Biochimica et Biophysica Acta, 311 (1973) 180–190

BBA 76350

STUDIES OF THE CHEMISTRY OF THE LUMINAL PLASMA MEMBRANE OF RAT BLADDER EPITHELIAL CELLS

B. KETTERER[a], R. M. HICKS[b], L. CHRISTODOULIDES[a] and D. BEALE[c]

[a]*The Courtauld Institute of Biochemistry*, [b]*The School of Pathology, Middlesex Hospital Medical School and* [c]*The A.R.C. Institute of Animal Physiology, Babraham, Cambridgeshire (Great Britain)*

(Received February 22nd, 1973)

SUMMARY

The lipid and protein components of the luminal plasma membrane of the rat bladder have been analysed.

The principal lipid components are cholesterol, phosphatidylcholine, phosphatidylethanolamine and cerebroside. Saponification showed the presence in the phospholipids of palmitic, stearic, oleic, linoleic, linolenic, eicosatrienoic and arachidonic acyl groups.

A range of proteins was separated according to isoelectric point and polypeptide molecular weight.

The amino acid composition of the luminal plasma membrane protein differed from that of other cell subfractions in having an unusually high proline content.

These factors are discussed in relation to both the unusual ultrastructure of the membrane and its low permeability to water and electrolytes.

INTRODUCTION

The 3–4-cell-thick layer of the transitional epithelium of the rat bladder acts as a barrier to the flow of water and ions between the isotonic extracellular fluid of the body and the hypertonic urine[1–3]. Morphological and physiological evidence indicates that this barrier is passive and that the efficiency of the barrier depends on the integrity of the thick luminal plasma membrane[3–5]. This membrane has ultrastructural characteristics which are probably related to its biological properties. When sheets of membrane are subjected to negative contrast staining a hexagonal array of subunits is revealed[6,7] each of which is a dodecamer in which the monomers have a 30 Å centre-to-centre spacing[8,9]. A hexagonal lattice substructure has also been observed in the mouse[10], the rabbit[11] and the pig and man (Warren, R. C. and Hicks, R. M., unpublished). In the mouse and the rabbit the subunits have been reported as hexamers. In pig and man, as in the rat, dodecamers have been seen and it has been shown that the number of monomers observable depends on the resolution of the electron microscope image (Warren, R. C. and Hicks, R. M., unpublished).

A method for the isolation of the bladder luminal plasma membrane of the

rat has been reported[7]. The present paper gives an account of chemical information obtained from the small quantities of luminal plasma membrane isolated by this method. The relevance of this information to the structure and function of this membrane is discussed. Some of this work has been reported in brief before[12,13].

MATERIALS AND METHODS

The preparation of the bladder luminal membrane.

The bladder luminal membrane was prepared according to the method of Hicks and Ketterer[29] using the scrapings of the luminal surface of the bladders of 10 Wistar rats as starting material. The final step in purification involved the sedimentation of a membrane-rich fraction through a discontinuous sucrose density gradient. Electron microscopic examination of pellets obtained from various density bands showed the luminal plasma membrane to be concentrated in a 30% (w/v) sucrose layer ($d=1.13$). A light membrane fraction was found in the layer above and a mixed fraction containing several types of cell organelle together with some luminal plasma membrane occupied the denser layer below. The luminal plasma membrane fraction could not be used for microanalysis direct from the gradient because of the presence of so much sucrose. A washed membrane pellet was obtained by resedimentation in water. This procedure removed most of the sucrose but also resulted in loss of membrane. The analyses described below show that the yield of membrane in this pellet was of the order of approx. 200 μg dry weight.

Chromatographic analysis of lipids

Analysis of the components of the lipid fraction was performed by thin-layer chromatography on Kieselgel G. (E. Merck A. G., Darmstadt, Germany). Either a chloroform–methanol (2:1, by vol.) extract of the pellet was applied to the silica previously activated at 120 °C, or the whole pellet itself was placed in the silica at the origin of a freshly spread thin-layer plate, allowed to dry at room temperature over dehydrated silica gel and then activated by standing in vacuum over P_2O_5 for several hours.

