Biochimica et Biophysica Acta, 291 (1973) 93–106

BBA 76063

SUBCELLULAR FRACTIONATION OF HUMAN LYMPHOCYTES

ISOLATION OF TWO PLASMA MEMBRANE FRACTIONS AND COMPARISON OF THE PROTEIN COMPONENTS OF THE VARIOUS LYMPHOCYTIC ORGANELLES

HELMUT DEMUS

Department of Neuropathology, Max-Planck Institute for Psychiatry, 8 Munich 40 (Germany)

(Received May 16th, 1972)

SUMMARY

1. A procedure was developed for the isolation and fractionation of human lymphocytes from tonsils. Nine subcellular fractions were obtained and partial characterization of each was performed by the determination of enzymic activities and the nucleic acid, cholesterol and phospholipid contents, as well as by electrophoresis and immunodiffusion studies.

2. Two plasma membrane fractions of different densities were isolated and termed "light" and "heavy" plasma membranes. The former was derived primarily from the crude mitochondrial fraction and was assumed to be composed of larger membrane vesicles. The specific activity of the 5'-nucleotidase showed a 14-fold and 13-fold increase in the two plasma membrane fractions, respectively, as compared with the homogenate. Furthermore, these fractions were distinguished by very high cholesterol:protein ratios (0.263 and 0.210) and phospholipid:protein ratios (0.706 and 0.608). Lactate dehydrogenase activity decreased 170-fold and 500-fold, respectively, in comparison with cytosol.

3. Human serum albumin was found in all cell fractions except ribosomes. It is assumed to be incorporated into lymphocytes by phagocytosis. Whole immunoglobulin molecules were present in the plasma membranes as well as in the other subcellular fractions. A minor protein fraction of molecular weight of about 45000 was found primarily in the plasma membranes and might be plasma membrane specific.

INTRODUCTION

Lymphocytes arouse increasing interest because of their key role in immunological reactions: the ability of the thymic-dependent lymphocytes to cause delayed hypersensitivity reactions, especially graft rejections and the ability of the thymic-independent lymphocytes to produce antibodies (for review see ref. 1). Furthermore, lymphocytes play a role in some human diseases, such as neoplastic disorders (lym-

phocytic leukaemia, myeloma, lymphoma), agammaglobulinaemia and autoimmune disorders, including possibly multiple sclerosis.

Considering the important role of the plasma membrane in these and many other interesting processes as recently reviewed[2], it is now thought desirable to work with purified plasma membranes for detailed investigations of surface structures, especially antigen receptors. Until the present time, however, a method for the subcellular fractionation of human lymphocytes was not available. Recently, the isolation of plasma membranes of pig lymphocytes from lymph nodes[3] and fractionation of some of the components[4] has been reported. Since it is difficult to procure a sufficient amount of human lymphocytes for a large-scale preparation, we used freshly removed tonsils for the isolation of lymphocytes. During the progress of our work it became apparent that there are significant differences between the sedimentation behaviour of the subcellular organelles of these two species (pig and human lymphocytes). We therefore developed a new method, which should also be applicable to human blood lymphocytes isolated by one of the known procedures[5].

MATERIALS AND METHODS

Isolation and subcellular fractionation of human lymphocytes

Freshly removed tonsils in a non-inflammatory state were placed immediately into a Dewar flask containing a 10 mM Tris–HCl–0.15 M NaCl buffer (pH 7.4) at 4 °C. All further processes were carried out at 0–4 °C. After having obtained six tonsils, they were freed from adherent connective tissue and blood vessels so that they appeared as greyish organs of a rigid consistency. They were then washed, weighed and minced with scalpels and razor blades in the above saline buffer, then filtered through a double layer of finest silk cloth (50 μm pore size) resting on a plastic sieve. The filtration was gently forced by stirring and pressure with a pestle. After this the filtrate was diluted with the above saline buffer in the proportion filtrate:buffer of 1:20 (w/v). Breakage of the cells was performed by means of a Potter–Elvehjem homogenizer (0.1 mm clearance) at about 1000 rev./min for 3 × 1 min. All stages of the procedure were examined in the light microscope after staining of the cell smears by the method of Pappenheim[53].

The procedure of subcellular fractionation of the homogenate is summarized in Fig. 1. Nuclei, crude mitochondria and microsomes were sedimented with the SS-34 rotor of the Sorvall centrifuge. The pellets of crude ribosomes were obtained by using the rotor 60 Ti of the preparative ultracentrifuge L2-65B of Beckman Instruments.

The pellets of crude mitochondria, microsomes and crude ribosomes were then each suspended in 10 ml of 10 mM Tris–HCl buffer (pH 7.4) with 55 % sucrose (v/w). In addition, the solution for the ribosomes contained 5 mM disodium EDTA. Careful resuspension of the pellets by means of the Potter–Elvehjem homogenizer was of the utmost importance because of the danger of clumping of the cell particles. The suspensions were put into three cellulose nitrate tubes (60 ml capacity) of the SW 25.2 rotor of the Beckman centrifuge. Each suspension was overlayed with a discontinuous density gradient of 10 ml of 41 % sucrose (w/v), 15 ml of 33 % sucrose (w/v), 15 ml of 24 % sucrose (w/v) and 10 ml of 15 % sucrose (w/v). All sucrose solutions were prepared with 10 mM Tris–HCl buffer (pH 7.4). After centrifugation at 75000 × g_{av} for 22 h, the interfacial bands were carefully removed with a syringe

