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DEPARTMENT OF THE ARMY
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Author: W. Frisch-Niggemeyer, Hygiene Institute of Vienna University (Hygiene Institut der Universitat Wien)

Title: The chemical structure of the receptor substance involved in the adsorption of TBE virus to erythrocytes (Die chemische Struktur der bei der Adsorption von TBE-Virus an Erythrozyten wirksamen Rezeptorsubstanz).

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The attachment of animal pathogenic viruses to the surface of their respective host cells appears to be a prerequisate for the penetration of the virus into the interior of the cell and thus also for the initiation of all of the processes involved with the infection. This adsorption involves more or less specific "receptor substances" (Cohen, 1963). The chemical structures of these receptor substances are not known with the exception of those for the myxoviruses. The investigation of the adsorption of virus to cells, from which they can be separated, is extremely difficult. On the other hand, the adsorption of such viruses to erythrocytes results in the easily measurable phenomenon of hemagglutination (HA). HA is produced by many viruses. Included among these are the following: psittacosis group, pox viruses, myxoviruses, adenoviruses, reoviruses, enteroviruses, and the arbovirus group (Rosen, 1964). These viruses can be specifically measured by means of this adsorption phenomenon. The present investigation is concerned with the chemical mechanism of the adsorption of TBE virus to erythrocytes.

Hemagglutination by arboviruses has been known to exist since 1950 (Sabin and Buescher, 1950) and a list of the arboviruses possessing this property was prepared by Casals (1961). According to Sabin and Buescher (1950), the sera of some mammals contain an inhibitor of HA. This can be almost completely removed by extraction with lipid-dissolving solvents (Sabin, 1951). Salminen (1960a) studied the conditions necessary for the adsorption of TBE virus to erythrocytes and was able to elute the virus again. He concluded that in his virus preparations, there were present nonspecific inhibitors which acted in a manner similar to that of the receptors

on the surface of the blood cells. Recently, Nicoli (1965) showed that erythrocytes, which had been extracted with lipid-dissolving solvents, were no longer capable of adsorbing various arboviruses. This and the fact that the extracted lipids themselves were strong inhibitors of hemagglutination, validate the conclusions of Salminen.

The chemical nature of the HA inhibitor substances with its lipid properties was first studied in detail by Porterfield and Rowe (1960). These investigators found that only the hemagglutination of the arboviruses of group B was inhibited. It was inhibited by alpha- and beta-lipoprotein extracts from serum as well as by the phospholipid fraction of erythrocytes which contained sphingomyelin. The inhibitory activity of egg lecithin was destroyed by lecithinase. Nicoli (1965) arrived at similar conclusions after chromatographic fractionation of HA-inhibiting lipids from erythrocytes. Only the lecithin-containing fraction showed a HA-inhibiting activity and this could be destroyed by lecithinase. On the other hand, Salminen et al. (1960a) concluded from analytical studies that the inhibition of HA by TBE virus was not the results of a single class of lipids but was due to a complex of cholesterol with certain fatty acids or with phosphatides. Experiments with purified lipids led to the same conclusion (Salminen et al. 1960b).

This completely contradictory situation appeared limited especially by the quality of the usual preparations of arbovirus-hemagglutinin, in that they are not stable at the pH value where the HA test is carried out using the method given by Clarke and Casals (1958). In this method, serial dilutions of the virus solution are prepared in a borate-saline buffer (pH 9) with albumin (BSA) added,

and then the erythrocytes are added. These were suspended in a phosphate buffer (VAD) whose composition is such that it will represent optimal conditions for the HA reaction. Also, most of the HA inhibition tests with lipid inhibitors were carried out by incubation of the substance to be studied with the virus in BSA at pH 9. However, for a critical evaluation of the effect of such substances which react with the virus in a manner similar to the cell receptor, there is no adequate procedure available. The hemagglutination of the arboviruses shows a clear dependence on the pH value (Movitsch, 1965), and the study of the activity of receptor-like substances must be carried out under competitive conditions, that is, at the pH optimum of the HA reaction. The instability of the hemagglutination effect of the TBE virus was studied by Frisch-Niggemeyer (1966). It was shown that under certain conditions, the decrease in the hemaggltuination capability was only slight at that pH optimum. A half-life of about an hour was found. This led us to develop a method which would allow one to measure the competitive action of certain lipid inhibitors at the pH optimum of HA of the TBE virus.