Cholesteryl esters, triglycerides, cholesterol and polar lipids were separated by the solvent system di-isopropyl ether–hexane (1:4, by vol.). This system was modified by Miss H. Sanders from that of Morris[14]. Human serum lipid was run in parallel as a standard.

A partial separation of polar lipids in one dimension was made with the solvent system chloroform–methanol–water (14:16:1, by vol.)[15]. A more complete separation was achieved using a two-dimensional system which separates all major phosphatides and cerebroside[16]. The solvent in the first dimension was chloroform–methanol–7 M NH_4OH (60:35:5, by vol.) and in the second was chloroform–methanol–7 M NH_4OH (35:60:5, by vol.). The plate was activated between the two runs by first drying it in air over dehydrated silica gel and then standing it *in vacuo* over P_2O_5 for several hours. Lipids were located by spraying the plate with conc. H_2SO_4 and charring on a hotplate. They were identified by comparison with standard phospholipids and ox-brain cerebroside obtained from Lipid Products Limited, Redhill. The presence of the hexose moieties of the cerebroside fraction was demonstrated by using a Molisch reagent modified from that of Devor[17].

The plate was sprayed with 0.4% α-naphthol in conc. H_2SO_4 followed by gentle heating. A purple colouration indicated the presence of hexose.

Estimation of phospholipid

Phospholipid was estimated by a micro-method based on that of Rouser, *et al.*[18]. Membrane pellets were extracted three times with chloroform–methanol (2:1, by vol.) and the extract was washed with one fifth its volume of 0.1 M NaCl in order to remove as much of the remaining sucrose as possible from the extract. The organic phase was made up to 6 ml with chloroform–methanol.

Samples of this extract were then transferred to test tubes, dried down in a stream of nitrogen and digested with 0.45 ml of $HClO_4$ (spec. gravity, 1.70) for 20 min in a sand bath under gentle reflux. When cool, the walls of the tube were rinsed with 2.5 ml of water and colour was developed by adding 0.5 ml 2.5% (w/v) ammonium molybdate, 0.5 ml 1% (w/v) ascorbic acid and 1.0 ml of water followed by heating in a boiling water bath for 5 min. Colour was estimated at 820 nm. Amounts of Na_2HPO_4 containing 0.1–0.5 μg P were treated similarly to provide a standard curve. A factor of 25 was used to give an estimate of phospholipid by weight.

Estimation of cholesterol

Cholesterol was estimated colorimetrically using the same lipid extracts as those used for phospholipid analysis. The method was based on that of Webster[19]. A suitable sample was dried down, as above, in a 3-ml stoppered tube, 0.4 ml of 0.1% (w/v) $FeCl_3 \cdot 6H_2O$ in aldehyde-free glacial acetic acid was then added and it was heated at 80 °C for 5 min. The contents of the tube were then quickly mixed with 0.3 ml conc. H_2SO_4 and allowed to cool in the dark for 20 min. Colour was read at 560 nm. Standards containing 1–5 μg cholesterol were treated similarly and all solutions were read against a reagent blank.

Fatty acid analysis of the lipid fraction

This was performed by Dr G. L. Mills by modifications of the method of Albrink[20]. A chloroform–methanol extract of a whole pellet was saponified in alcoholic KOH prepared by mixing 6 ml of 33% (w/v) KOH with 94 ml absolute ethanol and heating at 80 °C for 1 h. After acidification with HCl the fatty acids were extracted with ether, methylated with diazomethane and the methyl esters taken up into a 2-mm square glass fibre filter paper and placed in a Pye Argon gas chromatograph in which the stationary phase was 20% polyethyleneglycol succinate and the temperature 156 °C. Fatty acid methyl esters from the bladder luminal membrane were identified by comparison with standards obtained from Applied Science Laboratories, Inc., State College, Philadelphia.