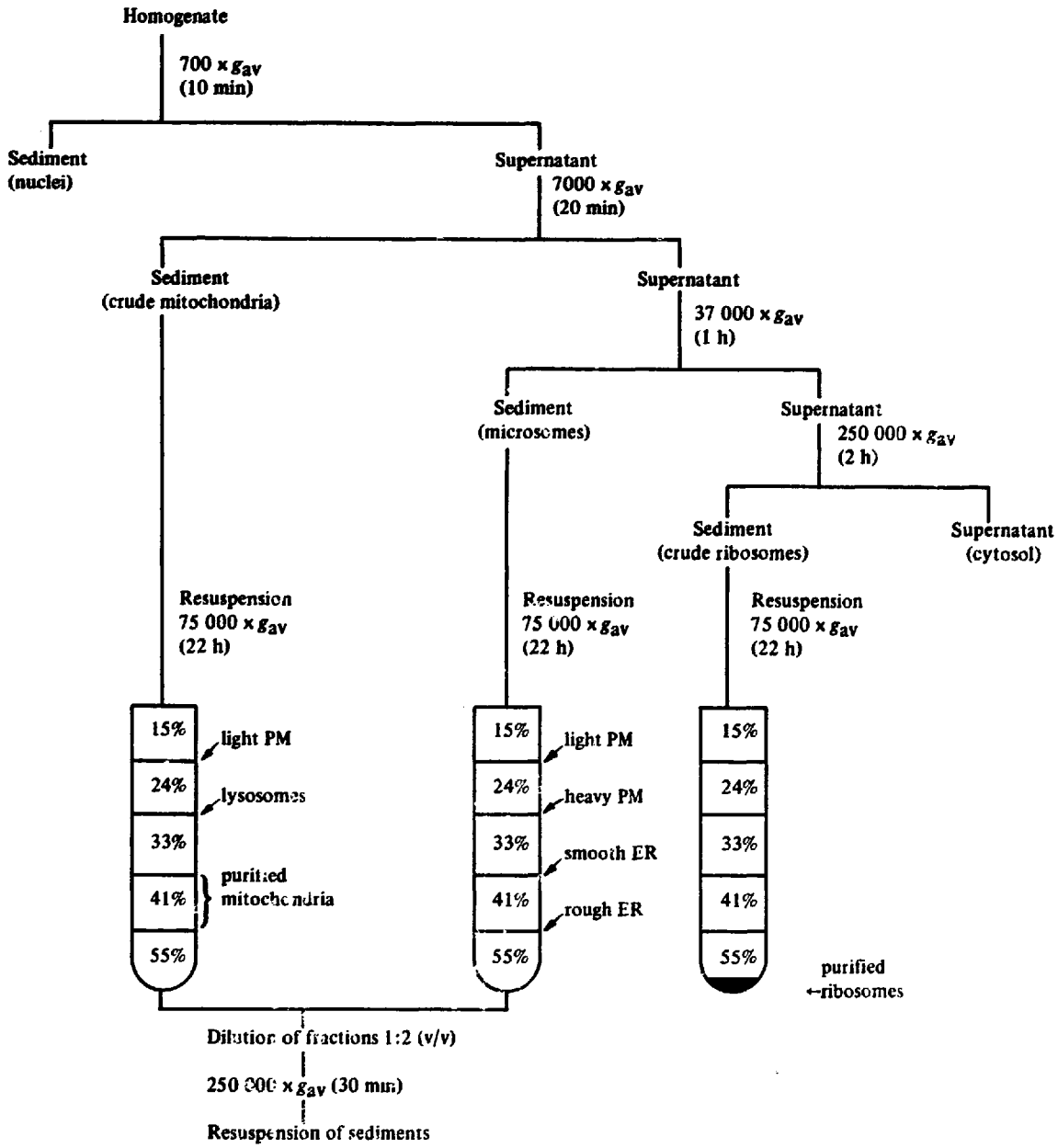

Fig. 1. Diagram of subcellular fractionation of lymphocytes. Terminology of organelles is based on the subsequent chemical characterization of the fractions. Further details are given in the text. Abbreviations: PM, plasma membrane; ER, endoplasmic reticulum; %, percentage of sucrose solutions.

equipped with a V_2A-needle bent to a right angle. Then the fractions were diluted 3-fold with 10 mM Tris–HCl buffer (pH 7.4) and spun down in the 60 Ti rotor at 250000 × g_{av} for 30 min, with the exception of the ribosomes already sedimented. The sediments were resuspended in 0.2 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and stored at −20 °C.

Preparation of human erythrocyte membranes

Human erythrocyte membranes were prepared as previously described[6] but with the use of Tris–HCl buffer instead of phosphate buffer and by washing the membrane pellet only 3 times instead of 10 times.

Analytical methods

Protein was determined by a modification of the method of Miller[7]. NaOH was omitted from the original solutions and the proteins were hydrolysed for 1 h at 100 °C with the corresponding amount of NaOH before the determination. Bovine serum albumin was used as a standard. The RNA content of fractions was measured as described by Munro and Fleck[8] using yeast RNA (Serva, Heidelberg) as standard. DNA was determined by the method of Ceriotti[9] using calf thymus DNA (Serva, Heidelberg) as a standard. The lipid phosphorus content of the membranes was assayed by the method of Norton and Autilio[10] after lipid extraction for 24 h at room temperature with chloroform–methanol–water (16:8:1, v/v/v) as described by Hess and Thalheimer[11]. Cholesterol was determined by the Liebermann–Burchard reaction using the procedure of Stadtman[12]. Cholesterol from Merck (Darmstadt) was used as a standard.

Assay of enzymic activities

The activity of the 5′-nucleotidase (EC 3.1.3.5) was measured as described by Michell and Hawthorne[13]. Glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of Swanson[14] modified by the use of acetate buffer instead of citrate buffer. This was done because of chelation of molybdate by citrate[15] during the Fiske–SubbaRow procedure[16] for the determination of enzymically released phosphate, the procedure which was used throughout. Two acid phosphatases (EC 3.1.3.2) were determined: *p*-nitrophenylphosphatase as described by Linhardt and Walter[17] and β-glycerophosphatase by the method of Gianetto and de Duve[18]. Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the procedure of Adams *et al.*[19] and lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer *et al.*[20] with addition of 0.1 % Triton X-100 to the buffer.

