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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

RED CELL RECEPTORS FOR ARBORVIRUSES

THE CELL LIPIDS*

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The interaction between arborviruses and lipids is characterized by the nature of the serological inhibitors [13]. The variety of the reactions associated with viral hemagglutination is well known.

We thus felt that the interaction of arborviruses and red blood cells should be reinvestigated, with particular emphasis on:

- 1° Identification of the receptors on the intact cell.
- 2° Isolation and analysis of the compounds responsible for this activity.

The inhibitory effect of total lipids, extracted from red cells, on the hemagglutination caused by arborviruses has already been demonstrated by Salminen [19] as well as by a preliminary study of Porterfield and Rowe [16] made with lipids from human erythrocytes. The significance of the separation of inhibiting lipids will be discussed from this point of view.

Without prejudging their final identification, the hemagglutinins of the arborviruses will, in this investigation, be

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identified with the virions. This seems justified in view of:

1° The persistence of the infectious capacity of the preparations.

2° The chromatographic behavior of the hemagglutinating and infectious properties which are the subject of parallel investigations carried out in this laboratory.

MATERIAL AND METHODS

1° Virological Techniques

a) Strain: Arboviruses of group A: Sindbis A.R. 339, Chikungunya S. 27; group B: West Nile B. 956, amaril virus, French neurotropic strain; group D: Bunyamwera M.R. 376.

b) Extraction of viral hemagglutinins by means of the saccharose acetone method from young mouse brain infected according to the Clarke and Casals method [3].

c) Determination of the inhibition power by incubation of variable amounts of the material to be tested with four doses of viral hemagglutinates. The tests were carried out at the optimum pH for hemagglutination under normal conditions (dilution of the virus with protein-borate buffer, pH 9, and dilution of the inhibiting material with isotonic phosphate buffer so adjusted that the mixture of the two buffers yields the desired pH). After verifying that there is no excess cold fixation, the inhibition is carried out for thirty minutes at room temperature.

Emulsification makes it impossible to obtain reproducible, quantitative measurements of the lipids. The orientation of the lipids at the interface of the two phases can cast some doubt on the qualitative results. It is thus important to make a careful water suspension after the evaporation of the solvent.

d) Titration of the residual hemagglutinins at 37°C against goose red cells.

2° Chemical Techniques

a) Extraction of cell lipids, at room temperature, with a 2:1 chloroform-methanol mixture, according to Folch's technique [7]. At each operation 10 ml of globular residue, previously washed three times with Alsever solution are extracted

for twenty minutes with twenty volumes of the mixture. The cells are agitated during this operation. The organic phase is collected. The residue is crushed and twice reextracted. The organic phases are combined and evaporated, under vacuum, at below 50°C. The non-lipid material is separated, after the residue has been taken up by a 3:1 ether-water mixture, by washing with acetone to which 1/4 vol NaCl (18%) has been added. The ether-acetone phase and the precipitate at the interphase are washed twice with the saline acetone, evaporated in a calibrated capsule and weighed [6]. The lipid material is finally taken up in 10 ml chloroform and kept at -30°C. The final yield is between 150 and 200 mg goose red-cell lipids. The extraction is incomplete and other lipids can be extracted at higher temperatures.

b) Separation of the lipids by chromatography on a silicic acid column (Mallinckrodt, 100 mesh), activated by heating for two hours at 110°C. 2 gm columns (fixation of 30 to 40 mg lipids) are necessary for a 20 gm analysis. Elution with 100 ml pure chloroform, then 100 ml of the following chloroform-methanol mixtures: 90:100; 80:20; 60:50; 40:60, finally with 100 ml pure methanol. 10 ml fractions collected in calibrated flasks, gravity flow. The chromatograms are made by weighing the lipids in each fraction.

c) Thin-layer chromatography on 0.25 mm thick silica gel, activated thirty minutes at 110°C. The phosphatides are separated. After trying several mixtures a chloroform-methanol water mixture (65:25:4) and the technique of Wagner [26] were chosen. The total lipids are determined by development with bromthymol blue or phosphomolybdic acid, the compounds containing choline with Draggendorf's reagent, the aminophosphatides with ninhydrin and the glycolipids with diphenylamine reagent [17, 25].

d) Alkaline hydrolysis of each fraction is carried out with alcoholic potash, reflux and two-hour long heating. In the non-saponified fraction cholesterol is titrated by Lieberman's reaction. After liberation of the fatty aldehydes with HgCl_2 , the acetals of the plasmalogens are developed with Schiff's reagent. After acidification of the aqueous phase and taking up of the fatty acids with ether, choline is determined in the watersoluble fraction by periodate formation, the sugars oxidation with HIO_4 -- by Schiff's reagent; serine and ethanolamine are characterized by ninhydrin.

e) Selective hydrolysis of lecithins and cephalines according to Hack's method [9] with 1 N aqueous KOH for eighteen hours at 37°C.

f) Titration of the lipid phosphorus, following Delsal [5], after mineralization with $H_2SO_4-H_2O_2$. Nitrogen is titrated by micro-kjeldahl with an Aminco apparatus, according to the technique of Grabar [8] Choline is titrated by Appleton's method [1].