Analysis of proteins

Two methods of separating the protein components of the membrane by disc electrophoresis in polyacrylamide gels were used.

In Method 1, proteins were separated according to their polypeptide molecular weights using the sodium dodecyl sulphate–8 M urea method of Shapiro *et al.*[21] incorporating modifications[22]. A pellet was dissociated into polypeptide chains

by incubating at 37 °C for 2 h in 0.1 M Tris (pH 7.3), 8 M urea, 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanolamine.

Gels, 5 cm long and made from 7% acrylamide were used. It was necessary to analyse low and high molecular weight polypeptides in separate runs. Current was applied for 8 or 16 h according to which separation was required.

Gels were fixed and stained for protein with naphthalene black according to Tasserson *et al.*[22]. After destaining gels were allowed to swell in 7.5% (v/v) acetic acid, and the absorbance of the various bands read by means of a Joyce-Loebl Chromoscan. The rate of electrophoresis of the various polypeptides derived from the membrane was compared with that of polypeptides of known molecular weight. If to be stained for carbohydrate instead of protein, the gel was washed with 12.5% trichloroacetic acid, rinsed in distilled water and then stood in 1% (w/v) HIO_4 in 3% (v/v) acetic acid for 50 min. The gel was subsequently washed with frequent changes of distilled water until no precipitate was obtained with silver nitrate in the washings. Finally it was placed in Feulgen stain–HIO_4–Schiff reagent for 50 min followed by three changes of freshly prepared 0.4% (w/v) sodium metabisulphite. The gel was stored in 7.5% (v/v) acetic acid.

In Method 2, proteins were separated according to their isoelectric points in a Triton X-100, 8 M urea, Ampholine system[23]. Dissociation of the proteins in the membrane was achieved by dissolving a pellet in 8 M urea, 5% (v/v) Triton X-100, 0.05 M K_2CO_3 and 1 mM *N*-ethylmaleimide[23–25]. In this technique disulphide links are left intact and disulphide bond interchange is blocked by the *N*-ethylmaleimide. Excess *N*-ethylmaleimide was removed by dialysis against 8 M urea, 5% Triton X-100 overnight. Gels had the composition 5% (v/v) of 40% (w/v) Ampholine pH 3–10, 8 M urea, 0.3% (v/v) Triton X-100, 5% acrylamide and 0.2% *N,N'*-methylene bisacrylamide. Gels were polymerized by making them 0.04% (w/v) with respect to ammonium persulphate. The cathode was at the top of the gel and the cathode solution was 5% (v/v) diaminoethane; the anode solution was a 20-fold dilution of conc. H_2SO_4. The sample was introduced as a layer at the top of the gel and electrophoresis was carried out at 100 V for 16 h.

The gel was fixed and freed of Ampholine by washing it four times with 5% (w/v) trichloracetic acid over the course of 24 h. Proteins were stained for 16 h in 0.1% (w/v) Coomassie Blue in acetic acid–ethanol–water (2:9:9, by vol.) and destained in acetic acid–ethanol–water (2:5:13, by vol.). Staining for carbohydrate was as described above.

Amino acid analysis

The amino acid composition of membrane fractions was determined in hydrolysates of membrane pellets prepared by heating the pellet in constant boiling HCl *in vacuo* for 24 and 48 h at 105 °C followed by chromatographic analyses on a micro-scale[26,27].

RESULTS

Chromatographic analysis of lipids

Thin-layer chromatography on Kieselgel G using the solvent system diisopropyl ether–hexane showed the major lipids present to be cholesterol and polar lipids.

Cholesterol esters and triglycerides were not detected. Analysis of polar lipids using the two-dimensional system separated three major components, namely phosphatidylcholine, phosphatidylethanolamine and cerebroside (see Fig. 1).