Polyacrylamide slab gel electrophoresis

For the preparation of polyacrylamide gels the following standard solutions were used: A, 50 mM sodium phosphate buffer (pH 7.0) with 1 % sodium dodecylsulphate; B, 0.4 ml *N,N,N′,N′*-tetramethylethylenediamine filled up to a volume of 100 ml with Solution A; C, 20 g acrylamide and 0.52 g *N,N′*-methylenebisacrylamide filled up to a volume of 100 ml with Solution A; D, 0.3 g ammonium persulphate filled up to 100 ml with Solution A. The polyacrylamide slab gel electrophoresis apparatus of Desaga (Heidelberg) was used. The 10 % separation gel consisted of 4 ml of Solution B, 20 ml of Solution C and 16 ml of Solution D. This was overlayed with a 5 % spacer gel of the following composition: 3 ml of Solution A, 2 ml of Solution B, 4 ml of Solution C and 7 ml of Solution D. Fractions with a protein content of about 50 μg dissolved in 15 μl 50 mM sodium phosphate buffer (pH 7.0) with 5 % sodium dodecylsulphate and 1 mM disodium EDTA but without 2-mercaptoethanol were filled into the pockets of the spacer gel. The density of the solutions was increased by the addition of sucrose. For molecular weight estimations, according to the procedure of Shapiro *et al.*[21], proteins from the collection MS-1 of Serva (Heidelberg) were used in amounts of 5 μg. Electrophoresis was carried out under the following conditions: 90-mA current for 3 h, using Solution A as the electrode buffer. Gels were stained with a solution of 0.25 % Coomassie Blue G-250 (Serva, Heidelberg) in water–

methanol–acetic acid (9:9:2, v/v/v) and destained in water–methanol–acetic acid (35:2:3, v/v/v).

Immunodiffusion of membrane proteins

Immunodiffusions in 2 % agarose gels were performed as described[22] but with 2 % Triton X-100 in the Tris–HCl buffer and without urea. These gels were stained with a solution of 0.06 % Coomassie Blue G-250 in water–methanol–acetic acid (70:9:2, v/v/v) and destained in water–methanol–acetic acid (35:2:3, v/v/v). Antisera against human γ-globulin and human serum albumin were from Behringwerke AG (Marburg).

RESULTS

From six tonsils of a wet weight of about 20 g, approximately 8 g cells were obtained, corresponding to a minced residue of 12 g. Cell smears (Fig. 2) indicate the

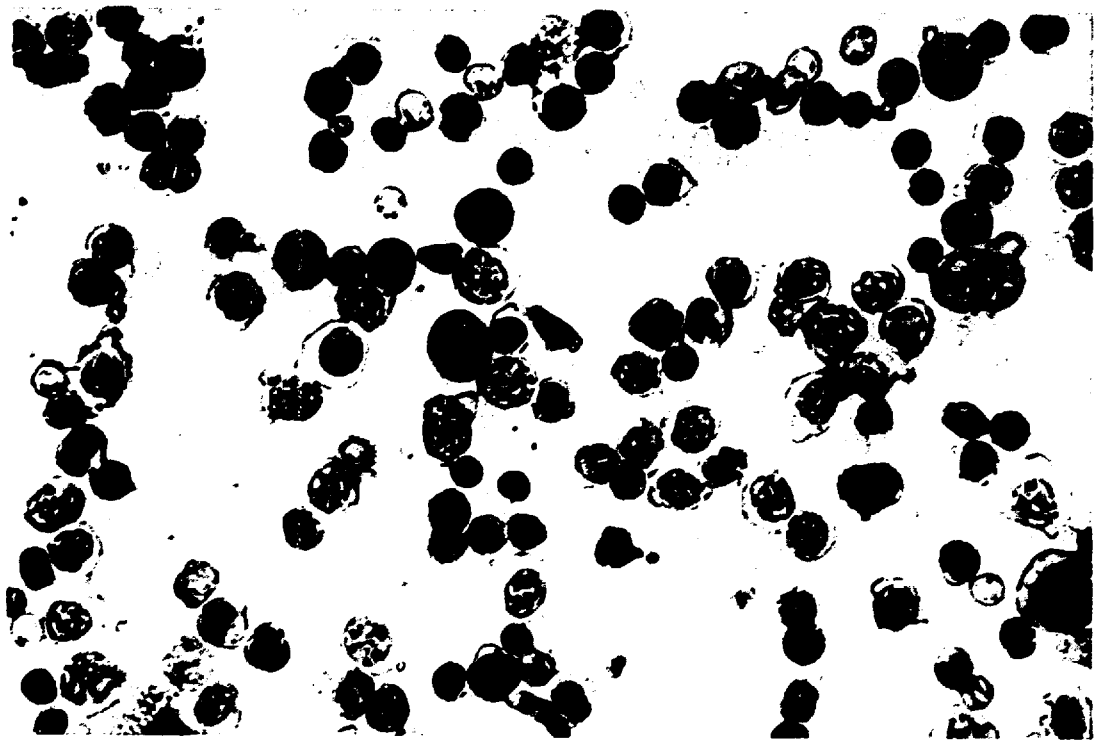

Fig. 2. Cell smear of the filtrate of minced tonsils. Staining by the method of Pappenheim[53]. 90 % of the cells consist of large and small lymphocytes and 10 % of erythrocytes, monocytes and plasma cells. × 100.

presence of about 90 % lymphocytes, consisting of a mixture of large and small lymphocytes. The remaining 10 % consist of erythrocytes, monocytes and plasma cells. Granulocytes are very rare. Reticulum cells, fibrocytes and epithelial cells were found exclusively in the residue. Most of the contaminating erythrocytes appeared in the nuclear pellet because they had not been disrupted by the homogenization procedure employed.

Various centrifugation processes were tested to find a satisfactory subcellular fractionation method. The use of sucrose and 1 mM $CaCl_2$ during differential centri-

TABLE I

CHEMICAL COMPOSITION OF SUBCELLULAR FRACTIONS FROM HUMAN LYMPHOCYTES

The origin of the fractions is described in Fig. 1. The protein weights are the total amounts obtained from tonsils of 20 g wet weight. The weight of the phospholipid was calculated by multiplying the weight of phosphorus by 25. The molecular weight of phospholipid was assumed to be 775. All figures are mean values of two fractionation processes

	Protein (mg total wt)	*DNA (μg/mg protein)*	*RNA (μg/mg protein)*	*Cholesterol (μg/mg protein)*	*Lipid phosphorus (μg/mg protein)*	*Phospholipid (μg/mg protein)*	*Cholesterol: phospholipid (molar ratio)*
Homogenate	1150	126	59	33			
Nuclei	255	**400**	64	17			
Crude mitochondria	85	21	56	72			
Purified mitochondria	55	23	60	69	12.9	324	0.43
Lysosomes	6	11	12	145	16.9	422	0.68
Light plasma membranes	2	8	20	**263**	**28.2**	**706**	**0.75**
Heavy plasma membranes	8.5	10	15	**210**	**24.3**	**608**	**0.69**
Smooth endoplasmic reticulum	16	19	70	106	15.7	393	0.54
Rough endoplasmic reticulum	7	11	159	43	11.5	287	0.30
Ribosomes	24	40	**360**	31			
Cytosol	400	4	20	5			

TABLE II

ENZYMIC ACTIVITIES OF SUBCELLULAR FRACTIONS FROM HUMAN LYMPHOCYTES

The specific activities are given as μmoles of product liberated per h per mg of protein at 37 °C. These are mean values of two fractionation processes.

