute two lipid monolayers in the case of citric acid-isolated nuclei.

INTRODUCTION

In comparison with mitochondria and other cell particles, little work has been reported on the lipid composition of isolated cell nuclei[1-15]. The available results are not easy to compare and also include a wide range of values for the lipid content of the cell nucleus. One of the most poignant reasons for this is the difficulty of isolating nuclei completely free from other cell particles rich in phospholipids. It will be shown that the isolated cell nucleus contains only a very small fraction of the cell lipid and hence even slight contamination with other particulate fractions will give a false picture of nuclear phospholipids.

Many methods have been reported for the isolation of cell nuclei[1, 15-22] and there are some excellent reviews on the subject[2, 23-25]. Methods employing organic solvents[20] to separate particles according to their densities, whilst having the advantage that little adsorption of other material on the nucleus occurs, are obviously of little use

in quantitative lipid studies owing to the extraction of lipid by the solvent. The main disadvantages of aqueous media are (a) extraction of protein and high-molecular-weight material from the nucleus, (b) adsorption on the nuclei of cytoplasmic constituents. In the present lipid studies, objection (a) is of little consequence. Several aqueous media have been reported which reduce objection (b) to a minimum: I, 1% citric acid[1,23]; II, sucrose–citrate (pH 6.0–6.2); III, 2.2 M sucrose[15].

Of these, citric acid was chosen here for the following reasons: (1) Several other workers have found it suitable for lipid work[6,10,11,14]. (2) It gives nuclei of high purity; it will be shown in this paper, by chemical and microscapic methods, that there is little contamination. (3) The low pH creates "granular" nuclei and protects them from damage and breakage during homogenisation. Working at low pH, however, has the disadvantage that enzyme methods cannot be used as criteria of purity. (4) The method gives a fairly high yield and is simple to carry out on a large scale. This is necessary in order to obtain enough lipid for analytical purposes. CHAUVEAU's method, whilst giving nuclei free from contamination (see MATERIALS AND METHODS), has the disadvantages that the conditions for isolating uncontaminated nuclei are more rigorous, (fine density and temperature control) and the method is not so easily applied to large-scale preparations.

The question of the structural arrangement of the lipid molecules within the nucleus has been investigated by a number of workers[27,28]. Studies of both osmium tetroxide, and potassium permanganate-fixed tissue have indicated that the nuclear membrane features two layers, each of which can be considered to be a unit membrane. Therefore covering most of the nuclear surface in the intact tissue there are two bimolecular leaflets of lipid with associated non-lipid components[26–29]. One other point of interest from the point of view of the lipid components of nuclear structures is that the nuclear membrane is at many points continuous with the endoplasmic reticulum[30]. Some evidence has been obtained in the present study, both by electron microscopy and chemical methods, that in the citric acid method of isolation, the outer membrane may be stripped off, whilst in other methods it may be left intact. It seems likely that the bulk of lipid is sited in the membrane, but evidence for its presence in chromosomes has been published by CHAYEN *et al.*[31]. The results obtained in the present study, while suggesting that lipid is confined to the membrane, cannot entirely rule out the possibility that some may be located in the chromosomes.

MATERIALS AND METHODS

Isolation of the nuclei

Isolation in citric acid: Nuclei were isolated routinely by the method of MIRSKY AND POLLISTER[1,23], using 1% citric acid. The details of the method were as given by ALLFREY[23] except for the following additions or modifications.

The nuclei were isolated from the livers of rats fed on the normal laboratory diet, up to 30 animals being used for each experiment. The non-perfused tissue was dried off on absorbent paper, sliced with scissors, and then homogenised in a Potter–Elvejhem type of perspex homogeniser, not in a Waring-blender as described by ALLFREY. The difference in diameter between the plunger and the vessel was 0.038 cm, the diameter of the plunger, 3.7 cm and the speed of rotation 470 rev./min.