The identity of cerebroside was confirmed by thin-layer chromatography in one dimension using the chloroform–methanol–water solvent system. A single fast-moving Molisch reagent-staining band which was obtained from the bladder luminal membrane had the same R_F as Molisch reagent-staining material in an ox-brain cerebroside preparation run in parallel.

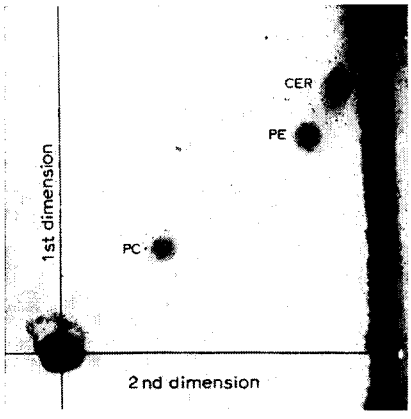

Fig. 1. Two-dimensional thin-layer chromatography of the polar lipids of the bladder luminal membrane. In this experiment whole membrane pellet was placed at the origin of a freshly prepared thin-layer plate. The plate was dried over silica gel and activated *in vacuo* over P_2O_5. The solvent run in the first dimension was chloroform–methanol–7 M NH_4OH (35:60:5, by vol.). The plate was dried, reactivated *in vacuo* and run in the second dimension using the solvent mixture chloroform–methanol–7 M NH_4OH (60:35:5, by vol.). Spots were revealed by charring with conc. H_2SO_4. The material at the origin was protein and that forming a ring around the origin, sucrose. PC, PE and CER are abbreviations for phosphatidylcholine, phosphatidylethanolamine and cerebroside, respectively.

Phospholipid and cholesterol content

The phospholipid content of three membrane preparations, each from 10 rat bladders, was 66, 88 and 86 μg. Values for the cholesterol content of the same three fractions were 25, 23 and 21 μg respectively. The mean molar cholesterol to phospholipid ratio from these three preparations was 0.6.

Fatty acid analysis of the phospholipid fraction

The membrane lipid fraction was saponified, methylated and the methyl esters of the following fatty acids identified and quantified: palmitic, stearic, oleic, linoleic, linolenic, eicosatrienoic and arachidonic acids. Their relative concentrations in moles per cent are shown in Table I. Chromatographic analysis of the lipid fraction has shown that there are only two sources of fatty acid groups, namely phospholipids and cerebroside. It is concluded that the fatty acids which were detected and estimated were derived from the phospholipid fraction, since not only were the mild conditions of saponification insufficient to release fatty acids from their amide link in cerebroside, but also the temperature of gas chromatography used was too low to resolve the longer chain acids characteristic of cerebroside.

TABLE I

FATTY ACID CONTENTS OF THE BLADDER LUMINAL MEMBRANE, THE ERYTHROCYTE GHOST AND THE LIVER PLASMA MEMBRANE OF THE RAT

Fatty acid		*Bladder luminal membrane** (*moles %*)	*Erythrocyte ghost*** (*moles %*)	*Liver plasma membrane**** (*moles %*)
Palmitic acid	(16:0)	12.2	44	22.9
Stearic acid	(18:0)	16.6	22	29.8
Oleic acid	(18:1)	18.7	18	10.2
Linoleic acid	(18:2)	24.9	14	13.1
Linolenic acid	(18:3)	3.5	—	—
Eicosatrienoic acid	(20:3)	5.9	—	0.9
Arachidonic acid	(20:4)	18.2	17	16.7
Others				6.4

* The present work.
** Taken from Maddy[43].
*** Based on the results of Wood[44].

TABLE II

AMINO ACID ANALYSIS OF THE BLADDER LUMINAL MEMBRANE FRACTION AND FRACTIONS ABOVE AND BELOW IT IN A SUCROSE DENSITY GRADIENT

Results are expressed as moles per cent of the amino acid residues determined.