	5'-Nucleotidase	*Glucose-6-phosphatase*	*Succinate dehydrogenase*	*Acid p-nitrophenyl-phosphatase*	*Acid β-glycero-phosphatase*	*Lactate dehydrogenase*
Homogenate	1.02	0.80	0.19	2.19	0.81	25.2
Nuclei	0.78	0.71	0.12	1.99	0.75	10.5
Crude mitochondria	3.77	1.19	0.98	13.40	3.27	6.4
Purified mitochondria	2.11	1.23	**1.06**	7.34	**3.29**	4.0
Lysosomes	8.36	0.87	0.05	**15.23**	3.05	0.2
Light plasma membranes	**14.60**	1.05	0.04	3.75	1.02	0.3
Heavy plasma membranes	**13.48**	0.98	0.01	6.08	1.51	0.1
Smooth endoplasmic reticulum	2.37	**1.82**	0.14	5.92	1.75	0.6
Rough endoplasmic reticulum	1.14	1.10	0.17	3.14	0.86	0.5
Ribosomes	0.20	0.34	0.00	0.82	0.38	0.4
Cytosol	0.63	0.80	0.01	1.38	0.76	**51.3**

fugation was found to be of no advantage. During the subsequent density-gradient centrifugation, the addition of Ca^{2+} was unsuitable because of strong association between the plasma membranes and endoplasmic reticulum. However, addition of EDTA failed to give better results since fragments of released endoplasmic reticulum contaminated the plasma membranes. On the other hand, the addition of 5 mM disodium EDTA was shown to be of great advantage in increasing the yield of a high percentage of free ribosomes, as indicated by their RNA content (RNA:protein ratio, 0.36) listed in Table I.

Tables I and II document the results of the subcellular fractionation procedure. 22 % of the total protein weight and 70 % of the total DNA weight sedimented within the nuclear fraction. This fraction exhibited a DNA:protein ratio of 0.4 and a DNA:RNA ratio of 6.25. Some difficulties were encountered in the separation of mitochondria and lysosomes from the plasma membranes. As can be seen in Table II the crude mitochondrial fraction contained a relatively high specific activity of 5'-nucleotidase which is assumed to represent a marker for plasma membranes. Therefore this fraction was subjected to further subfractionation.

Equilibrium density centrifugation of the crude mitochondrial fraction yielded three subfractions. Some nuclei found in the pellet were discarded. A wide zone between the 55 % and 33 % sucrose layers with a 5.6-fold increase of the specific activity of succinate dehydrogenase in comparison with the homogenate appeared to represent purified mitochondria. A high specific activity of acid *p*-nitrophenylphosphatase between the 33 % and 24 % sucrose layers and a higher content of cholesterol and phospholipid as compared with the mitochondria indicated the presence of lysosomes. However, a further marker for lysosomes, acid β-glycerophosphatase, exhibited a somewhat higher activity within the mitochondrial fraction, indicating a heterogeneity of the lysosomes. The third subfraction between the 24 % and 15 % sucrose layers showed a 14-fold increase in the specific activity of 5'-nucleotidase, and both a very high cholesterol content (263 μg/mg protein) and phospholipid content (706 μg/mg protein) with a cholesterol:phospholipid molar ratio of 0.75. This subfraction was termed the "light" plasma membrane fraction. It exhibited some contamination by endoplasmic reticulum, mitochondria, lysosomes and cytosol, as indicated by the enzyme activities of glucose-6-phosphatase, succinate dehydrogenase, acid phosphatases and lactate dehydrogenase. It was interesting to observe that the amount of this fraction changed in relation to the homogenization conditions: reduction of the number of revolutions per minute in the Potter–Elvehjem homogenizer resulted in an increase in the yield.

The microsomal fraction, which was obtained by middle-speed differential centrifugation (leaving most of the ribosomes in the supernatant) was also subjected to equilibrium density centrifugation. Some ribosomes which formed a pellet were discarded. The microsomal fraction contained only a small amount of the "light" plasma membrane subfraction between the 24 % and 15 % sucrose layers. The traces obtained here were combined with the fraction described above. A high specific activity of 5'-nucleotidase (13-fold increase in comparison with the homogenate) appeared also between the 33 % and 24 % sucrose layers. Moreover, this zone was also characterized by a high content of both cholesterol (210 μg/mg protein) and phospholipid (608 μg/mg protein) with a cholesterol:phospholipid molar ratio of 0.69. This zone was therefore termed the "heavy" plasma membrane fraction. It

exhibited a marked contamination by lysosomes, as indicated by the figures in Table II. Contamination by other organelles was very slight. The activity of the lactate dehydrogenase was reduced 500-fold in comparison with the supernatant indicating the presence of only negligible amounts of cytosol. Addition of 0.1 % Triton X-100 to the reaction buffer ensured that there was no latent enzyme activity in formed membrane vesicles.

Two endoplasmic reticulum fractions were also obtained from the microsomes. The relatively low specific activity of the glucose-6-phosphatase found throughout is due to the instability of this enzyme at 4 °C (ref. 14). Therefore only the residual activities could be measured. A so-called "smooth" endoplasmic reticulum fraction between the 33 % and 41 % sucrose layers exhibited a higher glucose-6-phosphatase activity but a lower RNA content (70 μg/mg protein). The "rough" endoplasmic reticulum fraction between the 41 % and 55 % sucrose layers was characterized by a lower glucose-6-phosphatase activity and by a higher RNA content (159 μg/mg protein). Both membrane fractions had a low content of cholesterol and phospholipids as compared with the plasma membrane fractions. Lactate dehydrogenase shows a very slight increase in its activity in the endoplasmic reticulum fractions. This is an indication of a slight contamination by free proteins rather than by adsorbed or enclosed proteins because the density of most proteins is of the same order as that of the endoplasmic reticulum membranes. Therefore the subcellular fractions of higher and lower density exhibited a lower specific activity of lactate dehydrogenase.