Materials and Methods

Virus

We employed strain No. 415 of the TBE virus which was originally isolated from ticks (Ixodes ricinus) (Radda, Loew, and Pretzmann, 1963). It was cultivated in suckling mouse brains. Three-day old mice were injected i.c. with 0.03 ml of a suspension of infected brain which had been diluted 1/100 with phosphate buffered saline (PBS) in 10 % horse serum. Usually, the mice were moribund

by the fourth day after infection and the brains were then removed. They were immediately cooled and then mixed with five volumes of borate-saline buffer, pH 9 (BS) in a "Lourdes" homogenizer at 10,000 rpm for about one minute. The homogenate was centrifuged at 4,000 x g for 30 minutes. To the still somewhat lightly turbid supernatant was added 1/7 volume of a 5 % solution of protamine sulfate ("Culpine" NBCo). This brought about the formation of a heavy precipitate. The mixture was held overnight in the refrigerator and then centrifuged at 4,000 x g for 15 minutes. To the supernatant was added an equal amount of penicillin and streptomycin and the mixture was incubated at 37°C. After 12 hours, an additional precipitate formed. After 7 days of incubation at 37°C, this precipitate was removed by centrifugation and the supernatant, which contained only the purified virus, was stored at 4°C. This treatment is a combination of the alkaline extraction of Clarke and Casals (1958) with the heat-inactivation procedue of Salminen (1962). Our preparation was not infectious and had an HA titer between 1,280 and 5,120. At 4°C, this decreased in about two months to half of the original value and was then stable up to a year. The sedimentation in sucrose density gradient was the same as that obtained for infectious TBE virus (Frisch-Niggemeyer, 1966).

Erythrocytes

All HA tests and HA inhibition tests were carried out using only goose erythrocytes (Porterfield, 1957). The cells were washed and standardized as described in the method of Clarke and Casals (1958). We employed a 0.2 % suspension of the erythrocytes.

HA Test and HA Inhibition Test

For the determination of virus concentrations, the HA titrations were carried out as described by Clarke and Casals (1958). Serial dilutions each containing 0.4 ml were prepared from the virus solution using BS buffer, pH 9, without albumin added. To these was added an equal volume of erythrocytes suspended in VAD, pH 6.4. After two hours at room temperature, the type of sedimentation was observed.

In order to compare the results with those of other authors, the HA inhibition test was also carried out at pH 9 according to the method of Clarke and Casals (1958). Two-fold, serial dilutions of the substance under study were prepared in BS buffer, pH 9. To each tube containing 0.2 ml of this diluted material was added 0.2 ml of virus suspension prepared in the same buffer. The virus suspension was diluted to contain approximately 4 HA units. After incubation overnight in the refrigerator, 0.4 ml of erythrocytes was added to each tube as previously described. After two hours at room temperature, the results were observed.

In the case of the HA test under competitive conditions, which was at pH 6.4 for the virus strain employed, all the solutions were held in an ice bath (Frisch-Niggemeyer, 1966). Serial dilutions of the substance under study, each containing 0.2 ml, were prepared in VAD, pH 6.4. To each tube was added 0.2 ml of ice-cold virus prepared in BS. The virus solution contained twice as much virus as before, that is, about 8 HA units. After incubation for only 30 minutes in the ice bath, 0.4 ml of erythrocytes was added to each

tube. In this case, the erythrocytes were suspended in a mixture of equal volumes of BS and VAD. After two hours at room temperature, the sedimentation was observed.

Homogenates

The organs, which were to be studied for the homagglutination inhibiting capability, were mixed with 9 volumes of buffer in a Potter-Elvehjem homogenizer until a fine suspension was obtained. This was easily accomplished with brain, liver, kidneys, and lungs. For muscle, a high-torque homogenizer of the "Lourdes Co." was employed. The homogenates were prepared either in borate-saline buffer, pH 9, or in VAD, pH 6.4. The suspensions employed in this manner were used as 1:10 dilutions, and 0.2 ml was used as the starting dilution for the study at pH 9. For the HA inhibition studies at pH 6.4, the suspension was further diluted 1:80 in VAD, pH 6.4. This corresponded to a starting dilution of 1:800.

Lipid Extracts

In order to prepare a good mixture of complex lipids from organs, the method of Folch et al. (1951) was employed. The organ was mixed in a Potter-Elvehjem or in a "Lourdes" homogenizer with 20 volumes of a mixture of chloroform and methanol in a ratio of 2:1. The homogenate was cooled with ice and allowed to stand for 30 minutes after which it was centrifuged for 10 minutes at 4,000 x g. The supernatant was dialyzed according to the method of Folch et al. (1957). A small beaker was placed in a large one with about 10 times as much volume. This was filled about 4/5 with distilled water.