RESULTS

1° Investigation of the Chemical Changes of the Red-Cell Surface

This investigation was carried out to determine the structures which could represent the viral receptors prior to attempt their isolation.

The different agents used modify, sometimes to a considerable extent, the sedimentation characteristics of the red cells and thus the character of the hemagglutination. The activity of each agent is determined by titrating the virus adsorption capacity of treated, washed red cells and comparing it with the values obtained for untreated cells, under normalized conditions (adsorption time: thirty minutes; red cell concentration determined photometrically and adjusted to a dilution of 120 times the cell residue. Identical reaction volumes are used).

The formal treatment of the red cells (goose or rooster) is carried out according to the technique of Daniel [4]. One volume of red cells is mixed with eight volumes of dilute formaldehyde -- diluted to 3% with phosphate buffer pH 7.2; agitated for 24 hours at $+4^\circ C$. Addition of two volumes of cold 30% formaldehyde, agitate again for 24 hours. Wash in 8 to 10 volumes of physiological saline. The adsorption capacity is not affected by this treatment. When used in hemagglutination, formaldehyde-treated cells give comparable, or slightly higher titers than untreated cells.

Trypsin and α -chymotrypsin are used in Ca^{++} buffers, 0.01 M, pH 7.9; reaction time 30 minutes at $28^\circ C$; final concentration from 0.1 to 1 mg/ml. In general the adsorption capacity increases with an increase in the enzyme concentration.

Neuraminidase is used as a filtrate from a Vibrio cholerae culture, for 30 minutes at $37^\circ C$. This interval avoids the action of hemolysine, left in the preparation.

The amount of sialic acid liberated is determined by means of Warren's method [27]. It does not change the adsorption capacity.

The periodate oxidation of the red cells is carried out by 0.001 M KIO_4 at pH 7. The course of the oxidation is followed by titration of the excess KIO_4 at 2250 Å [10 a]. The adsorption capacity is increased. At the same time a slight inhibitory power appears in the supernatant of the red cell washes. This indicates mainly that there is a structural modification of the cellular membrane by the treatment.

The phospholipase A of the venom of *Bitis gabonica* does not affect the adsorption capacity of the red cells; but this phospholipase is not directly hemolytic. This indicates that normally its substrate is not directly accessible.

Phospholipase D, from cabbage, is used at a concentration of 1 mg/ml, in a phosphate buffer pH 6.1, NaCl 0.15 M. It cleaves, classically, lecithins, cephalins and similar compounds, into choline, serine or ethanolamine. The preparation used in these experiments has a hemolytic effect on goose red cells. Treated red cells have, however, a slightly higher adsorption capacity than untreated controls.

Likewise, saponin, used at concentrations of 1/1000, slightly increases the adsorption capacity of red cells in spite of its hemolytic action.

On the other hand the residue of the extraction of red-cells with cold fat solvents (a 2:1 mixture of CHCl_3 and HCH_2OH) used as such or after desiccation and pulverization, has no adsorbant power when the same weight of red cells is used as in the previous experiments.

These results are essentially identical for all the viruses studied and are presented in Table I.

2° Spectrum of Normally Sensitive Red Cells

Arbovirus hemagglutinins agglutinate male and female goose erythrocytes, rooster erythrocytes, but not those of hens after sexual maturation. Their action extends to red cells of other animal species especially sheep, but the titers are much lower, vary from one virus to the next, and this material has not been kept for further investigation.

Reinvestigation of these already classic facts shows that the hemagglutination titers obtained with rooster erythrocytes are weaker than those obtained for goose erythrocytes, but graphs of the hemagglutinate activity as a function of pH can essentially be superimposed (Fig. 1).

Table I.

Adsorption Capacity of Treated Red Cells

(1) RÉACTIF	(2) SUBSTRAT	(3) CAPACITÉ D'ADSORPTION RÉSIDUELLE (%)
Formol	(4) Protéines	100
Trypsin : 0,1 mg/ml	Protéines	130
1 mg/ml	Protéines	140
α -chymotrypsin (0,3-1 mg/ml)	Protéines	160
Neutaminidase	(5) Acide sialique	100
KIO ₃	(6) Osés	132
Saponin	(7) Lécithines - Céphalines	107
Phospholipase D	(8) Lipides	0
CHCl ₃ , CH ₃ OH, 2 : 1		

(9) (*) La capacité d'adsorption résiduelle après traitement est exprimée en pourcentage de la capacité d'adsorption d'hématies non traitées.