Fig. 3 shows the results of electrophoresis of the various subcellular fractions of lymphocytes and of γ-globulin and serum albumin markers in a 10 % polyacrylamide gel. Molecular weights of the separated proteins were estimated by comparative electrophoresis of eight standard proteins carried out under the same conditions in another gel, resulting in a calibration line. An impressive feature of the electrophoretic picture is the appearance of three zones of different histones[23] in the molecular weight range of 13000–17000 in the nuclear fraction (No.1). Compared to this, the very faint zone of cytochrome *c* (mol. wt 12400) in the mitochondrial fraction (No. 2) is barely visible.

The two plasma membrane fractions (Nos 4 and 5) exhibit complex patterns of about 30 bands each. A faint zone in the molecular weight range of 22000 appears only in these two fractions and in the smooth endoplasmic reticulum (No. 6). However, if more protein material is used for the electrophoresis, this zone appears in all fractions. In contrast to this, a very faint zone in the molecular weight range of 45000 is visible only in the plasma membrane fractions and to a slighter degree within the smooth endoplasmic reticular and the lysosomal fractions (Nos 6 and 3). This zone can only be seen in these fractions, even if the electrophoretic conditions are altered. The occurrence of this zone to a weaker extent in the fractions Nos 6 and 3 could be explained by contamination with plasma membranes, as indicated by the enzymic determinations. A zone of molecular weight of 150000 is also of special interest and might point to the presence of γ-globulin[24]. It is present not only in the plasma membranes, but in all subcellular fractions, as can easily be seen by comparison with the γ-globulin marker (No. 10).

Ribosomal proteins[25] are to be seen in the "rough" endoplasmic reticular fraction according to its content of bound ribosomes. This reveals a striking difference between the "rough" endoplasmic reticular (No. 7) and ribosomal (No. 8) fractions on

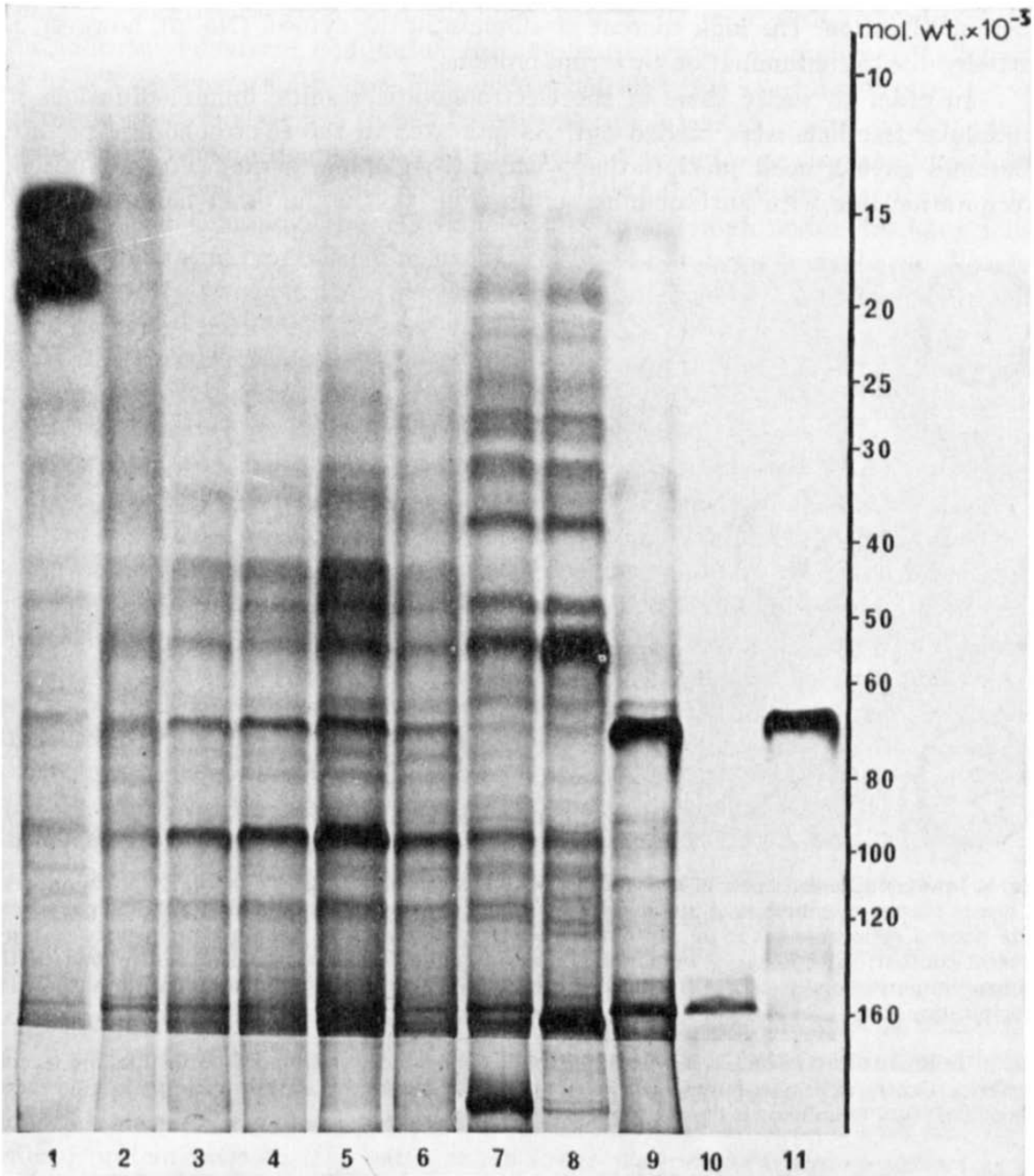

Fig. 3. Electrophoretogram of subcellular fractions of human lymphocytes (50 μg protein content) and marker proteins (5 μg γ-globulin, 10 μg albumin) in 10% polyacrylamide slab gel and 5% spacer gel with 50 mM sodium phosphate buffer (pH 7.0) containing 1% sodium dodecylsulphate as solvent. Molecular weight specifications were made possible by electrophoresis of eight calibrating proteins under the same conditions. Numbers: 1, nuclei; 2, mitochondria; 3, lysosomes; 4, light plasma membranes; 5, heavy plasma membranes; 6, smooth endoplasmic reticulum; 7, rough endoplasmic reticulum; 8, ribosomes; 9, cytosol; 10, human γ-globulin; 11, human serum albumin.

the one hand, and all other fractions in the molecular weight range of 14000–35000 on the other. Furthermore, the "rough" endoplasmic reticulum is characterized by a wide zone of very high molecular weight in the spacer gel, whereas the ribosomes exhibit a large band of molecular weight of about 170000 near the start of the separation gel. The albumin marker (No. 11) indicates the occurrence of a protein zone of molecular weight of 67000 in all cell fractions, but this zone is barely visible in the

ribosomal fraction. The high content of albumin in the cytosol (No. 9), however, is partially due to contamination by serum proteins.