	Bladder luminal membrane fraction*	*Fraction below it in the gradient*	*Fraction above it in the gradient*
Lysine	5.4	6.5	6.4
Arginine	4.7	4.8	5.1
Histidine	1.7	1.6	2.0
Glutaminic acid	10.4	11.1	10.5
Aspartic acid	8.9	9.0	9.1
Serine	6.5	9.5	7.7
Threonine	5.5	5.8	5.7
Glycine	8.7	10.2	8.1
Alanine	7.6	7.5	7.6
Valine	6.5	6.3	6.3
Leucine	8.9	8.5	9.9
Isoleucine	4.5	4.8	4.8
Phenylalanine	4.1	3.8	4.4
Tyrosine	4.1	4.5	5.8
Tryptophan	—	—	—
Proline	8.4	4.1	4.5
Methionine	2.2	2.0	2.1
Half-cystine**	1.9	—	—

* These values represent the mean of analyses of two fractions.
** In order to obtain an accurate value for half-cystine the sample was oxidized with performic acid prior to hydrolysis and half-cystine was estimated as $CySO_3H$.

Amino acid analysis

Table II shows the amino acid analyses of a luminal membrane fraction and the fractions above and below it in the sucrose density gradient. The luminal membrane fraction can be distinguished by its high proline content.

Calculation of the total weight of amino acid residues from the amino acid analyses of two luminal membrane preparations gave values of 78 and 70 μg. These values are close approximations to their polypeptide contents. Glucosamine was also detected in these analyses indicating the presence of mucoprotein.

Polypeptide analysis by disc electrophoresis in a medium containing sodium dodecyl sulphate–8 M urea

Fig. 2 represents a combination of scans of an 8-h and a 16-h run.

A number of polypeptides ranging in molecular weight from 10 000 to over 100 000 were separated. However, some material including mucoprotein was not dispersed and remained at the origin.

Separation of membrane proteins by isoelectric focusing disc electrophoresis in 8 M urea

Fig. 3 also shows a densitometric scan of a stained gel. About 10 proteins were separated with isoelectric points distributed more or less evenly on either side of neutrality. There are no proteins with isoelectric points at either extreme of the pH scale. There is however, material of such a high molecular weight that it is unable to penetrate far into the gel. This very large molecular weight material contained most of the mucoprotein.

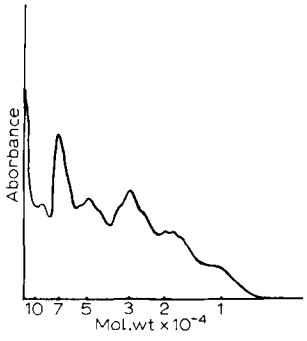

Fig. 2. Gel electrophoresis in sodium dodecyl sulphate–urea of the proteins of the bladder luminal membrane. This is a composite diagram of scans of membrane protein run in 7% polyacrylamide gels 8 M with respect to urea and 1% (w/v) with respect to sodium dodecyl sulphate for 8 and 16 h. The gels were stained with naphthalene black.

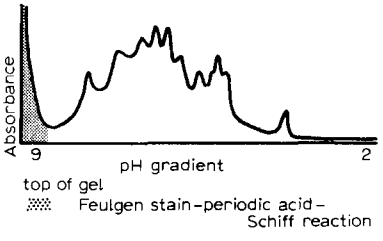

Fig. 3. Gel isoelectric focusing in Triton X-100-urea of proteins of the bladder luminal membrane. This is a scan of membrane proteins run in a 5% (w/v) polyacrylamide gel 0.3% (v/v) with respect to Triton X-100, 8 M with respect to urea and 5% (v/v) with respect to 40% (w/v) Ampholine pH 3–10. Sulphydryl groups were blocked with *N*-ethylmaleimide. Protein was stained with Coomassie Blue and carbohydrate with the Feulgen–HIO_4–Schiff reagent.