1 -- Reagent; 2 -- Substrate; 3 -- Residual* adsorption capacity; 4 -- Proteins; 5 -- Sialic acid; 6 -- Sugars; 7 -- Lecithins - cephalines; 8 -- Lipids; 9 -- *The residual adsorption capacity after treatment is given as percent of the adsorption capacity of non-treated red cells.

3° Inhibition Power of the Lipids Extracted from Red Cells

Total lipids, obtained from goose, rooster or hen red cells, have, when used at high enough concentration, a hemagglutinating effect. They also have, regardless of their origin, an inhibitory effect on the hemagglutination of goose red cells by arborviruses. In inhibition reactions preliminary incubation of lipids with hemagglutinine preparations results in a corresponding decrease in the hemagglutinating ability of these lipids. This is shown in Table II. The inhibition reaction is thus clearly related to an association of the inhibiting lipids with the viral hemagglutinines.

The inhibition reaction is very strongly pH dependent, the optimum is at the optimal pH of the hemagglutination reaction. These differences are even more marked than would appear from a reading of the crude results, since the determination of the inhibition requires that, during the second phase of the reaction, that the pH be adjusted to its optimum; during this time there could be a competitive inhibition. Variations for two types of viruses and three lipid fractions are shown in Fig. 2. The investigated viruses, group B (West Nile, amaril virus) seem to be less affected by this phenomenon than the virus of group A and the Bunyamwera virus.

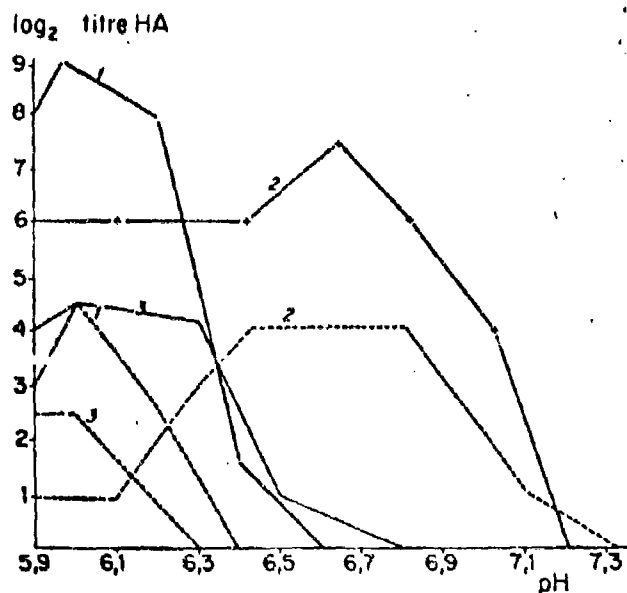


Fig. 1. Variation of the Hemagglutination Activity as a Function of pH and the Type of Cell.

1 = Sindbis, 2 = West Nile, 3 = Bunyamwera. Solid line: goose red cells. Broken line: rooster cells.

Table II.

Inhibitory Power of the Total Cell Lipids

(1) ESPÈCE D'ORIGINE	(2) TÉMOINS : LIPIDES SEULS POUVOIR HÉMAGGLUTINANT	(3) RÉACTION D'INHIBITION (LIPIDES + VIRIONS)	
		(4) Pouvoir hémagglutinant	Pouvoir (5) inhibiteur
Oie. (6).....	250	8	1 024
Coq. (7).....	2 048	8	2 048
Poule. (8).....	250	16	512

The titers of the hemagglutination are given as the reciprocal of the greatest dilution of the aqueous suspension of lipids which gives either a direct hemagglutination or inhibits viral hemagglutination.

1 -- Type; 2 -- Control: Lipids only, Hemagglutinant effect; 3 -- Inhibition Reaction (Lipids + Virions);

4 -- Hemagglutinant effect; 5 -- Inhibitory effect;
6 -- Goose; 7 -- Rooster; 8 -- Chicken.

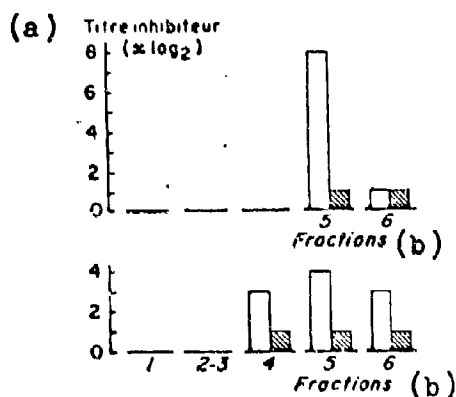


Fig. 2. Variation of the Inhibitory Power of Lipids as a Function of pH. Lipid fractions extracted from goose red cells. Inhibitory power studied: Above: towards Bunyamwera virus; Below: towards amaril virus, at the pH optimal for hemagglutination (white) and at pH 9 (broken line).

a -- Inhibiting titer; b -- Fractions.