In order to verify some of the electrophoretic results, immunodiffusions of subcellular fractions were carried out. As indicated in the electrophoretic picture, ribosomes gave a good precipitation with anti-γ-globulin serum (Fig. 4), but no precipitation line with anti-albumin serum (Fig. 5). On the other hand, dissolved

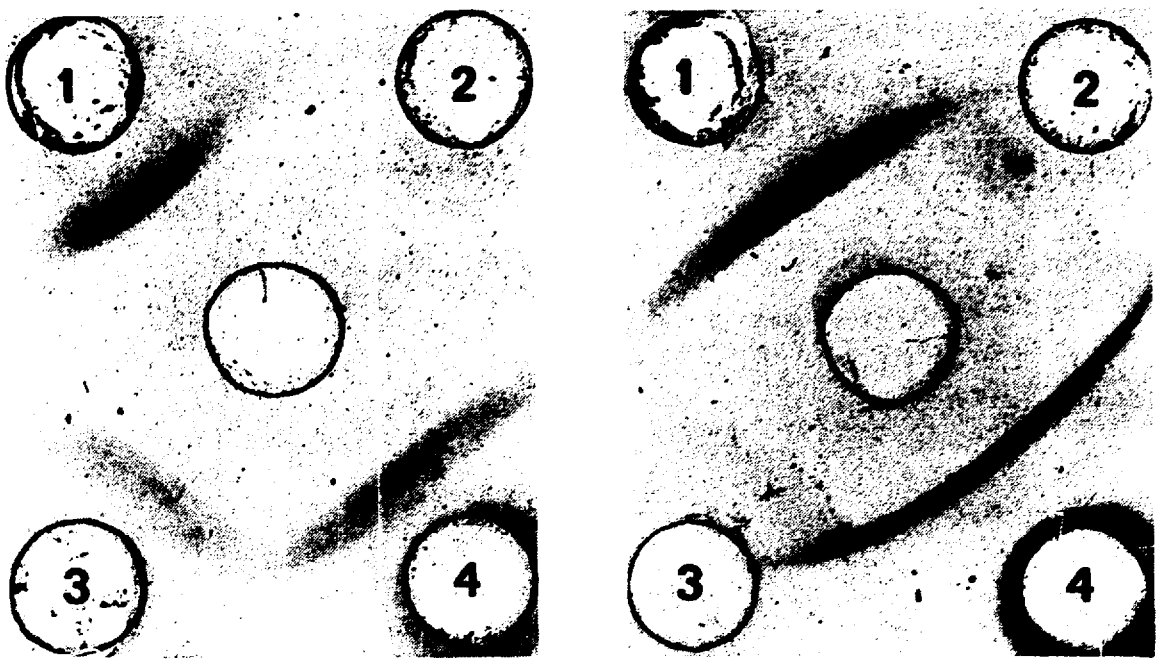

Fig. 4. Immunodiffusion in 0.1 M Tris–HCl buffer (pH 7.4) containing 2 % Triton X-100. Numbers: 1, heavy plasma membranes of human lymphocytes (120 μg protein content); 2, human erythrocyte plasma membranes (120 μg protein content); 3, ribosomes of human lymphocytes (120 μg protein content); 4, cytosol of human lymphocytes (30 μg protein content); centre well: anti-human immunoglobulin serum G from rabbit. Only the erythrocyte membranes do not form a precipitation line.

Fig. 5. Immunodiffusion under the conditions and with the cell fractions described in the legend to Fig. 4. Centre well: anti-human serum albumin from the rabbit. Erythrocyte membranes and ribosomes from lymphocytes do not form precipitation lines.

plasma membrane samples precipitated well with both antisera. In order to be certain that these results were not caused by protein contamination of the membranes, further control tests were performed. Experiments with human erythrocyte membranes did not result in precipitation lines. Ribosomes and plasma membranes of lymphocytes in a Tris–HCl buffer without Triton X-100 exhibited barely visible precipitation lines, indicating a stronger interaction between the organelles and the examined proteins. The cytosol, on the other hand, did not show this dependence on solubilization conditions but always gave strong precipitation lines.

DISCUSSION

One of the major problems during our work was obtaining a sufficiently pure lymphocyte population. Pure populations can be isolated using various procedures

such as separation on glass bead columns[26], sedimentation in density gradients with the addition of dextran[5], equilibrium density centrifugation on gradients of albumin[27] or ficoll[28], or separation by free flow electrophoresis[29]. For preparative purposes our filtration procedure seemed to be of particular advantage. A purity of 90 % can be obtained by using a double layer of silk cloth of 50 μm pore size, the finest cloth commercially available at the present time. Using the previous described method by Allan and Crumpton[3] for pig lymphocytes from lymph nodes, we have found considerable contamination by reticulum cells and fibrocytes from the tonsils when performing the homogenization procedure with a mincer and subsequent filtration through one layer of muslin cloth.

A striking difference between the sedimentation behaviour of subcellular organelles from pig and human lymphocytes was observed. For the differential centrifugation of cell organelles we found it necessary to use a 2–4-fold stronger centrifugal force than the conditions described by Allan and Crumpton[3]. These authors obtained the plasma membranes of pig lymphocytes between two layers of 30 % and 40 % sucrose. Under the same conditions, we found in this range the smooth endoplasmic reticular fraction not isolated by these authors. In this connection it appears interesting that Barber and Jamieson[30] isolated two plasma membrane fractions from human blood platelets with densities of 1.09 and 1.12 under isopycnic conditions. This is equal to sucrose concentrations of about 22 % and 28.5 % (ref. 31) and corresponds to the range where we found plasma membranes of human lymphocytes. In contrast to this, Evans[32] isolated two plasma membrane subfractions from mouse and rat livers with densities of 1.12 and 1.18, respectively, equal to about 28.5 % and 40.5 % sucrose and corresponding to the range where plasma membranes from pig lymphocytes were obtained.