With a pipette, the small beaker was filled to about 2/3 volume with the extract. Overnight in the refrigerator, the methanol and water-soluble components of the extract diffuse into the water whereas the chloroform remains behind with the soluble lipids. A flocculant layer forms at the interface. Water is removed as much as possible without disturbing the flocculant layer and then methanol is added until the flocculant substances are again dissolved.

The extracted is precipitated with a double volume of acetone. After two hours at 4°C, the precipitate is removed by centrifugation. The supernatant is dried in a rotary evaporator at 40°C and then taken up in about 1/10 of the original volume with chloroform. The solution is stored in the refrigerator.

In order to determine the concentration, an aliquot was dried first with a warm air stream and then over silica gel before weighing.

For the HA inhibition assay, an appropriate volume, containing usually 1 mg of substance, was pipetted into the tube of a Potter-Elvehjem homogenizer. After drying with a cold air stream, the material was suspended in 10 ml of buffer by means of the homogenizer blade. For experiments at pH 9, a borate-saline buffer of this pH value was used, and for experiments at pH 6.4, the corresponding VAD was employed. 0.2 ml containing approximately 20 µg of lipid was employed as the starting material for the dilution series.

Erythrocytes were extracted using the method of Rose and Oklander (1965): A volume of washed erythrocytes was hemolyzed by the addition of an equal volume of distilled water. After 15 minutes,

eleven volumes of isopropanol were added and the mixture was allowed to stand at room temperature for one hour with occasional shaking. After the addition of seven volumes of chloroform, the mixture was allowed to stand for an additional hour with occasional shaking. The mixture was then centrifuged for 30 minutes at 500 x g and the supernatant dried under vacuum at 40°C. The lipids wer redissolved in a mixture of chloroform and methanol in the ratio of 2:1, dialyzed, and treated further as described for the extracts of other organs.

In the preparation of phosphatides of cholesterol and neutral fats, the extracts were precipitated with six volumes of acetone. After standing overnight at 4°C, the precipitated could be collected by centrifugation at 4,000 x g for 10 minutes. The precipitated phosphatides were washed with acetone and dissolved in a small volume of chloroform.

Column Chromatography

A column with a diameter of 2 cm was employed. This was packed about 37 cm high with approximately 60 gm of silica gel, "Serva". Silica gel with a particle diameter of 100-200 μ was dried overnight at 110°C. Also, water-containing silica gel with a diameter of 50 - 100 μ was employed. This was obtained by exposing for 24 hours thin layers to an atmosphere saturated with water vapor at room temperature. To the column were applied 100 - 200 mg of the phosphatide mixture and elution was carried out at room temperature. For this purpose, a discontinous gradient of methanol in chloroform was employed. With a flow rate of 1.5 - 2.0 ml per minute, 15 ml fractions were collected. Generally, high quality reagents were employed. During

the course of the chromatographic procedure, phosphorous determinations were carried out and the compositions of several peaks were studied by means of thin-layer chromatography.

Thin-Layer Chromatography

As no method of single-dimension thin-layer chromatography was known to us that would give sharp enough separations of all of the phosphatides in question, we employed a modification of the two-dimensional thin-layer chromatographic technique described by Abramson and Blecher L964). Using the spreading apparatus of Stahl (1964), 20 x 20 cm plates were coated with a 0.25 mm thick layer of "Silica gel G". The plates were activated at 110°C for one hour. Approximately 100 µg of the lipid mixture was spotted on the plate. The first run was carried out using chloroform-methanol-water- con. ammonia in ratios of 65:30:4:2. The plate was dried in a warm air stream and the second run was carried out in chloroform-methanol-water-glacial acetic acid in ratios of 65: 30: 1: 5. After drying in an air stream, the spots were made visible by development with iodine vapor or by spraying with specific reagents. Using this technique, the following compounds could be identified: Cholesterol, phosphatidyl acid, cerebroside-sulfonate ester, cephaline phosphatidyl-serine, cephaline phosphatidyl-cholamine, lecithin, sphingomyelin, phosphatidyl inositol, lysolecithin, ganglioside, and several amino acids. A detailed description of this method will be published at another time.