DISCUSSION

In bladder luminal membrane preparations from 10 rats the total yield of solid derived from phospholipid, polypeptide and cholesterol was approx. 200 μg

dry weight. This amount of material has proved adequate for duplicate quantitative amino acid, cholesterol, phospholipid and fatty acid analyses on single membrane preparations. It has also been possible to carry out several protein separations by disc electrophoresis on the same membrane preparation. Qualitative studies indicate that cerebroside and the carbohydrate moieties of mucoprotein are important components of the membrane, but the respective quantities have not been determined in this study.

In Tables I and III some lipid parameters for the bladder luminal membrane are compared with those known for membranes with other specializations. Low permeability in membranes has been associated with high levels of cholesterol and saturated hydrocarbon in the hydrophobic phase[28–30]. However, the bladder luminal membrane differs from other more permeable membranes in its lipid component only by its apparent high cerebroside content. Cerebroside contributes saturated hydrocarbon to the hydrophobic region and it has also been suggested that its carbohydrate head group might orient other polar head groups at the surface and as a result, the neighbouring aqueous phase[31].

TABLE III

PROTEIN AND LIPID CONTENTS OF SOME PLASMA MEMBRANES

Except where otherwise indicated results in this table are taken from Korn[45].

	Protein/lipid (w/w)	*Cholesterol/phospholipid (mole/mole)*	*Major polar lipids*
Myelin	0.25	0.7–1.2	phosphatidylcholine, phosphatidylethanolamine, cerebroside
Bladder luminal membrane*	0.7	0.6	phosphatidylcholine, phosphatidylethanolamine, cerebroside
Liver cell**	1.0–1.4	0.3–0.8	phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin
Ehrlich ascites	2.2	—	—
Intestinal microvilli	4.6	0.5–1.2	—
Erythrocyte ghost	1.5–4.0	0.9–1.0	phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin

* The present work.

** Additional values from Dod and Gray[46], Coleman[47] and Touster *et al.*[48].

Gel electrophoresis of the bladder luminal membrane (see Figs 2 and 3) shows a protein component which, like that of other plasma membranes, is heterogeneous[24,32,33]. Apart from a large molecular weight mucoprotein it has no obvious distinguishing features. Amino acid analysis on the other hand does reveal an interesting difference between the protein component of this and other membranes. When the analysis is compared with that known for some 200 proteins[34] it is seen that while its content of most amino acids is average, it has an unusually high proline content (see Fig. 4). So much proline, if randomly distributed, would tend to limit α-helix formation[35,36]. Therefore, since plasma membranes are believed to be

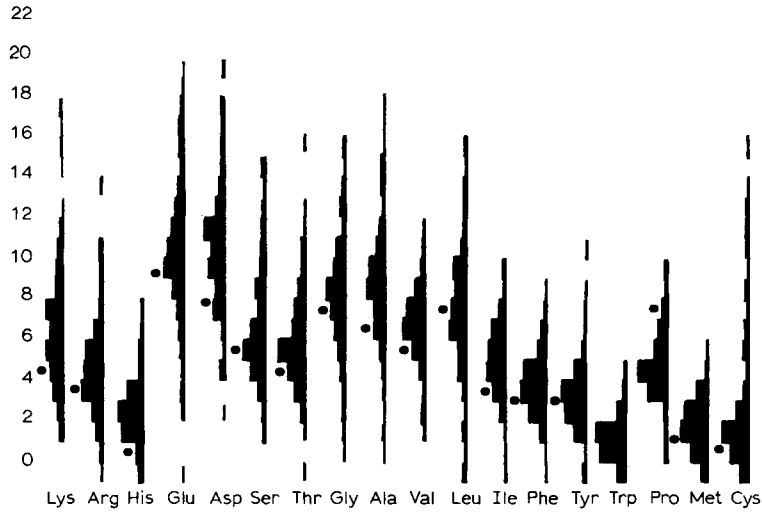

Fig. 4. A comparison of the amino acid analysis of the bladder luminal membrane with that of some 200 pure proteins. The frequency of content of each amino acid in the pure proteins is represented as a separate histogram. In each histogram the ordinate represents the content of each amino acid in moles per cent, simplified so that values falling between two whole numbers are considered together. Thus the first category is 0 mole %, the second 0–1 mole %, the third 1–2 moles % and so on. The abscissa represents the number of proteins from 0–100 in which each content is found. The analysis of the bladder luminal membrane is shown as spots superimposed upon the histograms.