4° Investigation of the Lipid Fractions of Goose Red Cells which are Responsible for the Inhibitory Effect

It is first ascertained that:

1° Lipids extracted from goose red cells with hot anhydrous ether, according to the method of Moskovitz and Calvin [12] are devoid of inhibitory activity.

2° That the lipids, eluted with chloroform from a silica column, are devoid of inhibitory activity; and that the inhibitory activity can be recovered in the fraction eluted by methanol (phosphatides).

3° That the inhibitory activity of the phosphatides is not increased when the cell glycerides and sterols are added.

The separation is carried out, after adsorption of the lipids on a silica column, by elution with chloroform followed by chloroform-methanol mixtures of increasing methanol concentration. The eluates are then grouped in six fractions as is shown in Fig. 3.

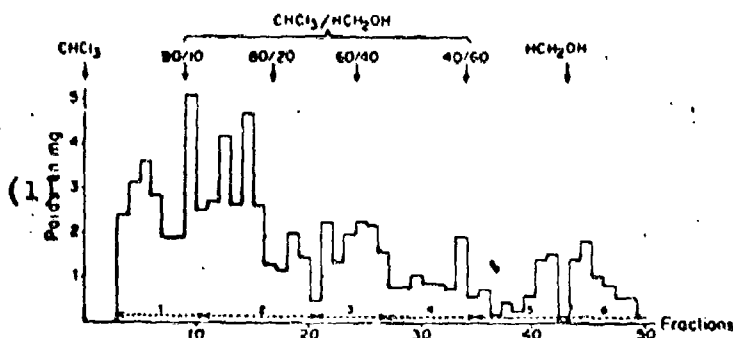


Fig. 3. Chromatogram of the Goose Red Cell Lipids on a Silicic Acid Column.

Column containing 20 gm silicic acid. 10 ml fractions. Elution with 100 ml fractions of solvent mixtures with increasing elution power.

1 -- Weight in mg.

The number of constituents of each fraction is investigated by thin-layer chromatography on silica gel and the identification of each group is confirmed by an investigation of the alkaline hydrolysis products. The results are practically identical for the three separations that were carried out -- one example of which is given in Fig. 4 -- and can be summarized as follows: no plasmalogen could be identified. Fraction 1 consists of two constituents, the more important one, the one with the highest R_f , essentially corresponds to cholesterol, and secondarily to cell glycerides. The second constituent is an undefined glycolipid. Fraction number 2 is more complex. It contains cholesterol and small amounts of two glycolipids associated with a cephalin. From the value of the R_f one cannot completely exclude the existence of compounds which resemble cardiolipids. Fractions 3 and 4 are identical and consist of two constituents, of which the one that is present in a greater amount is a cephalin; this cephalin is associated with a compound, having a weaker R_f and which is an analogue of lecithin. This compound is present as a trace in fraction 4; but the presence of choline could not be demonstrated in the hydrolyzate. Fraction 5 and 6 correspond to phosphatides which contain choline. Fraction 5 consists only of lecithin, in fraction 6 a sphingomyelin is associated with lecithin. This is demonstrated by the resistance of that compound to cold alkaline hydrolysis.

The inhibitory activity of these fractions is identical for the 5 investigated viruses when the optimum pH conditions prevail. This activity is associated with the phosphatidylcholines (fractions 5 and 6). Fraction 4 has only a very weak inhibitory activity which is probably due to the small amount of lecithin present in this fraction, since fraction 3, which also contains cephalin is devoid of any inhibitory activity.

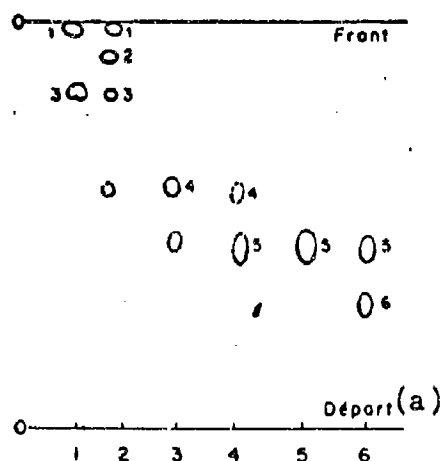


Fig. 4. Chromatography of the Lipid Fraction of Goose Red Cells on a Thin Layer of Silica Gel.

