MEANING OF LIPIDS IN THE IMMUNE SERUM

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Introduction

It is reported that the amount of lipids in the serum may in some cases increases by an immunization. Namely, Danysz-Michel and Laskownicki[1] found that the cholesterol increased in the serum of rabbits immunized with B paratyphosus. The same result was also obtained by Ionesco-Mihaesti and Damboviceanu[2] in the serum of horses immunized with the same B bacillus, and more over they found that the cholesterol content reached a maximum at the same time as the agglutination titer. Felton and Kaufmann found that substances, soluble in alcohol or ether, increased in the serum of various animals immunized with pneumococcus types 1 or 11.

On the contrary, however, Koldaw observed that the serum of horses immunized with tetanus, diphtheria, or typhoid bacilli contained the same amount of cholesterol as normal serum. Marie[3] found that various immun-horse-serum contained less cholesterol than normal.

The effect of the removing alcohol- and ether-soluble substances from antiserum, without apparent denaturation of the proteins, was studied by Hartley.[4] He obtained the results that the antihorse serumprotein sera of rabbits and the antidiphtheria serum of horses were bereft the precipitating or flocculating power by extraction with alcohol and ether. Horsfall and Goodner[5] found that agglutinating power of the antiserum was restored to a considerable extent by the addition of suspension of lipids to the extracted serum.

In this studies I attempted to know the behaviour of various fraction of serum lipids during an immunization of animals, to clear that it may be essential to the antibody properties or not if the changes of any fractions of serum lipids are perceived acutualy.

Experiment

I. The lipids content of hemolysin serum.

Rabbits were used for an immunization, excluding those in which the serum, prior to the immunization, gave a strong or complete hemolysis in a dilution of 1:25 in an hour under the condition of the test. Before the immunization the lipid content of the blood serum of rabbits were estimated. Twelve hours after the last diet the blood was taken from the rabbits and serum was separated by standing in a room temperature.

The methods of determinations of lipids are as follows[6], [7].

a). The extraction of the serum.

To 70 ml. of boiling alcohol-ether mixture (3:1) in a 100 ml. messflask, 1 ml. of serum is added under constant shaking. It was boiled for several seconds. After cooling it was diluted to a mark with alcohol-ether mixture and filtrated.

b). Total lipid.

30 ml. of this extract was taken and evaporated to dryness and then dissolved in a small amount of ether and diluted to 15 ml. 5 ml. of this etheral solution was dried on a water-bath and added to it a certain amount of Nicloux-reagent and oxidized by heating on a boiling water-bath for two hours. After cooling, 20 ml. of water and 1 ml. of 40 per cent solution of pottasium iodie were added and titrated with N/25 sodium thiosulphate solution using few drops of 1 per cent starch solution as an indicator. Number of ml. of sodium thiosulphate solution required is indicated as A.

c). Elimination of phospholipids.

10 ml. of the etheral solution in the above experiment was taken in an Ellenmeyer flask. After a concentration on a water-bath to syrupy, a small amount of acetone was added to it and further concentrated. Then few drops of alcoholic solution of magnesium chloride were added to it and let stand for 30 minutes at a room temperature and filtrated. The filtrate was diluted with ether to 10 ml. 5 ml. of this filtrate was taken and treated as described as above and titrated with sodium thiosulphate solution. Number of ml. of sodium thiosulphate solution required is indicated as B.

d). Elimination of free cholesterol.

5 ml. of the above ether solution was taken and 5 ml. of acetone and 1 ml. of 0.2 per cent alcoholic solution of digitonine were added and evaporated to dryness on a water-bath. The residue was dissolved in ether and

filtrated. The filtrate was titrated in the same manner described as above. Number of ml. of sodium thiosulphate solution required is indicated as C.

e). Saponification.

20 ml. of the alcohol-etheral extract in a section of (a) was taken. Few drops of satulated solution of sodium hydroxide was added and saponificated on a boiling water-bath. Then it was neutralized with 1 mol phospholic acid and warmed for several minutes under an addition of 8 ml. of petroleum ether. The petroleum ether fraction was transfered in a flask and evaporated to dryness. The residue was dissolved in ether and diluted with ether to 10 ml. 5 ml. of this solution was used for titration with sodium thiosulphate as above. Number of ml. of sodium thiosulphate solution required is indicated as D_1.

f). Elimination of total cholesterol.

To 5 ml. of the above solution, 5 ml. of acetone and 1 ml. of 0.2 per cent alcoholic solution of digitonine was added and evaporated on a water-bath. The residue was dissolved in ether and filtrated. The filtrate was titrated in the same condition described as above. Number of ml. of sodium thiosulphate solution required is indicated as D_2.

Calcuration of each lipids content depends upon the following formuras.

1. $\text{phospholipid} = \frac{(B-A)\times T}{2.82}\times\frac{1}{2.5}\times\frac{100}{0.1} = (B-A)\,T\times 142$ mg/dl.

2. $\text{free cholesterol} = \frac{(C-B)\times T}{3.92}\times\frac{1}{2.5}\times\frac{100}{0.1} = (C-B)\,T\times 102$ mg/dl.