rich in α-helix[37–39], either the bladder luminal membrane is different in this respect or its proline content is concentrated in one or more proteins having some sort of open structure. One such protein may be the large molecular weight mucoprotein. If this protein occurred as a compact molecule it should be observable in the electron microscope. Perhaps therefore it is present as an unfolded structure having a relatively large area and so disposed in the membrane that its carbohydrate component is at the extracellular surface, the location believed to be general for plasma membrane glycoproteins[40].

The basis for the low flux of ions and water through this membrane remains to be fully explained. Ordered water at the surface may well play an important role and many components in the membrane could participate in this phenomenon. It is possible that the regular ultrastructure with its implied regular molecular architecture may be important in this regard but very little is known about the structure of water at the interfaces of any biological membranes[41].

The basis of the apparently simple ultrastructure is also baffling. The monomers composing the dodecameric subunits appear similar to each other and have dimensions falling in the range for those of protein molecules. For example, it can be calculated that if the monomers represented polypeptide spheres 30 Å in diameter, they would have a molecular weight of approx. 11 000 (ref. 42). Or if the monomers are rods which span the 120-Å thickness of the membrane, as seems likely from freeze-fracture studies of the bladder luminal membrane of the rabbit[11], their molecular weight would be approx. 70 000 if composed of pure polypeptide. However the protein analysis is complex and not simple, as would be expected if these repeating

structures were similar protein molecules. The monomers may well contain protein, but other components of the membrane are also probably involved in their structure.

Much more detailed information is required to answer some of these questions raised by the chemical observations it has been possible to make on the small amount of material obtained from the rat. An alternative source of material giving much larger yields is being sought.

ACKNOWLEDGEMENTS

This work was assisted by a generous grant to B. K. and R. M. H. from the Cancer Research Campaign. We thank Dr A. D. Bangham, Dr R. Coleman and Miss Hazel Sanders for helpful discussion.