Silica gel 0.25 mm. Eluant 65:25:4, CHCl_3 , HCH_2OH and H_2O . Developing agent: Phosphomolybdic and 110°C . 1 = cholesterol, glycerides. 2-3 = Glycolipids. 4 = cephalins; 5 = lecithins; 6 = spingomyelin.

a -- Start.

The results of the separations and the investigation of the inhibitory activity of the separate fractions are presented in Table III.

The identification of the inhibiting fraction is completed by an investigation of the resistance of the inhibitory activity to various treatments.

Aliquots of each of the preceding fractions or an amount of total lipids corresponding to 0.10 mg of phosphatide are evaporated to dryness and taken up in 1 ml of phosphate buffer, corresponding to the investigated virus. This mixture is subjected for 14 hours, at 37°C to the following treatments:

1° Action of phospholipase A from *Bitis gabonica* venom.

2° Action of phospholipase D from cabbage (0.05 ml of a 1/1000 solution).

3° 1 ml of an aqueous solution of 1 N potassium hydroxide. At the end of the incubation time the lipids are taken up in 1 ml of chloroform. The resulting emulsion is broken up by centrifuging. The organic phase is separated and evaporated. The lipids are taken up in 1 ml of suitable phosphate buffer. A control aliquot is incubated under the same conditions and subjected to the same operations. The results are given in Table IV.

Table III.

Inhibitory Activity of the Chromatographic Fractions of Goose Red Cell Lipids

FRACTION (1)	P/N	P/CHOLINE	(a) CONSTITUANTS	Rf (2)	Qmi (3)
1.....	---	---	(b) Glycérides stérols	0,08	---
			(c) Glycolipides	0,71	---
2.....	0,11	---	(b) Glycérides stérols	0,08	---
			(c) Glycolipides	0,78 - 0,71	---
3.....	1,17		(d) Céphaline	0,62	---
			(e) Lécithine (?)	0,46	---
4.....			(d) Céphaline	0,62	2,1 µg
			(e) Lécithine (?)	0,46	
5.....	1,1		(e) Lécithine	0,46	0,029 µg
6.....	1,68	1,05	(e) Lécithine	0,46	
			(f) Sphingomyéline	0,31	0,040 µg

(1) Fractions obtenues par chromatographie des lipides totaux sur colonne d'acide silicique.
 (2) Rf établis sur couche mince de silicagel, avec le mélange éluant CHCl_3 , HCH_3OH , H_2O , 65 : 25 : 4.
 (3) Les quantités minima inhibitrices (Qmi) sont exprimées en µg de P vis-à-vis de 4 doses hémagglutinantes de virus amaril. Les fractions 1, 2, 3 sont dépourvues de pouvoir inhibiteur.

a -- Constituents; b -- Sterol glycerides; c -- Glycolipids; d -- Cephalin; e -- Lecithin; f -- Sphingomeylin; (1) -- Fraction obtained after chromatography of the total lipids on silicic acid column; (2) -- Rf obtained from thin layer [chromatography] on silicagel using a 65:25:4 mixture of CHCl_3 , HCH_3OH and H_2O as eluant; (3) -- Minimum inhibitory quantities (Qmi) are expressed in µg P against 4 hemagglutinant doses of amaril virus. Fractions 1, 2, 3 are devoid of inhibitory activity.

Table IV.

Modification of the Inhibitory Activity of Lipids
After Various Treatments

(1) USPÈCES	(2) NATURE DES LIPIDES	(3) TÉMOIN	PHOSPHOLIPASE A	PHOSPHOLIPASE D	KOH
	(7)				
(4)	1. totaux	64	—	16	1
Oie	fraction 4	> 16	> 16	> 16	0
	fraction 5	> 16	8	0	0
(5)	fraction 6	> 16	> 16	0	0
Coq ...	1. totaux	64	32	64	4
(6) Poule ..	1. totaux	128	128	16	2
	(7)				

The inhibitory titers are expressed as the reciprocal of the highest dilution which completely inhibits hemagglutination.

1 -- Type; 2 -- Nature of Lipids; 3 -- Control; 4 -- Goose; 5 -- Rooster; 6 -- Hen; 7 -- Total.

These results must be interpreted by taking the following into account: during hydrolysis with cold potassium hydroxide only the sphingomyelins remain unaffected: the reactions are checked by thin layer chromatography.

Phospholipase D is a phosphodiesterase which liberates choline and ethanolamine from lecithins, cephalins, lysolecithins and glycerophosphorylcholines.

Phospholipase A hydrolyses fatty acids in β position (Moore and Williams [11]).

5° Investigation of the Lipids Fractions of Rooster and Hen Red Cells

Under the experimental conditions used the yield of lipids extracted from rooster and hen red cells (about 50 mg) is smaller than those extracted from goose cells.

