rinse away mucus in the epididymal lumen. The specimens were postfixed in osmium tetroxide for 2 h, dehydrated in an ascending acetone series and dried by the critical point method using carbon dioxide. They were then coated with gold by ion sputter coating method and viewed in a Hitachi field emission scanning electron microscope (HFS-2). The remaining half of the specimens was fractured by frozen resin cracking method prior to the critical drying.

Results and discussion. In the epididymal ducts of the vasectomized animals, there are fragmented or disintegrated spermatozoa in a high concentration. The macrophages are present here and there among these disintegrated spermatozoa. The macrophages vary in shape from spherical to flattend, probably depending upon functional stage, and possess short ridge-like microvilli loosely distributed on their surface to which the fragmented portions of disintegrated spermatozoa adhere (figure 1). The fractured surfaces of the specimens reveal various stages of spermiophagy by the macrophage. Macrophage seen in figure 2 is relatively irregular in shape and engulfes in bulk such fragments of disintegrated spermatozoa as head and tail by flap-like cytoplasmic extensions. Figure 3 shows a surface of another macrophage fractured through the nucleus. This macrophage is more spherical than that in figure 2 and has no prominent cytoplasmic process. Its cytoplasm contains a number of fusiform inclusions suggestive of ingested sperm heads in addition to many spherical bodies presumably corresponding to lysosomes or residual bodies. The macrophage shown in figure 3 may be in more advanced stage of phagocytosis than that in figure 2. There is no morphological evidence for uptake of spermatozoa by epithelial cells in any region of the epididymal duct. The results indicate that disposal of disintegrated spermatozoa in the epididymal duct of the vasectomized Japanese monkey may be managed mainly, although not only, by macrophages appeared in the lumen as the results of the reports on this problem so far. The origin of the macrophage remains still obscure[1, 2, 4].

Recombination of integral and peripheral protein fractions from human red cell membrane with homologous lipids

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Summary. Integral and peripheral protein fractions from human red cell membranes were recombined with a total red cell lipid extract and with homologous lipids in varying mixtures, by dialysis from 2-chlorethanol solutions. The 2 protein fractions were compared for lipid binding capacity and for selectivity towards individual lipids.

Red blood cell membrane proteins and lipids can be dissociated and recombined, in a variety of experimental conditions, to form membrane-like structures which lack enzymatic activities[1–5]. Membranes which are formed by dialysing 2-chlorethanol (2-CE) solutions of membrane proteins and lipids against neutral aqueous buffer, possess but few intramembranal particles[6]. It has been shown, however, that the membrane protein structure is preserved sufficiently to retain lipid-binding specificity[7].

The membrane proteins have been classified as integral and peripheral[8, 9]. While the peripheral proteins interact weakly with lipids, the integral proteins exhibit strong hydrophobic interactions with the lipid bilayer whom they penetrate[8–11]. In this report we present a comparative study on the lipid-binding capacity and selectivity for lipid of the integral and peripheral protein fractions from human red cell membranes. We compared, for each protein fraction, the binding of charged and uncharged phospholipids and of neutral lipid, both from a total red cell lipid extract and from varying mixtures.

Materials and methods. Ghosts were prepared from human blood stored in acid-citrate-dextrose for 22–23 days according to the method of Fairbanks et al.[12], the 5 mM sodium phosphate buffer (pH 8) being supplemented with trasylol (19 kIU/ml). Peripheral proteins were obtained by successively extracting the ghosts with 1 mM EDTA[12] and H_2O at pH 11[13]. The proteins in the combined extracts were precipitated with ammonium sulphate at 50% saturation, freed from ammonium sulfate by dialysis, lyophilized and stored as dry powder at 4 °C. The peripheral proteins contained 0.0197 μmoles N-acetylneuraminic acid per mg protein. Acrylamide gel electrophoresis showed the predominance of protein bands I, II and V (figure).