REFERENCES

1 Blandy, J. P. (1964) *Ann. R. Coll. Surg. Engl.* 35, 287–311
2 Hicks, R. M. (1965) *J. Cell Biol.* 26, 25–48
3 Hicks, R. M. (1966) *J. Cell Biol.* 28, 21–31
4 Hicks, R. M. (1966) *J. Cell Biol.* 30, 623–643
5 Porter, K. R., Kenyon, K. and Badenhausen, S. (1967) *Protoplasma*, 63, 262–274
6 Hicks, R. M. and Ketterer, B. (1969) *Nature* 224, 1304–1305
7 Hicks, R. M. and Ketterer, B. (1970) *J. Cell Biol.* 45, 542–553
8 Warren, R. C. and Hicks, R. M. (1970) *Nature* 227, 280–281
9 Warren, R. C. and Hicks, R. M. (1971) *J. Ultrastruct. Res.* 36, 861–874
10 Vergara, J., Longley, W., and Robertson, J. D. (1969) *J. Mol. Biol.* 46, 593–596
11 Staehlin, L. A., Chlapowski, F. J. and Bonneville, M. (1972) *J. Cell Biol.* 53, 73–91
12 Hicks, R. M., Ketterer, B. and Beale, D. (1968) *Biochem. J.* 109, 41P
13 Ketterer, B. and Hicks, R. M. (1971) *Biochem. J.* 122, 66P
14 Morris, L. J. (1963) *J. Lipid Res.* 4, 357–359
15 Mehl, E. and Jatzekewitz, H. (1964) *Z. Physiol. Chem.* 339, 260–276
16 Skidmore, W. D. and Entenman, C. (1962) *J. Lipid Res.* 3, 471–475
17 Devor, A. W. (1950) *J. Am. Chem. Soc.* 72, 2008–2012
18 Rouser, G., Siakotos, A. N. and Fleischer, S. (1966) *Lipids* 1, 85–86
19 Webster, D. (1962) *Clin. Chim. Acta* 7, 277–284
20 Albrink, M. J. (1959) *J. Lipid Res.* 1, 53–59
21 Shapiro, A. L., Viñuela, E. and Maizel, J. V. (1967), *Biochim. Biophys. Res. Commun.* 28, 815–820
22 Tasserson, J. G., Diringer, H., Frohwirth, N., Mirvish, S. S. and Heidelberger, C. (1970) *Biochemistry* 9, 1636–1644
23 Gronow, M. and Griffiths, G. (1971) *FEBS Lett.* 15, 340–344
24 Neville, Jr., D. M., (1967) *Biochim. Biophys. Acta* 133, 168–170
25 Neville, Jr. D. M. (1968) *Biochim. Biophys. Acta* 154, 540–552
26 Spackman, D. H., Stein, W. N. and Moore, S. (1958) *Anal. Chem.* 30, 1190–1206
27 Beale, D. and Kent, C. M. (1968) *The separation of carboxymethyll cysteine, aminoethyl-cysteine, homoserine and homoserine lactone in protein hydrolysates using the modified Technicon microcolumn technique, 6th Colloq. Amino Acid Analysis*, Technicon Instruments Corporation, Chertsey, Surrey
28 de Gier, J. and van Deenen, L. L. M. (1961) *Biochim. Biophys. Acta*, 49, 286–296
29 de Gier, J., van Deenen, L. L. M. and van Senden, K. G. (1966) *Experientia* 22, 20–21
30 Finkelstein, A. and Cass, A. (1967) *Nature* 216, 717–718
31 Clowes, A. W., Cherry, R. J. and Chapman, D. (1971) *Biochim. Biophys. Acta* 249, 301–317
32 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) *Biochemistry*, 10, 2606–2617
33 Neville, Jr., D. M. and Glossman, H. (1971) *J. Biol. Chem.* 246, 6335–6338

34 Reeck, G. (1970) in *Handbook of Biochemistry*, (Sober, H. A., ed.) pp. C281–287, Chemical Rubber Co., Cleveland
35 Perutz, M. F., Kendrew, J. C. and Watson, H. C. (1965) *J. Mol. Biol.* 43, 669–678
36 Wu, T. T. and Kabat, E. A. (1971), *Proc. Natl. Acad. Sci. U.S.* 68, 1501–1506
37 Ke, B. (1965) *Arch. Biochem. Biophys.* 112, 554–561
38 Wallach, D. F. H. and Zahler, P. H. (1966) *Proc. Natl. Acad. Sci. U.S.* 56, 1552–1559
39 Lenard, J. and Singer, S. J. (1966), *Proc. Natl. Acad. Sci. U.S.* 56, 1828–1835
40 Winzler, R. J. (1970) *Int. Rev. Cytol.* 29, 77–125
41 Drost-Hansen, W. (1971) in *Chemistry of the Cell Interface, Part B*, (Brown, H. D., ed.), pp. 2–183, Academic Press, N.Y.
42 Cohn, E. J. and Edsall, J. T. (1943), in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p. 382, Reinhold, New York
43 Maddy, A. H. (1966) *Int. Rev. Cytol.* 20, 1–65
44 Wood, R. (1970) *Arch. Biochem. Biophys.* 141, 174–182
45 Korn, E. D. (1969), *Annu. Rev. Biochem.* 38, 263–288
46 Dod, B. J. and Gray, G. M. (1968) *Biochim. Biophys. Acta* 150, 397–404
47 Coleman, R. (1968) *Chem. Phys. Lipids* 2, 144–146
48 Touster, O., Aronson, N. N., Dulaney, J. T. and Hendrickson, H. (1970) *J. Cell Biol.* 47, 604–618