100 ml of 1% citric acid was used for every 10 g of liver and about 6–10 strokes

of the plunger were required for complete homogenisation. The centrifuge was the MSE "13000" refrigerated centrifuge (Measuring and Scientific Equipment (London)) and the corresponding speeds were: centrifugation before first filtration, 4000 rev./min (2000 × *g*) for 10 min; centrifugation before second filtration, 4000 rev./min (2000 × *g*) for 5 min; final centrifugation 1200 rev./min (300 × *g*) for 5 min. The nuclei were normally washed 6 times by successive homogenisation with 0.2% citric acid and centrifugation at 1200 rev./min. Recoveries ranged from 30 to 50% as judged by DNA recovery.

Isolation in 2.2 M sucrose: As a comparison, nuclei were also prepared by the method of CHAUVEAU, as modified by O'BRIEN[32]. The medium consisted of 2.2 M sucrose–0.002 M $MgCl_2$–0.05 M sodium glycerophosphate. The specific gravity, which is critical, was 1.273. The animals were fasted for 20 h previous to death, in order to deplete the cells of glycogen; this has the effect of lowering the specific gravity of the cytoplasm, and makes the method more sensitive. The fresh livers from 3 rats were weighted, freed from all pieces of hair etc. and then dried thoroughly on pieces of tissue paper. The sliced tissue was then homogenised in 20 volumes of the above medium in a perspex Potter–Elvejhem homogeniser (details above); 3 strokes of the plunger were required for complete homogenisation. The homogenate was then centrifuged at 40000 × *g* (18250 rev./min) in the MSE "40000" ultracentrifuge. The nuclei were obtained as a transparent pellet at the bottom of the tube, and were not subjected to any washing procedure. All operations were carried out at 4°.

Chemical composition of the nuclei

Chemical analyses were carried out on 9 samples of nuclei isolated in citric acid. All investigations of individual phospholipids by paper chromatography were on nuclei isolated in this medium. Only 3 analyses for DNA, whole lipid and protein were carried out on sucrose-isolated nuclei for purposes of comparison.

Several methods were employed. In cases where nuclei were analysed for DNA, lipid and protein, the scheme due to SCHNEIDER[33] was used. The nuclear sediment from 30–40 g of tissue was homogenised in 5 ml 10% trichloroacetic acid to precipitate protein, nucleic acid, and lipid material, and then washed 4 times with 5 ml of 10% trichloroacetic acid to remove acid-soluble phosphorus compounds. The sediment was washed several times with distilled water to bring the pH up to 6–7.

Extraction of lipids. Lipids were obtained by extracting 4 times with 10 ml 95% ethanol and then making a further two extractions with chloroform – methanol (2:1, v/v). It was found that the same amount of lipid was extracted by the SCHMIDT–THANNHAUSER[34] procedure (successive extractions with acetone, ethanol, chloroform, ethanol – ether (3:1, v/v), and ether), and also by simply extracting with chloroform – methanol (2:1, v/v) alone.

The lipid extracts were taken to dryness *in vacuo* (bath temperature 40°) and the residue of lipid and protein dried by successive evaporations with acetone. Chloroform was added and after extracting for some hours the residual protein was filtered off. The amount of phospholipid was calculated from phosphate analyses by the method of KING[35].

In some cases the whole tissue and the nuclear sediment were extracted for lipids by homogenising directly in chloroform – methanol (2:1, v/v). The extract was

washed with 0.2 volume of 0.9 % sodium chloride, the solvent layer taken to dryness *in vacuo* and the residue extracted with chloroform.

DNA analysis: The trichloroacetic acid-precipitated residue, after lipid extraction by the SCHNEIDER technique, was washed with 5 ml cold 5% trichloroacetic acid and extracted with 5 ml 5 % trichloroacetic acid at 90° for 15 min. DNA was estimated in the filtered extract by the diphenylamine reaction[33].

Other analyses: Protein was estimated by the biuret reaction[36] and total cholesterol by the Liebermann–Burchard reaction[37].