The integral protein fraction was prepared from the residual ghost fragments by extraction with 8 M urea-1 mM EDTA (pH 8) – 1% β-mercaptoethanol[13]. Following dialysis, the pellet was delipidated[13]. Integral proteins were stored as a dry powder at 4 °C. This fraction contained 0.1222 μmoles N-acetylneuraminic acid per mg protein, and residual phosphatidylserine. Acrylamide gel electrophoresis shows the predominance of band III in this fraction (figure).

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Lipids were extracted from human red cell membranes with chloroform-methanol[14] and the extract washed with 0.58% NaCl solution[15]. Fractionation of the total lipid was performed on alumina (Al_2O_3) columns[16]. Elution of the column with organic solvents of increasing polarity yielded the cholesterol fraction, the phosphatidylcholine (PC)-sphingomyelin (SM) fraction and the phosphatidylethanolamine (PE) fraction. Phosphatidylserine (PS) was prepared from calf brain[17,18]. All columns were monitored

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Table 1. Relative amounts of protein and lipid in recombinates from integral and peripheral red cell membrane proteins

Lipids	Mixture in 2-CE	Recombinates Integral proteins			Peripheral proteins		
	Pl/Pr w/w	Pl/Pr w/w	Chol/Pr w/w	Chol/Pl w/w	Pl/Pr w/w	Chol/Pr w/w	Chol/Pl w/w
Total lipid extract*	1	0.78 ± 0.22(7)	0.35 ± 0.1(7)	0.45 ± 0.1(7)	0.93 ± 0.1(8)	0.47 ± 0.1(8)	0.52 ± 0.17(8)
	2.5	1.39	0.48	0.34	1.37	0.67	0.49
	2.5	1.53	0.42	0.27	1.46	0.90	0.62
PC-SM-Chol.*	1	1.13 ± 0.09(6)	0.31 ± 0.1(6)	0.28 ± 0.04(6)	0.76 ± 0.06(6)	0.26 ± 0.04(6)	0.34 ± 0.04(6)
PE-Chol.*	1	1.09 ± 0.1(4)	0.30 ± 0.13(4)	0.28 ± 0.13(4)	0.87 ± 0.04(5)	0.27 ± 0.12(5)	0.33 ± 0.18(5)
	5	1.78	0.20	0.11	–	–	–
	10	–	–	–	1.22	0.16	0.13
PS-Chol.**	1	1.61 ± 0.05(4)	0.87 ± 0.17(4)	0.54 ± 0.11(4)	1.28 ± 0.1(4)	0.66 ± 0.06(5)	0.53 ± 0.04(4)
	5	4.62	1.88	8.41	3.64	1.40	0.39
	7	–	–	–	5.08	2.53	0.50

For the standard phospholipid/protein ratio = 1, results are given as means ± SD for the number of experiments indicated in parenthesis. Significance of differences is given in the text. * In recombination with this lipid mixtures an upper layer of free lipid was separated following centrifugation in a sucrose gradient. ** No free lipid was separated following sucrose gradient centrifugation, except in the experiments with peripheral proteins in ratios 5 and 7. Abbreviations: Pl, Phospholipid; Pr, protein; Chol., cholesterol; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Table 2. Recombination of integral and peripheral proteins with red cell lipid extracts. Phospholipid distribution