Phosphorous Determinations

This determinations was carried out using the micromethod of Gerlach and Deuticke (1963) which is a modification of the well known method of King (1932). The extinctions were measured in a Zeiss PMQ-2 spectrophotometer at 820 mμ. The values were linear for 0.1 to 10.0 ug of phosphorous and 1 μg of phosphorous gave a value of 0.175.

Results

Our hemagglutination inhibition studies with THE virus indicated that the homogenate of mouse brain at pH 6.4 was 100 times more effective than a similar homogenate at pH 9. A dilution of 1:100,000 still inhibited the hemagglutination of the THE virus. Extracts with chloroform-methanol (2:1) were likewise 100 times more effective at pH 6.4 than at pH 9. As little as 1 ug/0.8 ml was effective as an inhibitor under these conditions. On the other hand, extracted brain homogenates showed no decrease in their inhibition capabilities at pH 6.4 as compared to pH 9. The residual inhibition activity of the extracted homogenates at pH 6.4 was only about 1/500 th that of the original homogenates. The HA inhibiting activity of mouse brain homogenates can be attributed to a substance which is lipid in nature, can be extracted, and is still active in solution. However, this substances does not appear to be responsible for the inhibition at pH 9 since at this pH value, the inhibiting capabilities of the original and the extracted homogenates are identical.

The results of Salminen et al. (1960b) can be reproduced but the complex of cholesterol with palmitic acid or with lecithin was twice as active at pH 6.4 than at pH 9.0. It appears, therefore,

that this complex is not identical with those substances which can be extracted from the brain by the use of lipid-dissolving solvents.

The HA inhibition titer of serum which contains antibodies was not increased if one compared the activity at pH 6.4 with that obtained at the pH of the usual method. This was, of course, to be expected and reminds of us of the fact that the HA inhibition due to antibodies is the result of a mechanism which is completely different from that involving extracted lipid which is active at the pH optimum of the HA reaction. These results also indicate that our method for measuring HA inhibition does not decrease the value of the older method of Clarke and Casals (1958) as long as this latter method is used for the titration of antibody. A comparison of the HA inhibition of various substances at pH 9 and at pH 6.4 is summarised in Table I.

If one compares the HA inhibition by extracts from various organs, it is seen that per µg lipid, no significant differences are observed (Table II). For additional investigations on inhibitors, however, brain appeared to be the best starting material since the quantity of extracted lipid recovered was much greater than with the other organs that were examined. In addition, there are almost no neutral fats in the brain. Also, homogenates as well as lipid extracts from brains of various animals did not differ very much in their activity with regard to HA inhibition. However, in all the cases studied, the HA inhibition activity was approximately 100 times greater at pH 6.4 than at pH 9 (table III).

Table I

Minimum concentrations of different substances which will inhibit hemagglutination at pH 9 and at pH 6.4

Substance	pH 9	pH 6.4	Increase in activity
brain homogenate (mouse)	1:320	1:102,400	300 X
lipid extract of above	60µg/0.8 ml	0.3 µg/0.8 ml	200 X
extracted homogenate	1:320	1:160	0
antiserum (human)	1:640 +	1:1.280 -	2 X
cholesterol	1/8 µM/0.8 ml	1/40 µM/0.8 ml	5 X
lecithin	1 µM/0.8 ml	1 µM/0.8 ml	0
palmitic acid	1 µM/0.8 ml	1/20 µM/0.8 ml	20 X
Cholesterol + lecithin	1/10 µM/0.8 ml	1/20 µM/0.8 ml	2 X
cholesterol + palmitic acid	1/128 µM/0.8 ml	1/2 µM/0.8 ml	2 X

Table II

Hemagglutination inhibition activity of lipid extracts from various sources

Brain (rat)	0.15 µg/0.8 ml
liver (rat)	0.07 µg/0.8 ml
kidney (rat)	0.30 µg/0.8 ml
lung (rat)	0.07 µg/0.8 ml
muscle (rat)	0.15 µg/0.8 ml
erythrocytes (goose)	0.30 µg/0.8 ml
erythrocytes (human)	0.15 µg/0.8 ml

Table III

Hemagglutination inhibition activity of homogenates and lipid extracts prepared from brains from various animals