Analysis of phospholipids

Individual lipids were estimated as water-soluble esters by the chromatographic method of DAWSON[38], but it has been necessary to modify the procedure slightly when dealing with samples of nuclear lipid. When ethanol is added in the treatment of the lipid prior to hydrolysis the cephalins precipitate out from the nuclear lipid, whereas in extracts from whole tissue the solution remains perfectly clear. Consequently, less ethanol (3.3 ml) was used and the amount of carbon tetrachloride increased until the precipitated cephalin redissolved (5 ml).

The chromatographic solvents used here to separate the hydrolysis products of alkali-labile lipids have been: I, phenol, saturated with water – acetic acid – ethanol (100:10:12, v/v); II, methanol – formic acid – water (80:15:5, v/v). The papers were developed by spraying with ninhydrin (0.2 % in ethanol) to locate serine- and ethanolamine-containing compounds, followed by the phosphate spray of HANES AND ISHERWOOD[39]. After drying thoroughly the papers were developed in ultraviolet light. The phosphate content of each spot was determined by cutting out the spot and digesting the paper with perchloric acid as described by DAWSON.

Only the alkali-labile fraction has been studied in detail.

All the analyses described above were carried out on the nuclear sediment and an aliquot of the original liver homogenate.

Establishment of the purity of the nuclear fraction

The nuclear sediment was examined by three methods to investigate possible contamination and in each case compared with nuclei isolated in 2.2 M sucrose.

Light microscopy: Nuclei were stained with crystal violet.

Electron microscopy: Small samples of the nuclear sediment and the original homogenate were fixed in osmium tetroxide and embedded in araldite.

Enzymic methods: Succinate dehydrogenase (succinate:O_2 oxidoreductase EC 1.3.99.1), thiosulphate sulphurtransferase (EC 2.8.1.1 formerly known as rhodanese) and acid phosphatase (EC 3.1.3.2) activities were assayed as a measure of contamination by mitochondria and glucose 6-phosphatase (EC 3.1.3.9) as guide to microsomal contamination.

Surface properties of the nuclear lipid extract

The area occupied by a lipid film was investigated using a Langmuir trough. Weighed amounts of two samples of nuclear lipid were dissolved in petroleum ether and applied to the water surface by means of a micrometer syringe. Force–area curves were constructed and the limiting area for close-packing of the molecules determined.

TABLE I

THE CHEMICAL COMPOSITION OF THE RAT-LIVER NUCLEUS

Method of isolation	Whole liver		Nucleus									
	Lipid-phosphorus*	Cholesterol (mg/mg lipid-phosphorus)	Lipid-phosphorus*	DNA-phosphorus*	Protein*	Cholesterol (mg/mg lipid-phosphorus)	Lipid-phosphorus (as % of whole cell lipid-phosphorus)	Lipid-phosphorus / DNA-phosphorus	Lipid-phosphorus / Protein	Protein / DNA	µg lipid-phosphorus per nucleus*** $\times 10^9$
1% citric acid	1.1	2.3	0.0095	0.18	6.5	2.5	0.9 ± 0.1***	0.049*** ± 0.003	0.0015*** ± 0.0001	3.6	4.7 ± 0.3***
2.2 M sucrose	1.2	—	0.0299	0.23	18.6	—	2.5	0.13	0.0018	8.1	12.3

* mg/g wet tissue; values for nucleus calculated for 100% recovery on DNA basis.
** Calculated from nuclear DNA-phosphorus content of $9.47 \cdot 10^{-10}$ mg (see ref. 40).
*** Mean values for 9 experiments, with standard deviation of the mean.