Protein fraction	Pl/Pr in 2-CE w/w		Phospholipid (%) PE	PI*	LPE*	PA	PS**	PC	SM	LPC	Ratio PC + LPC/SM
Integral	1	Lipid extract	26.3	1.1	0	0.9	13.3	30.2	26.3	1.9	1.22
		Recombinate	19.3	10.3		0	16.2	35.0	16.9	2.3	2.21
		Upper layer	12.2	5.4		0	7.4	32.9	40.1	2.0	0.87
	1	Lipid extract	27.0	0.7	0	1.2	11.8	30.0	27.6	1.8	1.15
		Recombinate	16.7	1.0	11.3	2.9	11.2	33.9	20.2	2.7	1.81
		Upper layer	11.7	15.3		0	9.7	25.0	35.7	2.5	0.77
	2.5	Lipid extract	26.3	1.1	0	0.9	13.3	30.2	26.3	1.9	1.22
		Recombinate	14.7	12.7		0	10.2	37.3	20.8	4.3	2.00
		Upper layer	12.3	9.1		0	11.2	27.9	36.9	2.7	0.83
Peripheral	1	Lipid extract	26.3	1.1	0	0.9	13.3	30.2	26.3	1.9	1.22
		Recombinate	15.4	14.2		0	7.1	35.5	25.1	2.7	1.52
		Upper layer	15.9	7.1		0	6.8	28.2	39.5	2.3	0.77
	1	Lipid extract	26.3	0		3.6	19.6	29.7	20.8	0	1.40
		Recombinate	10.8	15.5		0.2	2.8	26.7	26.9	17.1	1.63
		Upper layer	12.6	13.7		0	1.3	22.7	40.4	9.3	0.79
	2.5	Lipid extract	26.3	1.1	0	0.9	13.3	30.3	26.3	1.9	1.22
		Recombinate	16.2	15.4		0	9.6	31.4	24.4	3.0	1.41
		Upper layer	15.1	11.4		0	9.6	29.8	30.4	3.7	1.10

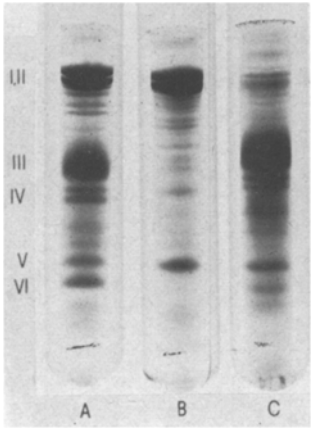
* PI and LPE separate poorly on their layers. When individually assayed, the values are listed in the respective colomns. When assayed together, the values appear as sum of PI + LPE. The increase in PI + LPE in recombinates and upper layer derives from PE hydrolysis during recombination (see corresponding decrease in PE values). ** Partial hydrolysis during recombination lowers the PS values in the recombinates of peripheral proteins and their upper layer. Residual PS firmly associated with the integral proteins increases the PS values in the respective recombinates. Abbreviations: Pl, phospholipid; Pr, protein, 2-CE, 2-chlorethanol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine.

Table 3. Recombination of integral and Peripheral proteins with phosphatidylcholine-sphingomyelin-cholesterol mixture
Phospholipid distribution

Protein fraction		Phospholipid (%) PC	SM	LPC	PS	Ratio PC/SM
Integral	Lipid mixture	27.0	60.0	13.0	0	0.45
	Recombinate	34.5	57.1	6.2	2.1*	0.60
	Upper layer	20.4	72.3	7.2	0	0.28
	Lipid mixture	30.7	54.7	14.6	0	0.56
	Recombinate	34.3	56.1	4.9	4.7*	0.61
	Upper layer	25.7	67.5	6.8	0	0.38
Peripheral	Lipid mixture	27.0	60.0	13.0	0	0.45
	Recombinate	27.7	65.5	6.8	0	0.42
	Uppar layer	32.2	61.9	5.9	0	0.52
	Lipid mixture	30.7	54.7	14.6	0	0.56
	Recombinate	38.4	59.4	2.2	0	0.65
	Upper layer	27.6	63.8	8.6	0	0.43

* Residual PS is associated with the integral protein fraction after delipidation. Abbreviations as in table 2.

by TLC. The total lipid extract contained 0.54 mg cholesterol per mg phospholipid. The lipid fractions were supplemented with 0.5 mg cholesterol per mg phospholipid. For recombination, protein and lipid solutions in freshly distilled 2-CE[5] were mixed yielding a solution which contained, in standard conditions, 1 mg/ml protein, 1 mg/ml phospholipid, and the corresponding amount of cholesterol (0.54 or 0.5 mg/ml). A number of experiments were performed with mixtures of increased lipid concentration. Following dialysis against 10 mM Tris-HCl (pH 7.6) containing 10 mM $CaCl_2$ and 1 mM $MgCl_2$, insoluble material appeared and was collected by centrifugation at 9.5×10^5 g max. min[5]. About 90% of the protein together with 96% of the lipid were recovered in the sediments. The sediments obtained after recombination were suspended in 2 ml of the dialysis buffer, sonicated at maximal intensity at 0 °C for 30 sec and layered over 28 ml of a linear sucrose gradient (13–50% sucrose in the same buffer). The gradients were centrifuged at 24.000 rpm in an SW 25.1 rotor of a Spinco Model L ultracentrifuge for 18 h at 4 °C. The recombinates and the floating upper lipid layer were separately collected and analyzed. All samples were dialyzed against bidistilled water prior to the assays.