Material	pH 9	pH 6.4
Homogenate		
white laboratory mouse	1: 320	1: 102,400
field mouse	1: 640	1: 204,800
guinea pig	1: 2,500	1: 250,000
cow	1: 640	1: 50,000
cat	1: 2,500	1: 250,000
monkey	1: 5,000	1: 250,000
Extract		
white laboratory mouse	60 µg/ 0.8 ml	0.30 µg/ 0.8 ml
field mouse	60 µg/ 0.8 ml	1.25 µg/ 0.8 ml
guinea pig	10 µg/ 0.8 ml	0.07 µg/0.8 ml
cow	50 µg/ 0.8 ml	0.60 µg/ 0.8 ml
cat	20 µg/ 0.8 ml	0.60 µg/ 0.8 ml
monkey	20 µg/ 0.8 ml	0.60 µg/ 0.8 ml

In order to characterize further the HA inhibiting component, lipid extracts from mouse brain were subjected to various fractionation methods. By mixing the extracted lipid with acetone at room temperature, cholesterol and cholesterol ester could be extracted in this manner. At pH 6.5, these substances were only weakly active (10 µg / 0.8 ml) and, as compared to their activity at pH 9.0, the characteristic increase in activity was not observed. By precipitation with six volumes of acetone, as described under "Materials and Methods", the phosphatides could be separated. Their HA inhibiting activity was comparable to that of the entire extract (0.4 µg /0.8 ml). A further extraction of this material with ether or with ethanol did not give clear results. This was similar to the situation

observed by Ansell and Hawthorne (1964) who treated brain with acetone, ethanol, and petroleum ether.

For additional separations, column chromatography was employed. Using activated silica gel, "Serva" 100 - 200 μ, a separation into three peaks could be achieved (Fractionation I, Fig. 1). The characterization of the several peaks was carried out qualitatively by means of two-dimensional thin-layer chromatography.

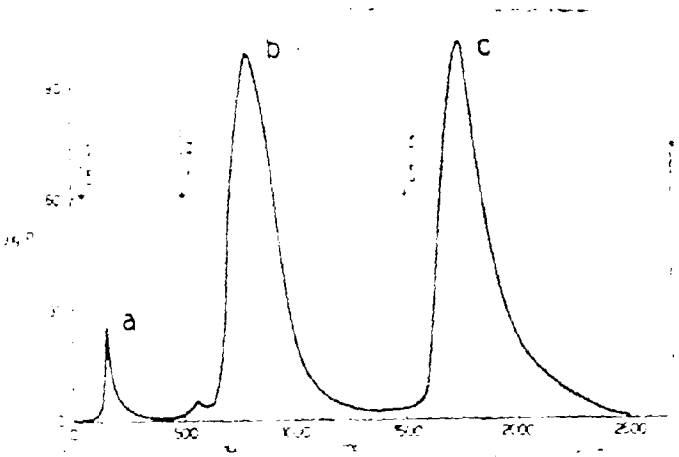

Fig. 1. Fractionation I: chromatography of phosphatides from mouse brain on activated silica gel. (a) phosphatidic acid, cerebroside, cerebroside ester; (b) phosphatidylcholamine; (c) phosphatidylserine, inositol, lecithin, sphingomyelin.

With chloroform-methanol (4:1), phosphatidic acid, cerebroside, and the sulfonic acid esters could be eluted. Chloroform-methanol (3:2) eluted phosphatidylcholamine. The same solvent system at a ratio of 1:4 eluted lecithin, sphingomyelin, phosphatidylserine and inositol. Also, a small amount of lysolecithin was recovered. This compound, however, could not be detected in the original extract and was, therefore, considered to be an artifact. A strong HA inhibiting

activity could be demonstrated only for those substances which were present in the last peak. The use of moist silica gel, "Serva" 50-100 μ, gave a somewhat different picture (Fractionation II, Fig. 2). With chloroform-methanol (4:1), phosphatidic acid, cerebroside, and their esters were again eluted. The same solvent system at a ratio of 3:2, however eluted phosphatidylserine and the inositol along with phosphatidylcholamine. An increase in the methanol concentration to 50 % resulted in lecithin and sphingomyelin being eluted from the column. In this experiment, none of the material found in the several peaks were capable of inhibiting hemagglutination of the THE virus. However, a mixture of the substances found in peaks a, b, c, and d was found to be as strong an inhibitor as was the original extract. If HA inhibition by combinations of just two of the peaks was tested, it was found that only those pairs demonstrated a measurable inhibitory capability that were combinations of peaks b (cephalin and inositol) with lecithin or with sphingomyelin. During Fraction I, we had obtained a pure phosphatidylcholamine. However, a mixture of this with lecithin or with sphingomyelin was not capable of surpressing HA of the virus (Table IV). As a result, it must be concluded that either phosphatidylserine or an inositol in combination with lecithin or with sphingomyelin is essential for inhibition of hemagglutination by THE virus at the pH optimum of this reaction.