The extracted lipids are separated chromatographically on a silicic acid column into six homologous fractions. Their distribution, as percent of total lipids, is shown in Fig. 5 where it is compared with goose red cell lipids. Identification is made by thin layer chromatography (Fig. 6). The classes of identified compounds are identical with those described for goose red cells. But even though the conditions

for the column chromatography have been reproduced as faithfully as possible, the separation is less efficient.

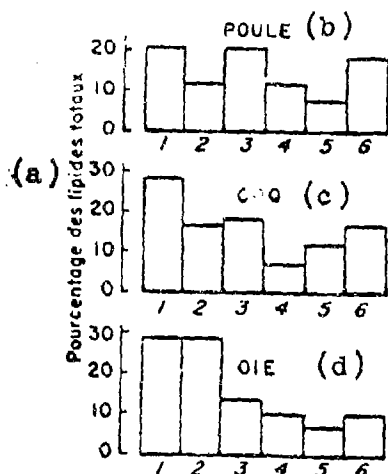


Fig. 5. Distribution of the Lipid Fractions Separated on a Silicic Acid Column Classified According Species.

The main constituents of the fractions are: 1 = cholesterol glycerides; 2 = cholesterol, glycolipids; 3 = cephalines; 4 = lecithin cephalins; 5 = lecithins; 6 = sphingomyelin.

a -- Percent of total lipids; b -- Hen; c -- Rooster; d -- Goose.

The identification of the inhibitory fractions is verified by subjecting the total lipids to various treatments as defined (Table IV). The results are less precise than for the goose lipids because of the faulty separations. But:

1° The inhibitory activity of the fractions increases with an increase of the methanol concentration in the eluent.

2° The maximum inhibitory activity is found in the fraction which contains the greatest amount of phosphatidylcholine.

3° The action of phospholipases and KOH is parallel for the three types, and it seems permissible to assume that the compounds responsible for the inhibitory activity of the three types of cells are the same.

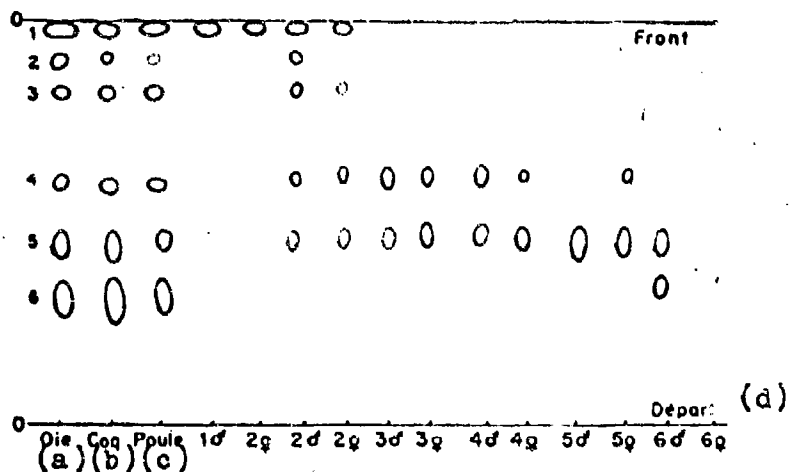


Fig. 6. Chromatography of Lipid Cell Fractions of Rooster and Hen on Thin Layer Silica Gel.

Same conditions as for Fig. 4.

a -- Goose; b -- Rooster; c -- Hen; d -- Start.

.6° Study of the Virus-Erythrocyte Bond

The conditions necessary for hemagglutination, particularly as far as pH is concerned, are known since the beginning of these investigations [3] (Fig. 1). The temperature conditions appear non-critical for the viruses investigated.

The absence of virus adsorption, outside the optimum pH zone, is verified, after tentative adsorption, by titrating the virus in the supernatant, and also by agglutination tests of the erythrocytes by the specific immunoserum of the virus under investigation.

The attempts to rupture the bonds and to elute viruses previously adsorbed under optimal conditions have been carried out at various temperatures: 4°, 28° and 37°C:

1° Using Fauconnier's elution technique [2] for viruses fixed on formaldehyde-treated red cells by repeated washing with distilled water.

2° Varying the pH by washing with a series of buffer solutions, either toward the alkaline pH zone (pH 6 → pH 9), or toward the acid zone (pH 5.8 → 5.3) following Salminen's technique [18 a].

Contrary to Salminen's results [18 a] no elution was obtained under these conditions. This corresponds to Hale's findings [10]. None of the preliminary treatments, described in the first paragraph (α -chymotrypsin and in particular phospholipases) could change these results.