TABLE II

PHOSPHOLIPID CONTENT OF RAT-LIVER NUCLEI

Author	Isolation method	µg lipid-phosphorus* per nucleus $\times 10^9$	Lipid phosphorus / DNA-phosphorus
CHAUVEAU *et al.*[15]	2.2 M sucrose	104	0.11
REES AND ROWLAND[3]	0.25 M sucrose–1.8 mM Ca^{2+}	590	0.81
BARNUM *et al.*[9],**	Homogenisation in 0.85% NaCl; Isolation in 2% citric acid	46	0.048
DOUNCE[7]	Citric acid (pH 3.8)	83***	0.088
MCINDOE AND DAVIDSON[11]	5%, 0.2% citric acid	35	0.037
Present work:	1%, 0.2% citric acid	47	0.049
	2.2 M sucrose–0.002 M $CaCl_2$–0.05 M sodium glycerophosphate	123	0.13

* REES AND ROWLAND give this figure as such in their paper; all other figures in this column are calculated on the assumption that the nucleus contains $9.47 \cdot 10^{-10}$ mg DNA-phosphorus[40].
** Mouse liver.
*** DOUNCE gives values as percent lipid; figures are worked out on the basis that 90% of this is phospholipid.

RESULTS

Chemical composition of nuclei

Nuclei isolated in citric acid: Details of the determination of phospholipid, DNA, and protein are given in the MATERIALS AND METHODS section. The chemical composition is given in Table I. The amount of phospholipid is low, being only about 1 % of the whole liver phospholipid-phosphorus. Figures expressed as milligrams of each constituent/g of wet tissue vary widely from animal to animal and depend on age and diet, so that more reliance should be placed on ratios using DNA as a standard. The relative constancy of the phospholipid-phosphorus/DNA ratio over 9 experiments is thought to be additional evidence that nuclei are free from contamination with other cell particles. Such contamination is unlikely to occur always to the same extent; hence the amount of lipid present compared to DNA would be expected to vary considerably. The figure for phospholipid-phosphorus per nucleus is calculated on the assumption that each contains $9.47 \cdot 10^{-10}$ mg DNA-phosphorus[40].

A few values for nuclear lipid which have been quoted in the literature and which are readily comparable with those obtained in this study are summarised in Table II.

The composition of individual phospholipids in the nucleus is summarised in Tables III and IV. It can be seen that the pattern of phospholipids in the nucleus

TABLE III

PHOSPHOLIPID FRACTIONS OF RAT-LIVER NUCLEI AND WHOLE LIVER BY THE METHOD OF SUCCESSIVE HYDROLYSIS

Figures are expressed as percent of total phospholipid-phosphorus.

Fraction	*Nucleus*	*Whole liver*
1 Labile to mild alkali	92.6	94.0
2 Labile to mild acid (plasmalogen)	1.1	0.5
3 Stable to mild acid and alkali (sphingomyelin)	6.3	5.5

TABLE IV

PHOSPHOLIPID COMPOSITION OF THE RAT-LIVER NUCLEUS AND WHOLE LIVER

Compound	*Phosphorus as percent of total phospholipid-phosphorus*	
	*Nucleus(5)**	*Whole liver(4)**
Phosphatidylcholine	52.2	57.9
Phosphatidylethanolamine	25.1	23.7
Phosphatidylserine	5.6	4.1
Phosphatidylinositol	4.1	6.9
Cardiolipin	—	2.6
Phosphatidic acid	Trace?	Trace
Plasmalogen	1.1	0.5
Sphingomyelin	6.3	5.5
Unidentified**	1.2	—

* Number of determinations.

** This component remained at the origin in two-dimensional paper chromatograms of the alkali-labile hydrolysis products of nuclear lipids.

is very similar to that in the whole cell. The major difference is the absence of cardiolipin, a lipid which has been shown to be primarily, if not exclusively, in the mitochondrial fraction[41,42]. Plasmalogen figures, though small, represent a greater percentage of total phospholipid than in the whole cell. The only other significant difference observed was the greater insolubility of the cephalin fraction of the nuclear lipid when ethanol was added to a carbon tetrachloride solution of the lipids. This might point to differences in fatty acid composition (see DISCUSSION).