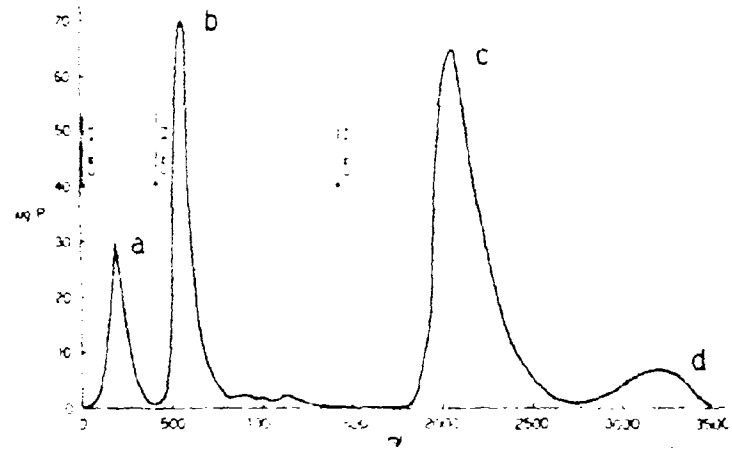

Fig. 2. Fractionation II: Chromatography of the phosphatides from mouse brain on moist silic gel. (a) phosphatidic acid, cerebroside, cerebroside ester; (b) phosphatidylcholamine, phosphatidyl-serine, inositol; (c) lecithin; (d) sphingomyelin.

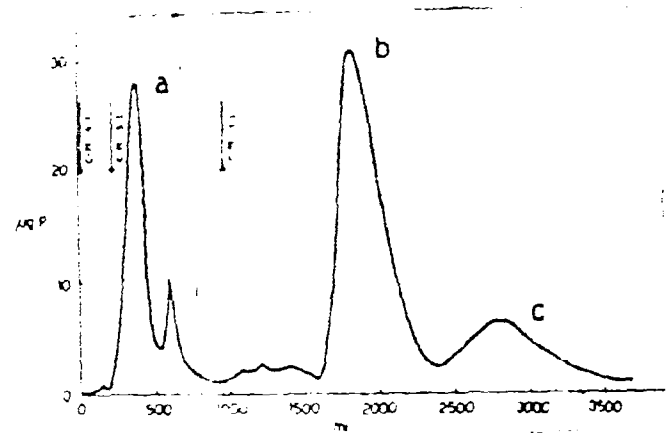

Fig. 3. Fraction III. Chromatography of the substances in Peak c of fractionation II on moist silica gel: (a) phosphatidylserine, inositol; (b) lecithin; (c) sphingomyelin.

Table IV

Hemagglutination inhibiting activity of the lipids and their combinations obtained by chromatography

	1st Exp.	2nd Exp.
Fractionation I (Phosphatides from mouse brain)		
a (cerebroside*)	—	—
b (phosphatidylcholamine)	—	—
c (lecithin, sphingomyelin, inositol, P-serine)	++	++
I (a b)(cerebroside* phosphatidylcholamine)	n.d.	—
Fractionation II (phosphatides from mouse brain)		
a (cerebroside*)	—	—
b (cephalin**), (inositol)	±	—
c (lecithin)	—	—
d (sphingomyelin)	—	—
II (a b c d)		
II (a b)(cerebroside, cephalin, inesitol)	n.d.	±
II (a c)(cerebroside, lecithin)	±	±
II (a d)(cerebroside, sphingomyelin)	n.d.	—
II (b c)(cephalin, inositol, lecithin)	+	+
II (b d)(cephalin, inositol, sphingomyelin)	+	+
II (c d)(lecithin, sphingomyelin)	—	—
Ib IIc (phosphatidylcholamine, lecithin)	—	—
Ib IId (phosphatidylcholamine, sphingomyelin)	—	—
Fractionation III (substances from Fractionation I, peak c)		
a (phosphatidylserine, inositol)	—	—
b (lecithin)	—	—
c (sphingomyelin)	—	—
III (a b c)	++	++
III (a b)(phosphatidylserine, inositol, lecithin)	++	n.d.
III (a c)(phosphatidylserine, inositol, sphingomyelin)	+	n.d.
III (b c)(lecithin, sphingomyelin)	—	n.d.
Ia IIIa (cerebroside, phosphatidylserine, inositol)	±	±
Ia IIIb (cerebroside, lecithin)	±	n.d.
Ia IIIc (cerebroside, sphingomyelin)	±	n.d.
Ib IIIa (cephalin**; inesitol)	—	n.d.
Ib IIIb (phosphatidylcholamine, lecithin)	—	n.d.
Ib IIIc (phosphatidylcholamine, sphingomyelin)	—	n.d.