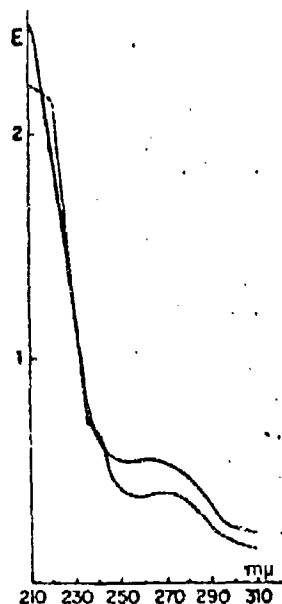


Fig. 7. UV Spectrum of Material Extracted from Goose Red Cells.

Material extracted by Calvin's method (solid line) and by that of Philipson and Bengtsson (dotted line).

It is, however, possible to elute the viruses by extracting a erythrocyte suspension, which adsorbed the virions from a borate protein buffer, with an organic solvent (2:1 chloroform-methanol). After separation of the two phases the free virions appear in the aqueous layer.

7° Isolation of Lipoproteins from the Cell Membrane

In these experiments methods derived from that of Calvin [12] and of Philipson and Bengtsson [14] were used. After hemolysis the first method essentially consists of solubilization of a portion of the protein material at pH 9, extraction by ether according to Kumagawa, and finally resolubilization of the material at pH 7, with agitation during 14 hours at +40°C.

The UV spectra of the two materials prepared are essentially the same (Fig. 7).

The material extracted by the first method has an inhibitory activity towards arborviruses. The minimum inhibitory quantity is 21 μ g of protein, under the normal test conditions (volume of the inhibitor 0.2 m., 4 hemagglutinating virus units).

DISCUSSION

1° The investigation of the chemical changes on the surface of the red cell constitutes a classical means of getting to know its structure [24]. This route, however, presents the problem of the number of receptors which are present on the

surface, a number which is certainly very large but unknown at the outset, and which thus presents the problem of quantitative elimination. The results of the present study are, however, too clearcut and thus do not need to be discussed at length. The loss of the activity of the receptors after treatment with organic solvents is corroborated by the fact that virions fixed on red cells can be extracted with these same solvents: these two phenomena emphasize the essential role that lipids play in the structure of the red-cell receptors. This was already predicted by Salminen [19].

In passing it should be noted that there is an increase in the adsorption capacity after certain treatments, especially with proteases. Like the classic phenomenon occurring in the study of blood group antigens [24] it must in this case too be considered as corresponding to the unmasking, on the surface, of a certain number of receptor sites.

2° The spectrum of the sensitive red cells seemed suitable to supply some additional information. The first studies of Clark and Casals [3] used one day old chick red cells. Porterfield [15] demonstrated the sensitivity of goose red cells, Salminen [18] that of rooster red cells contrasting them with the absence of the hemagglutination of hen red cells. The observation of Salminen [22] that treatment of roosters with estrogens makes their red cells non-agglutinable, permitted a feasible interpretation of these phenomena.

The question of the serum lipids of hen and rooster, of the egg and of the cell has in effect been investigated by the Scandinavian scientists in relation to the ovular function [23]. Paralleling the observations of Salminen, the treatment of roosters with estrogens leads to:

1° A decrease of all the normal lipid constituents of serum.

2° A very rapid increase of the lighter lipo-proteins.

3° Apparition of lipoprotein M₂ or lipovitellin, and of phosphoprotein x₁ or phosphovitin.

In the same manner the brooding period in geese brings about a decrease of the normal serum lipid inhibitors and the non-agglutinability of the red cells [28].

The apparent equivalence of the composition of the erythrocyte lipids in both sexes, the preponderance of inhibitory lipids in non-agglutinating hen red cells, seem to support the

fact that the observed differences are due not so much to the absence of the receptive fraction as to an architectural difference of the membrane which masks potential receptors. The transfers of lipids between serum lipoproteins and erythrocytes are a well established fact. The fixation, at the red cell level, of new serological fractions, devoid of inhibitory activity, and thus of receptor activity (phosphoprotein) seems a plausible mechanism which can explain the non-agglutinability after estrogen treatment and in females.

3° The realization that cell membrane lipids play an important role in the receptor sites of arborviruses naturally led to the isolation of these lipids.

First it was shown that the inhibiting fractions were always identical, for the arborvirus studied, regardless of their serological group (A, B, D). These findings contradict those of Porterfield [16] who, in an investigation carried out in Africa with a single virus A (W.E.E.) did not find any specificity. The quantitative differences between viruses, when they exist, are small and difficult to interpret.

The identification of the inhibitory fractions is based on the following considerations deduced from the study of goose red cells and extended, by analogy to rooster and hen red cells.

1° Fractions which contain neutral fats (cholesterol) and glycolipids are devoid of any inhibitory activity.