Nuclei isolated in sucrose: In the case of nuclei isolated in 2.2 M sucrose the lipid-phosphorus/DNA ratio is considerably higher and the phospholipid-phosphorus is approx. 2.5% of the whole cell lipid-phosphorus. The figure for nuclear protein is considerably higher than that quoted by CHAUVEAU[15], although it falls within the range quoted in the literature[43,44]. A comparison with the figure for citric acid nuclei, confirms the general view that large amounts of protein are lost from nuclei isolated in this medium.

Microscopic studies on the nuclei

Light microscopy of nuclei isolated in citric acid: A small sample of the sediment was stained with crystal violet; the nuclei appeared mainly as discrete particles, nearly circular, with well preserved shape. Nearly all the material took up the stain. Only occasionally were "clumps" or aggregates observed. Relatively few strands of membranous material, broken nuclei etc. were seen.

Light microscopy of nuclei isolated in 2.2 M sucrose: The sediments in the tube after centrifugation were vastly different in appearance. The citric acid nuclei appeared usually as a pure white "powdery" opaque sediment (sometimes discoloured by a brownish material), whereas the sucrose nuclei formed a translucent jelly, very difficult to see; however under the light microscope, they took up the crystal violet stain and had a very similar shape and appearance to citric acid nuclei, except that they had more tendency to "clump" in large aggregates.

Electron microscopy of nuclei isolated in citric acid: The electron micrographs revealed very little contamination of the nuclear material. There were no whole cells, mitochondria, or erythrocytes: small fragments of granular material occasionally observed between intact nuclei appeared to have come from a few disrupted nuclei. The individual nucleus had a well-rounded shape and a sharply defined surface, but there was no indication of a discrete double membrane such as is normally observed in sections of intact tissue. Within the membrane, the distribution of stained material was diffuse. A sample of the crude homogenate before centrifugation was examined by electron microscopy and here again the nucleus could be observed in a "granular" form with a definite bounding surface, which in no case appeared as a double structure (see Fig. 1).

Electron microscopy of nuclei isolated in 2.2 M sucrose: With 2.2 M sucrose nuclei, as in the case of the citric acid nuclei, little or no contamination was found. Some of the nuclei were broken and had their contents spilling out. In the interior of the nucleus there appeared to be a much greater concentration of stained material. The shape of the nuclei was rather different; in general these nuclei were not circular but rather elongated. They had "clumped" together much more and this aggregation seemed to govern their shape. The appearance of the membrane was also very different It was considerably thicker and in most cases its double structure could be easily seen