The specific activity of 5′-nucleotidase, which is a recognized marker of the plasma membrane, was increased 14-fold and 13-fold, respectively, over the homogenate in our so-called "light" and "heavy" plasma membrane fractions. This increase of 5′-nucleotidase activity is of the same order as found for plasma membranes of pig lymphocytes[3] and rat liver cells[33–35]. Furthermore, the two plasma membrane fractions possessed very high cholesterol:protein ratios (0.263 and 0.210, respectively) and phospholipid:protein ratios (0.706 and 0.608, respectively) as compared with the endoplasmic reticular fractions. Allan and Crumpton[3] found a similar cholesterol:protein ratio (0.217) but a much lower phospholipid:protein ratio (0.438) in the plasma membranes of pig lymphocytes. For this reason their cholesterol·phospholipid molar ratio was very high (1.01). We calculated cholesterol:phospholipid molar ratios of 0.75 and 0.69 for the two plasma membrane fractions of human lymphocytes, respectively. A ratio of the same order was found for plasma membranes of guinea pig liver[36], whereas the ratios reported for plasma membranes of rat liver varied between 0.26 (ref. 37) and 0.8 (ref. 38).

Our "light" plasma membrane fraction between 15 % and 24 % sucrose sedimented more quickly during differential centrifugation than the "heavy" plasma membrane fraction, thereby contaminating the crude mitochondrial fraction. Furthermore, the "light" plasma membrane fraction exhibited slightly more contamination by endoplasmic reticulum, mitochondria and cytosol than the "heavy" fraction. Finally, the yield of the "light" fraction was reduced under stronger homogenization conditions. These observations indicate that the "light" fraction consists of larger

vesicles with a density closer to that of whole cells. In accordance with this view, Barber and Jamieson[30] found larger vesicles in the "light" subfraction of human blood platelet membranes than in the "heavy" subfraction. In contrast to the "light" fraction, the "heavy" plasma membrane fraction was contaminated to a higher degree by lysosomes, a problem which we were unable to overcome. During differential sedimentation lysosomes sedimented only slightly faster and during isopycnic centrifugation most of them exhibited the same density as "heavy" plasma membrane vesicles. The different behaviour of the two acid phosphatases studied was interesting, indicating that lysosomes are not enzymically homogeneous[39]. When one considers that cytoplasmic and nuclear acid phosphatases[40] could also be present, the situation becomes more difficult to interpret. An increase in acid phosphatase activity of the same order compared to the homogenate was found in plasma membranes of rat liver[41]. A mitochondrial contamination of the same order as we found in our plasma membrane preparations has been described before[33-35,42].

The presence of serum albumin in all cell fractions (with the exception of ribosomes), as indicated by immunodiffusion, may be an interesting feature. Significant contamination of the membrane fractions by the supernatant can be ruled out by considering the 500-fold reduction of lactate dehydrogenase activity in the "heavy" plasma membrane fraction in comparison with the activity of the cytosol. The lack of albumin in human erythrocyte membranes, which were washed only 3 times after haemolysis, is a further assurance that plasma membranes did not adsorb supernatant proteins. Recently, R. S. Smith *et al.*[43] also found serum albumin in washed human lymphocytes and carried out a quantitative determination by a radioimmunochemical technique. J. L. Smith *et al.*[44], on the other hand, detected synthesis of various proteins but not of albumin in human lymphocytes. Considering all of these results, the assumption that albumin in the lymphocytes derives from phagocytosis would be strongly supported. Both phagocytosis and antibody production in the same lymphocytes has recently been described[45].

The presence of γ-globulin was indicated in all subcellular fractions by polyacrylamide gel electrophoresis and demonstrated in the plasma membrane and ribosomal fractions by immunodiffusion. It has been known for a long time[46] that synthesis of γ-globulin is carried out in human lymphocytes; however, it has been only in the last few years that immunoglobulin determinants have been recognized as part of the plasma membranes of lymphocytes. It was assumed that primarily light chains were present in human lymphocytic surface structures. Staining by immunofluorescence of surface heavy chains remained at an insignificant level over the background, whereas light chains stained well[47]. The authors attempted to explain this in terms of synthesis of light chains in excess over heavy chains or of inaccessibility of the heavy chain determinants for staining. Bert *et al.*[48] found that the addition of anti-light-chain sera reduced the electrophoretic mobility of human lymphocytes whereas the addition of individual antisera to γ, α or μ heavy chains failed to modify the electrophoretic mobility. A significant reduction in mobility was obtained when the cells were treated with a mixture of the three anti-heavy-chain sera. The authors concluded that individual lymphocytes may carry all heavy chain specificities on their surface and that treatment with only one monospecific antiserum causes insufficient change in surface charge. Greaves[49] demonstrated, by the "rosette" formation test, the existence of both μ and γ heavy chains on the surface of mouse lymphocytes a few

days after immunization. Recently, Baur *et al.*[50] isolated heavy and light chains after reduction and alkylation of radioactively labelled surface immunoglobulins from human lymphoma cells and mouse myeloma and spleen cells. Our results now indicate that the plasma membranes of normal human lymphocytes also contain whole immunoglobulin molecules. The possible existence of free heavy and light chains cannot be proved in the complex protein pattern of our electrophoresis. Subfractionation of the membranes combined with immunological examination will show whether free immunoglobulin chains occur or not.

Further attention will be given to the faint protein zone of molecular weight of about 45000 which might be plasma membrane specific. As shown with erythrocyte plasma membranes, most monomeric membrane proteins are to be found in this range of molecular weight[6]. Examination of plasma membrane proteins will be carried out with special attention to immunological functions. It is as yet unknown whether lymphocytic receptors represent whole immunoglobulin molecules, free immunoglobulin chains or possibly other, still undetected molecules. One interesting approach for the isolation of antigen receptors is the use of an affinity label[51]. Furthermore, investigations will include differences between thymic-dependent and independent lymphocytes, as suggested by the difference in θ-isoantigen described by Ra[52] for mouse lymphocytes.