Acrylamide gel electrophoresis of the protein fractions used for recombination. A, red cell membranes; B, peripheral proteins; C, integral proteins. The main polypeptide bands are numbered according to Fairbanks et al.[12].

Protein was measured by the method of Lowry et al.[19], with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate was performed according to Weber and Osborn[20] and Maizel[21] as described before[4]. Sialic acid was estimated according to Warren[22] and cholesterol was determined by the method of Zlatkis et al.[23]. Lipids were extracted according to Marinetti et al.[14]. Lipid phosphorus was determined by the method of Bartlett[24] and phospholipid was estimated by multiplying the lipid phosphorus content by 25. The individual phospholipids were separated by 2 dimensional TLC and estimated as described by Condrea et al.[25].

Results and discussion. The sucrose gradients achieved separation of a protein-lipid recombinate, a sediment of unrecombined protein and, in most cases, a floating layer of unrecombined lipid. The sedimented protein presented the same pattern on SDS-acrylamide gels as the soluble fraction. As seen in table 1, the phospholipid/protein (Pl/Pr) ratios in recombinates derived from mixtures of different lipid composition were variable, the highest values being obtained with PS-cholesterol. In standard conditions (Pl/Pr = 1 in 2-CE), PS-cholesterol recombined completely with both protein fractions as indicated by the absence of a free lipid layer in the gradient. With the integral proteins the recombination was complete even at a 5fold increase in lipid concentration, suggesting a very high affinity for PS. The recombinates of the integral proteins had higher Pl/Pr mean ratios than those of the peripheral proteins both when obtained from PS-cholesterol ($p < 0.001$), from PE-cholesterol ($p < 0.001$) or PC-SM-cholesterol ($p < 0.001$), but not from a total lipid extract (table 1). The higher affinity for lipid of the integral proteins, which is in agreement with their hydrophobic character, was best evidenced in recombination with concentrated anionic phospholipid.

The amounts of cholesterol recovered in recombinates suggest that the binding of cholesterol from lipid mixtures depends on the accompanying phospholipids. Thus, in the recombinates obtained from a total lipid extract, or from PS-cholesterol mixtures the cholesterol/phospolipid (chol/Pl) mean ratios were close to the original values of 0.54 and 0.50 respectively. In the recombinates obtaned from PC-SM and PE supplemented with cholesterol, the chol/Pl mean ratios were significantly lower ($p < 0.001$) (table 1).

We further investigated whether there is specificity in binding of individual phospholipid species by the proteins. As shown in table 2, in the recombinates of a red cell lipid extract with both protein fractions the percentage of SM was lower and that of PC higher than in the original lipid extract. A neglible hydrolysis of PC occurred during the procedure, as illustrated by the low lysophosphatidylcholine (LPC) values recovered from the gradients; we included this value for the calculation of

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the (PC+LPC)/SM ratios. These were increased in the recombinates and correspondingly decreased in the upper lipid layer. It is noteworthy that the high affinity for PS shown by both protein fraction when recombined with the single phospholipid was not manifested in the recombinates obtained from total lipid extracts, suggesting that the binding of a individual phospholipid species is influenced by the presence of other lipids.