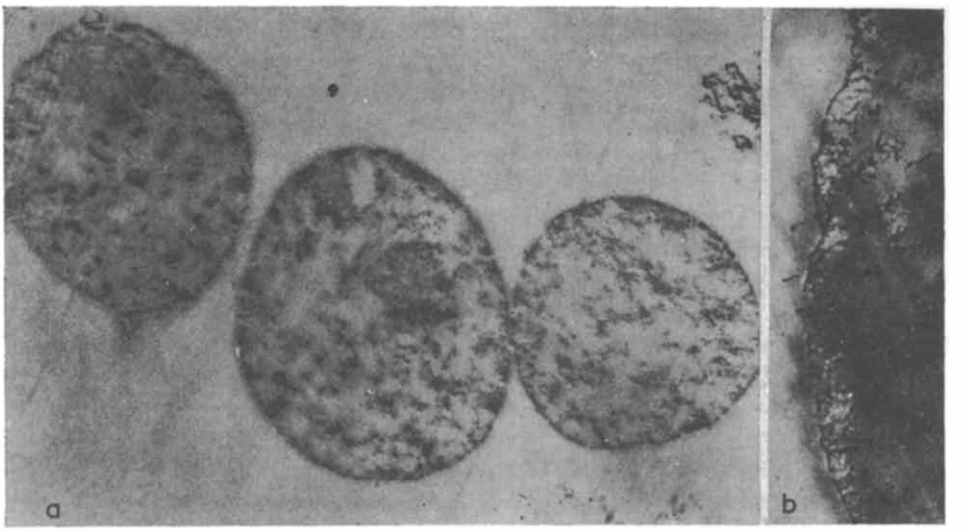

Fig. 1. (a) Rat-liver nuclei isolated in citric acid. OsO_4-fixed; araldite-embedded. Magnification: 7500 times. (b) Portion of the membrane of a citric acid-isolated nucleus. Magnification: 20000 times.

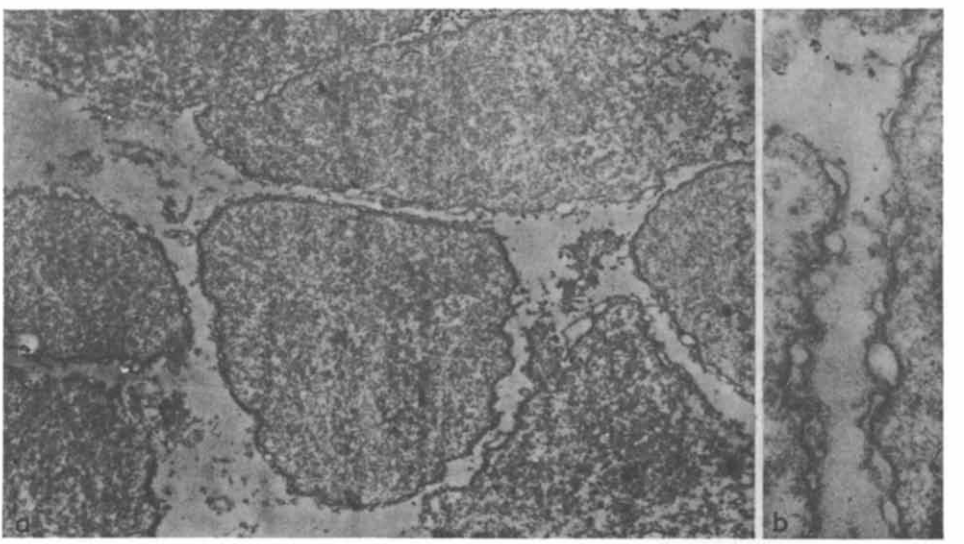

Fig. 2. (a) Rat-liver nuclei isolated in 2.2 M sucrose–0.002 M calcium chloride–0.05 M sodium glycerophosphate; OsO_4-fixed, araldite-embedded. Magnification: 7500 times. (b) Portion of the membrane of a sucrose-isolated nucleus. Magnification: 20000 times.

In some cases the outer layer of the double structure was "peeling away" and appeared as a strand attached to the nucleus in only one or two places (Fig. 2).

Enzyme studies

In the case of nuclei isolated in citric acid the low pH of the medium destroyed succinate dehydrogenase and rhodanese activities in mitochondrial "blanks" and so

these methods could not be used. Assays of sucrose nuclei showed zero succinate dehydrogenase and acid phosphatase activity and the nuclear preparation contained 0.44 % of the total glucose 6-phosphatase activity of the homogenate.

DISCUSSION

"It may very possibly be that lipids are foreign to nuclei in general"; such was the conclusion of MIESCHER AND SCHMIEDEBERG after their researches into the chemical composition of salmon-sperm heads[45]. Various studies, including the present one, show that in fact the isolated nuclei do contain lipid material, both phospholipid and neutral fat, although the amount is very small. The amount reported by different workers varies widely according to the method of isolation of nuclei and of extraction of the lipid, and is very sensitive to contamination. It is probable that such lipid as there is, is intimately bound to protein as lipoprotein[46,47] so that methods of lipid extraction are of importance.