ACKNOWLEDGEMENTS

I am grateful to Dr J. Simon for making available the facilities of the Division of Immunopathology. My thanks go to Dr J. Simon and to Dr E. Mehl for their useful discussions. I wish to acknowledge the excellent technical assistance of Miss Antje Hartmann and Miss Ruth Ziegler. I am indebted to Dr W. Michels, head of the ear, nose and throat department of the Städtisches Krankenhaus München-Schwabing for generous supplies of freshly removed tonsils. The investigations were supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Roitt, I. M., Greaves, M. F., Torrigiani, G., Brostoff, J. and Playfair, J. H. L. (1969) *Lancet* ii, 367–371
2 Fischer, H., Rüde, E. and Sellin, D. (1970) *Naturwissenschaften* 57, 507–513
3 Allan, D. and Crumpton, M. J. (1970) *Biochem. J.* 120, 133–143
4 Allan, D. and Crumpton, M. J. (1971) *Biochem. J.* 123, 967–975
5 Böyum, A. (1968) *Scand. J. Clin. Lab. Invest.* 21, Suppl. 97, 51–76
6 Demus, H. and Mehl, E. (1970) *Biochim. Biophys. Acta* 203, 291–297
7 Miller, G. L. (1959) *Anal. Chem.* 31, 964
8 Munro, H. N. and Fleck, A. (1966) in *Methods of Biochemical Analysis* (Glick, D., ed.) Vol. 14, Wiley–Interscience, New York, pp. 113–176
9 Ceriotti, G. (1952) *J. Biol. Chem.* 198, 297–303
10 Norton, W. T. and Autilio, L. A. (1966) *J. Neurochem.* 13, 213–222
11 Hess, H. H. and Thalheimer, C. (1965) *J. Neurochem.* 12, 193–204
12 Stadtman, T. C. (1957) *Methods Enzymol.* 3, 392–394
13 Michell, R. H. and Hawthorne, J. N. (1965) *Biochem. Biophys. Res. Commun.* 21, 333–338
14 Swanson, M. A. (1950) *J. Biol. Chem.* 184, 647–659
15 Emsande, J. and Ephrussi, B. (1964) *Science* 144, 854–856
16 Roske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–400
17 Lohnhardt, K. and Walter, K. (1962) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.) pp. 779–785, Verl ; Chemie, Weinheim/Bergstr.

18 Gianetto, R. and de Duve, C. (1955) *Biochem. J.* 59, 433–438
19 Adams, C. W. M., Davison, A. N. and Gregson, N. A. (1963) *J. Neurochem.* 10, 383–395
20 Bergmeyer, H. U., Bernt, E. and Hess, B. (1962) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., ed.) pp. 736–740, Verlag Chemie, Weinheim/Bergstr.
21 Shapiro, A. L., Vinuela, E. and Maizel, Jr., J. V. (1967) *Biochem. Biophys. Res. Commun.* 28, 815–820
22 Demus, H. and Mehl, E. (1970) *Biochim. Biophys. Acta* 211, 148–157
23 de Lange, R. J. and Smith, E. L. (1971) *Ann. Rev. Biochem.* 40, 279–314
24 Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishausen, U. and Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 78–85
25 di Girolamo, M. and Cammarano, P. (1968) *Biochim. Biophys. Acta* 168, 181–194
26 Shortman, K. (1969) in *Modern Separation Methods of Macromolecules and Particles* (Gerritsen, T., ed.), pp. 91–103, Wiley-Interscience, New York
27 Shortman, K. (1968) *Aust. J. Exp. Biol. Med. Sci.* 46, 375–396
28 Gorczynski, R. M., Miller, R. G. and Phillips, R. A. (1970) *Immunology* 19, 817–829
29 Zeiller, K., Holzberg, E., Pascher, G. and Hannig, K. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 105–110
30 Barber, A. J. and Jamieson, G. A. (1970) *J. Biol. Chem.* 245, 6357–6365
31 de Duve, C., Berthet, J. and Beaufay, H. (1959) *Progr. Biophys.* 9, 325–369
32 Evans, W. H. (1970) *Biochem. J.* 116, 833–842
33 Stein, Y., Widnell, C. and Stein, O. (1968) *J. Cell Biol.* 39, 185–192
34 Berman, H. M., Gram, W. and Spirtes, M. A. (1969) *Biochim. Biophys. Acta* 183, 10–18
35 Nigam, V. N., Morais, R. and Karasaki, S. (1971) *Biochim. Biophys. Acta* 249, 34–40
36 Coleman, R. and Finean, J. B. (1966) *Biochim. Biophys. Acta* 125, 197–206
37 Ashworth, L. E. A. and Green, C. (1966) *Science* 151, 210–211
38 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rümke, P. (1964) *Biochim. Biophys. Acta* 90, 126–145
39 Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R. and de Duve, C. (1959) *Biochem. J.* 73, 628–637
40 Love, R., Studzinski, G. P. and Walsh, R. J. (1969) *Exp. Cell. Res.* 58, 62–72
41 Henning, R., Kaulen, H. D., Stoffel, W. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351, 1191–1199
42 Coleman, R., Michell, R. H., Finean, J. B. and Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* 135, 573–579
43 Smith, R. S., Longmire, R. L., Reid, R. T. and Farr, R. S. (1970) *J. Immunol.* 104, 367–376
44 Smith, J. L., Lawton, J. W. M. and Forbes, I. J. (1967) *Aust. J. Exp. Biol. Med. Sci.* 45, 629–643
45 Noltenius, H. and Chahin, M. (1969) *Naturwissenschaften* 56, 140
46 Ortega, L. G. and Mellors, R. C. (1957) *J. Exp. Med.* 106, 627–640
47 Hellström, U., Zeromski, J. and Perlmann, P. (1971) *Immunology* 20, 1099–1111
48 Bert, G., Massaro, A. L., di Cossano, D. L. and Maja, M. (1969) *Immunology* 17, 1–6
49 Greaves, M. F. (1971) *Eur. J. Immunol.* 1, 186–194
50 Baur, S., Vitetta, E. S., Sherr, C. J., Schenkein, I. and Uhr, J. W. (1971) *J. Immunol.* 106, 1133–1135
51 Hill, W. C. (1971) *J. Immunol.* 106, 414–420
52 Raff, M. C. (1969) *Nature* 224, 378–379
53 Romeis, B. (1968) Mikroskopische Technik, 16th edn, R. Oldenbourg Verlag, München–Wien, p. 341