The phospholipid distribution in the experiments using the PC-SM-cholesterol mixture is given in table 3. PC-SM-cholesterol mixture used in the recombination experiments contained initially 13–15% LPC (see 'Lipid Mixture' table 3), an amount which was not well recovered from either the recombinates or the upper lipid layer. However, since our results (table 2) and those of others[7] show that there is no significiant hydrolysis of PC during the recombination procedure we were able to compare the PC/SM ratios. In 2 experiments with integral proteins and 1 out of 2 with peripheral proteins, we found an increase in PC/SM ratio in the recombinates, paralled by a decrease in the PC/SM ratio in the free lipid upper layer.

Phospholipid distribution in recombinates obtained with a total red cell lipid extract and with a PC-SM-cholesterol mixture showed, for both protein fractions, a preference for binding of PC at the expense of SM. This finding is in agreement with the results obtained by Kramer et al.[7]. The data presented herein confirm the conclusion of Kramer et al.[7] and Wehrli et al.[6] that in membrane recombinates obtained by dialysis from 2-CE solutions, the protein structure is preserved sufficiently to retain lipid binding capacity and specificity.

Delayed-type skin reactions in bursectomized or thymectomized chickens[1]

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Summary. Chickens can easily be induced to develop delayed-type skin reactions to oxazolone when animals are sensitized 7 days before the challenge. The reaction is quantitated by assessing the increase in wattle thickness: maximum reactions occur 24 h after challenge. The reaction is inhibited by neonatal thymectomy or bursectomy; these findings therefore suggest also an important B-derived component in delayed hypersensitivity to oxazolone.

Oxazolone-induced skin reaction in the mouse has proved to be a complex and informative model in elucidating cellular events in delayed-type hypersensitivity. Thymus-derived cells are necessary to induce contact sensitivity and both these and bone-marrow cells, probably monocytes[2], are needed for developing in vivo manifestations of the delayed hypersensitivity. Whereas thymus-derived cells are essential to develop delayed reaction to oxazolone in the mouse[3], there is no definite evidence about a similar function of bone-marrow-derived lymphocytes. Some authors suggested a B-cell participation in this reaction because they observed a proliferation of these cells in the draining lymph-nodes which show germinal center development peaking at 8 days from sensitization[4,5].

The function of this proliferation in the reaction remains unclear, because mice lethally irradiated and reconstituted with B-cells do not develop contact sensitivity to oxazolone[6]. In a preliminary report, we demonstrated by skin test that chickens develop oxazolone hypersensitivity and neonatally irradiated and thymectomized chickens, like thymectomized mice[6], show a depressed skin reaction to oxazolone[7].

The problem of B-cell participation in the delayed-type hypersensitivity can be studied further with advantage in birds, since they possess a separate organ, the bursa of Fabricius, site of production of B-lymphocytes. The results presented seem to show that also the bursa of Fabricius is important in the development of contact sensitivity to oxazolone in chickens.

Materials and methods. Male Hubbard chicks obtained from Nutrix (Palermo) were bursectomized or thymectomized within 5 h after hatching. Some thymectomized chickens, as well as unoperated birds, were irradiated with 550 rads as previously described[8]. Autopsies were performed on all operated birds at the end of the experiment, and chickens showing thymic residues were excluded from the results. Chickens were sensitized with 33 mg/kg of oxazolone (ethoxymethylene-2-phenyl-oxazolone, BDH chemicals Ltd) in 0.5 ml of absolute ethanol applied to the previously shaved anterior region of the neck. Control animals received only absolute ethanol. Chickens were challenged 7 days later with 25 mg of oxazolone in olive oil applied to the 2 wattles. The reaction was tested with a screw gauge micrometer by measuring the increase of the wattle thickness at 6, 24 and 48 h after challenge. Wattle thickness was expressed as percent average of increase. Results were analyzed by Student's t-test.

Results and discussion. Our initial experiment reconfirms the previous finding[7] that chickens can be easily induced to develop contact sensitivity by painting the wattles with the sensitizing agent, and quantitated by assessing the increase in wattles thickness; maximum reactions occur 24 h after challenge, smaller increases in wattles thickness are also detected 6 and 48 h after challenge. Reactions are characterized by wattle swelling accompanied by slight ischemia and occasionally at 48 h by detachment of skin and/or necrosis. By contrast, oxa-

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