This study shows that the pattern of phospholipids in the nucleus is very similar to that in the cell as a whole. Such a pattern seems to be characteristic of all compartments of the liver cell and of several other tissues, suggesting an intrinsic advantage of such a mixture, possible due to the resultant electrical charge distribution of the molecules. The evidence, so far, is that cardiolipin (diphosphatidylglycerol) is the only lipid to occupy a unique site in the cell[41,42]. In another study GETZ AND BARTLEY have demonstrated, both for individual phospholipids and for fatty acid composition, this reflection of a similar pattern in all parts of the cell[13,42]. In this respect, the present difference in solubility between nuclear lipid and whole cell lipid is interesting. The difference does not seem to be due to the solubilising influence of triglyceride on the alcohol-insoluble phospholipids in the case of whole cell lipids, since neither microsomal nor mitochondrial lipid extracts form a precipitate when small amounts of ethanol are added to carbon tetrachloride solutions and these fractions contain little glyceride. Therefore, such solubility differences may be due to greater saturation in the fatty acids of the nuclear phospholipids, although GETZ AND BARTLEY's work[13] suggests that this is not the case. These workers appear to have isolated nuclei in 0.25 M sucrose, but do not give evidence for the purity of their fraction, so that it is difficult to make a comparison. Work is in hand in this laboratory to study the fatty acid composition of nuclei isolated in citric acid. It is interesting to note that solubility differences have been observed before in nuclear lipids[14]. It should be pointed out here that data obtained from Langmuir trough experiments during this study would indicate a cross-sectional area per phospholipid molecule of 83 Å; this is rather lower than values already quoted in the literature for natural phospholipid mixtures[48] and might possibly be another indication of the greater saturation of the fatty acids of these lipids.

In regard to the question of the location of the lipid within the nucleus, it is of interest to relate the amount of lipid found in the nuclear fraction to the amount that can be accommodated in membrane layers at the surface.

If the nuclei are assumed to be approximately spherical and of average diameter $11.0 \cdot 10^{-4}$ cm (see ref. 22), then the surface area per nucleus is calculated to be $3.8 \cdot 10^{-6}$ cm^2.

The amount of DNA present in a single nucleus has been calculated to be

$9.47 \cdot 10^{-13}$ g (see ref. 40). The amount of lipid per nucleus can therefore be deduced from the relative amounts of lipid and DNA in the nuclear fraction (see Table I) and the figure obtained is $1.3 \cdot 10^{-12}$ g. (Phospholipid + cholesterol; this neglects any triglyceride which is likely to be in small amount.)

If the lipid component of the nuclear membrane is assumed to form layers similar to those obtained by spreading on the Langmuir trough then the "limiting area" covered by $1.3 \cdot 10^{-12}$ g of lipid is $7.96 \cdot 10^{-6}$ cm^2. Relating this now to the surface area of the nucleus, the lipid available would be capable of providing 2.1 monolayers over the whole surface of the nucleus.

In the case of nuclei isolated in sucrose, the lipid content is 2.65 times greater than that for the citric acid nuclei. The number of layers would therefore be $2.65 \times 2.1 = 5.6$.

In view of the assumptions made in these calculations, an accurate figure for the number of lipid monolayers which would be present at the nuclear surface is not to be expected. Nevertheless, the figure of 2.1 obtained for the citric acid nuclei strongly suggests the presence of a single bimolecular layer. The electron micrographs are in keeping with this in the sense that a boundary layer appears to be present but there is no indication of a double layer.

With sucrose nuclei on the other hand, the micrographs show a double structure which is usually interpreted as two unit membranes or four lipid monolayers. The lipid figure of 5.5 layers may be due to the fact that parts of the endoplasmic reticulum are still attached to sucrose nuclei, which might be expected if nuclear membrane and endoplasmic reticulum form one continuous structure in the living cell[30]. It seems reasonable to conclude from the present data, that the bulk of nuclear phospholipid is in the membrane and that citric acid removes the outer unit membrane surrounding the nucleus.

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