* Also phosphatidic acid and cerebroside ester
** phosphatidylcholamine and phosphatidylserine

++ very strong reaction; + strong reaction; ± weak reaction; — no reaction; n.d. not determined.

If the HA inhibiting material, which is present in peak c of Fractionation I, is chromatographed on moist silica gel, "Serva" 50-100 μ, the following results are obtained: with chloroform-methanol (4:1), no phosphorous-containing compounds were eluted. Chloroform-methanol (3:2) eluted phosphatidylserine along with the inositols. The same solvent system at a ratio of 1:1 eluted lecithin and sphingomyelin from the column (Fractionation III, Fig. 3). None of these materials alone was active as an inhibitor of HA, but a combination of all of them was again completely active. Thus, the phosphatides from mouse brain could be separated into five groups by the employment of two different chromatographic separations on silica gels of different activities. None of these groups alone were capable of inhibiting hemagglutination of the virus. However, if several of the groups were combined together, several of these pairs could be demonstrated to be active as inhibitors. Also, in these experiments, a strong inhibition was found to be associated only with those combinations in which phosphatidylserine and phosphatidylinositol were paired with lecithin or with sphingomyelin (Table IV).

Because of the small amounts available, an attempt was not made to separate the substances found in peak a of Fractionation III from each other. However, phosphatidylserine, phosphatidylinositol, and phosphatidylinositol-diphosphate could be purchased (Koch-Light Ltd.). Of these substances, only phosphatidylinositol-diphosphate (triphosphoinositol) showed a strong capability of inhibiting hemagglutination of the TBE virus when in was combined with lecithin

or with sphingomyelin. Such combinations were tested repeatedly for their ability to inhibit HA, and the triphosphoinositol was found to be an essential component while lecithin and sphingomyelin could be replaced by cetyl trimethyl ammonium bromide (Cetavlon) without loss of activity (Table V). Phosphatidylinositol was not an inhibitor of HA of the virus. The commercial preparation of phosphatidylserine from bovine brain alone showed a strong HA inhibitory effect which was not intensified by the addition of lecithin. This behavior contradicts completely our findings with substances which can be extracted and fractionated from mouse brain.

Table V

Hemagglutination inhibition effect of phosphatidylinositol-diphosphate combined with several trimethylammonium compounds

smallest amoung of equi-molar combination which is still active at pH 6.4		
P-inositol-dp + lecithin	P-inositol-dp + sphingomyelin	P-inositol-dp + Cetavlon
0.08 µg/0.8 ml	0.08 µg/0.8 ml	0.02 µg/0.8 ml
0.04 µg/0.8 ml	0.04 µg/0.8 ml	0.04 µg/0.8 ml
0.08 µg/0.8 ml	0.15 µg/0.8 ml	0.03 µg/0.8 ml
0.15 µg/0.8 ml		
0.04 µg/0.8 ml		

Discussion

In contrast to the methods described by other authors, the technique that we have presented here is capable of measuring the HA inhibition activity of various substances at the same pH as the pH which is optimal for HA. Materials which could be extracted from various organs with lipid-dissolving solvents, were found to

be 100 times more active under these conditions than when their activity was tested outside the pH range of HA. As a result, it is very obvious that the mechanism of their action is analogous to the function of the virus receptor on the surface of erythrocytes. It is conceivable that our extracts contain the entire receptor substances since extracted erythrocytes are no longer capable of adsorbing arboviruses (Nicoli, 1965).

Our finding that the HA inhibiting activity of different organ extracts is the result of the activity of two different substances explains divergent results. During the course of our studies on the purification of lipid extracts by solvent fractionation, it was found that if any one of the fractions, which represent the partner in the HA inhibition pair, is sufficiently "contaminated" with the other, then HA inhibition can be observed. In the course of additional purification steps, most of this activity is lost. Only the chromatographic method yields a substance which is sufficiently pure to be use for further experimentation.