2° The fraction which contains, almost pure cephalin, is devoid of inhibitory activity. This activity appears in the next fraction at the same time as an adequate amount of a compound, which chromatographically behaves like lecithin.

3° Destruction of the inhibitory activity treatment with cold potassium hydroxide -- destruction which is complete for goose red cells and almost complete for rooster and hen red cells -- eliminates sphingomyelin, which is not destroyed by this treatment

4° The maximum inhibitory activity is found in the fraction which contains pure lecithin, and only those fractions which contain lecithin have an inhibitory effect.

The minimum inhibitory quantity (expressed as lecithin P) for the amaril virus is 0.029 μ g, or 47×10^{-10} moles/ml. This figure approaches that of Porterfield of 38×10^{-10} moles for dengue virus and the lecithins of human cells.

The inhibiting lipids thus consist essentially of phosphatidyl-choline and our results do not agree with Porterfield as far as the activity of the sphingomyelins is concerned -- this activity seems strongly diminished. The hypothesis of a non-identified phosphatide which exists as a trace and which has the same chromatographic behavior as the true phosphatidyl-cholines is unlikely because of the necessity to assume that in spite of its low concentration it is eluted over a wide elution range and by the action of the phospholipases.

The identification of the active inhibitory site does present some ambiguity. The investigations of Porterfield has already demonstrated the destruction of the inhibitory activity by the action of phospholipase C from Clostridium perfringens (phosphorylcholinesterase).

Phospholipase A is inactive. Phospholipase D has a real and regular effect on the fractions which contain pure lecithin or lecithin associated with sphingomyelins. This effect is less regular when the total lipids are present because of the variety of substrates.

The functional groups constituted by choline or by the phosphoryl radical thus seem to represent the active inhibitory site. This hypothesis has the advantage of explaining the equivalence in the activity of the various lecithin even though, because of steric effects, the original cells are not hemagglutinated by arborviruses.

There exist virus (lipovirus) lipid interactions [29] and the lipid mixtures described by Salminen [20, 21] seem to be an example of this phenomena. A priori there does not exist a substance and its existence as a natural receptor. The conditions and the limits of the activity of the inhibitory effect of isolated lipids are, however, comparable to that of the hemagglutination itself, and the significance of these lipids is beyond any doubt. The isolation of the active lipid fractions, however, does not prejudice the real intact structure of the receptor in the globular membrane. It has thus been possible to isolate an inhibitory, soluble material which is probably of a lipoprotein nature.

The nature of the bond between virions and erythrocytes is, as a matter of fact, not elucidated. The narrow pH conditions necessary for the hemagglutination reaction and the inhibition reaction by active lipids, and the fact that to maintain the inhibitory activity the phosphorylcholine group of the lecithins must remain intact suggest an ionic type bond. The phenomena, however, can not be described in such simple

terms because even though the adsorption cannot be carried out outside the suitable pH zone it is not reversible by a simple variation in pH.

It is possible that this reaction takes place in two successive steps: the first ionic, pH-dependent and allowing the correct orientation of the virion with respect to the receptor site: the second pH-independent, forming weak intermolecular bonds between the virions and the cell receptors.

No positive argument, however, has been provided in support of this hypothesis.

CONCLUSIONS

This investigation seems to have established that:

1° Cell lipids play an important role in hemagglutination because they probably are the constituents of the receptor site.

2° The active lipid fraction which is identical for all the viruses investigated, consists of phosphatidylcholines.

The presence of inhibitory fractions in hen cells, which are normally non-agglutinable, the difficulties encountered in eluting by simple ionic effects emphasize the existence of a structure at the receptor site. This site cannot be reduced to a simple ionized phosphatidylcholine group, though the integrity of the latter is shown to be indispensable for the maintenance of the inhibitory activity.

The protein nature of the site of viral hemagglutinine is also proven by its sensitivity towards proteolytic enzymes, specific according to the virus groups, as has been established in this laboratory. The first step in the hemagglutination reaction can thus be described as a virus protein-cell lipid interaction.

The existence of a second non-ionic step precludes any conclusion on the nature of the final bond which is established between virion and receptor sites.

SUMMARY

Red Cell Receptors for Arborviruses The Cell Lipids

1° A study of the behavior of red cells subjected to various chemical or enzymatic treatments demonstrates the presence of lipids in the cell site receptor of arborviruses.

2° Inhibiting lipids are present in red cells, either agglutinable or non-agglutinable by arboviruses.

3° The responsible fractions seem to be phosphatidylcholines. They are the same for all the viruses studied.

The pH conditions for inhibition are analogous to those of hemagglutination.

These facts suggest certain hypotheses on the actual nature of the red cell receptor site, and of the bonds between this site and the virions.

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