The mutual activity of phosphatidylinesitol-diphosphate (fig. 4) with lecithin or with sphingomyelin can also explain the results obtained by Porterfield and Rowe (1960). These authors found that lecithin obtained chromatographically from erythrocytes was not an inhibitor whereas sphingomyelin and other phosphalipids containing choline demonstrated a clear inhibition of HA. In our opinion, there was probably contamination with phosphatidylinositol-diphosphate. Porterfield and Rowe were of the opinion that the HA inhibiting activity of their preparations was the result of a still unidentified phospholipid which was present in many fractions.

Phosphatidylinositol-diphosphate was not mentioned in their publication.

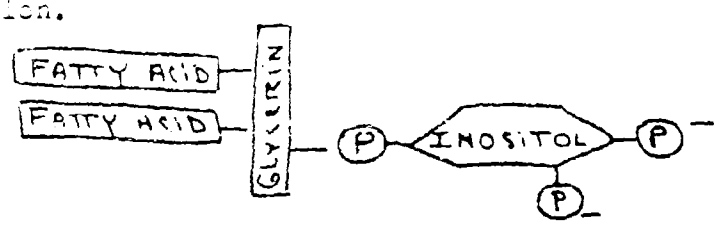

Fig. 4. Phosphatidylinositol-diphosphate (triphosphoinositol)

Our results, however, are in agreement with the conclusions of the studies of Nicoli (1965): from erythrocyte lipids, he obtained by chromatography six fractions of which three contained lecithin. Of these only two, however, were active as inhibitors of HA. Our guess is that these contained phosphatidylinositol-diphosphate. This compound, however, was never mentioned by Nicoli. The fact that the hemagglutination inhibition activity of these fractions was destroyed by phospholipase D is not unreasonable since this enzyme is also capable of acting on lecithin.

If one considers the fact that the induction of HA inhibiting activity by triphosphoinositol has an absolute requirement for the presence of a surface-active trimethylammonium compound, then a striking parallel must be mentioned: Thompson and Dawson (1964) characterized an enzyme that cleaves phosphatidylinositol-diphosphate hydrolytically into a diglyceride and inositol triphosphate. The activity of this enzyme is usually very weak but it is activated by certain surface-active substances such as Cetavlon, stearylamine, palmitoylcholine, and cetyl pyridinium bromide. In the presence of

physiological concentrations of NaCl, lecithin was also a strong activator of this enzyme. Thompson and Dawson demonstrated that Cetavlon will react with the triphosphoinositol in such a way that the complex formed assumes a colloidal structure. This causes to some extent an orientation of the inositol molecule. The specific enzyme can apparently only attach itself to the substrate when it is found in this orientated state.

We believe that our own results can be intepreted through a quite identical mechanism for the adsorption of TBE virus onto the erythrocyte surface and for the inhibtion of this reaction by a surface-active trimethylammonium compound. Even in 1925 it was assumed that the surface of the erythrocyte was composed of a double layer of lipid molecules orientated parallel to each other. Their polar "heads" were orientated outwardly while the nonpolar "tails" were orientated towards the interior of the membrane (Gorter and Grendel, 1925). Later investigators showed this theory to be essentially correct, and the modern concept of the so-called "unit membrane" was proposed in a review by Robertson (1966). The double lipid layer is covered partly with protein and partly with mucopolysaccharides and appears to be present in all cells. We assume that the TBE virus can attach to a certain lipid receptor molecule which is positioned in a certain way in this double layer. This substance or similar substances in solution, however, can only compete with the receptors in the cell wall when they have been orientated in a similar state through the colloidal-forming activity of certain trimethylammonium compounds. Only then are they capable of binding to the virus which would have attached to the membrane of the erythrocyte. Experimentaly, this is observed as inhibition of hemagglutination.

Summary

A technique is described which allows one to measure the capacity of certain substances to inhibit the hemagglutination of TBE virus under competitive conditions. When tested with this method, brain homogenates and extracts obtained from them with lipid solvents were more than 100 times as active as when investigated with the older techniques. Using solvent fractionation and different chromatographic procedures, the active factors could be isolated and were shown to consist of phosphatidylinositol diphosphate in combination with lecithin or with sphingomyelin. These choline-containing bases could be substituted by cetyltrimethylammonium bromide. A theory is suggested which assumes that proper alignment of receptor molecules and inhibitors is necessary for their reaction with the surface of the virus particle.

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