3. $\text{total cholesterol} = \frac{(D_2-D_1)\times T}{3.92}\times\frac{1}{2.5}\times\frac{100}{0.1} = (D_2-D_1)\,T\times 102$ mg/dl.

4. $\text{neutral fat} = \left\{(B_1-C)\times T\times\frac{1}{2.5}\times\frac{100}{0.1} - \text{cholesterol ester}\times 3.92\right\}\times\frac{1}{3.60}$

$$= \left\{(B_1-C)\times T\times 1000 - \text{cholesterol ester}\times 9.8\right\}\times\frac{1}{9}\ \text{mg/dl.}$$

5. $\text{total fatty acid} = \frac{(B_1-D_2)\,T}{3.60}\times\frac{1}{2.5}\times\frac{100}{0.1} = (B_1-D_2)\,T\times 111$ mg/dl.

B_1 is the number of ml. of the sodium thiosulphate required in a blind test.

Few weeks after a taking of blood for the determination of lipids in a normal state the rabbits were immunized. To generate the hemolysin serum rabbits were immunized with sheep red corpuscles. Sheep corpuscles freed from serum were washed saline solution three times and suspended in physiological saline solution in a concentration of 3 per cent. The rabbits were immunized three times each with one ml. of the suspension with intervals of five days between each injection. Five or seven days after the last

injection the blood was taken out for an experiment. In general, by an immunization, a titer of hemolysin riched to 1000 or 2000 times. The results of determination are as follows.

Table I.

The lipids content of the serum of rabbits before an immunization.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
1	2.3	white/♂	364	211	72	130	162	54	108
2	2.1	white/♀	331	229	69	122	140	45	95
3	2.2	white/♂	347	147	70	125	152	62	90
4	2.6	white/♀	434	213	134	120	180	66	114
5	2.3	white/♂	403	138	52	140	210	72	138
6	2.4	white/♀	427	190	110	127	190	48	142
7	2.3	white/♂	357	210	65	112	180	93	87
8	2.3	white/♂	311	192	71	110	130	36	94
average			371.7	191.2	80.3	124.2	168.0	59.5	108.5

Table II.

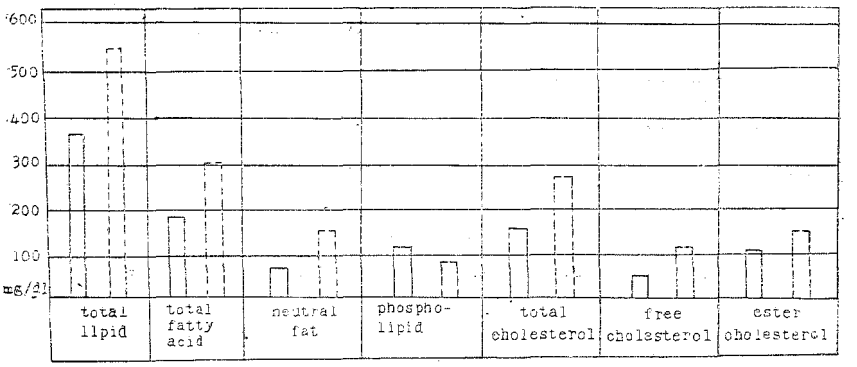
The lipids content of the rabbits immunized with sheep red corpuscles.

No. of rabbit	body weight (kg.)	color and sex	hemolysin titer	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
1	2.5	white/♂	640	456	329	142	34	280	112	168
2	2.4	white/♀	1000	563	320	160	83	320	160	160
3	2.4	white/♂	480	530	260	189	86	255	120	135
4	2.8	white/♀	1700	504	433	134	110	260	130	130
5	2.5	white/♂	1000	672	251	232	130	310	124	186
6	2.6	white/♀	640	590	280	159	134	307	92	205
7	2.6	white/♂	1300	550	312	150	100	300	120	180
8	2.5	white/♂	640	457	270	140	56	261	110	151
average				540.2	306.8	163.2	91.6	286.6	121.0	164.3

The results of the two investigations are most conveniently shown in Fig. 1.

Fig. I.

Comparison of the lipids contents of normal- and immunized-rabbits serum.



real line: normal serum
blocken line: hemolysin serum.

From these experiments we know that in the hemolysin serum

1). Phospholipids decreased compared with normal serum.
2). Free cholesterol, cholesterol ester, neutral fat and total fatty acids increased and consequently the total lipid increased remarkably.

II. The lipids content of rabbits immunized with eggalbumin.

Rabbits were immunized three times with 1 ml. of 10 per cent eggalbumin solution, each times, with intervals of five days between each injections.

At the 7th day after the last injection, blood was taken out and the titer of the precipitins were determined by Uhlenfut's method. Serums, which precipitin titer rise over 10000 times, were used for analysis of lipids.

The results are as follows.

Table III.

The lipids content of the serum of rabbits before an immunization.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospho lipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
9	2.1	brown/♀	322	209	67	113	142	61	81
10	2.3	white/♂	297	167	63	98	136	59	77
11	2.2	white/♂	314	182	64	110	140	54	86
12	2.9	white/♀	362	212	81	120	161	71	90
average			323.7	192.5	68.7	110.2	144.7	61.2	83.5

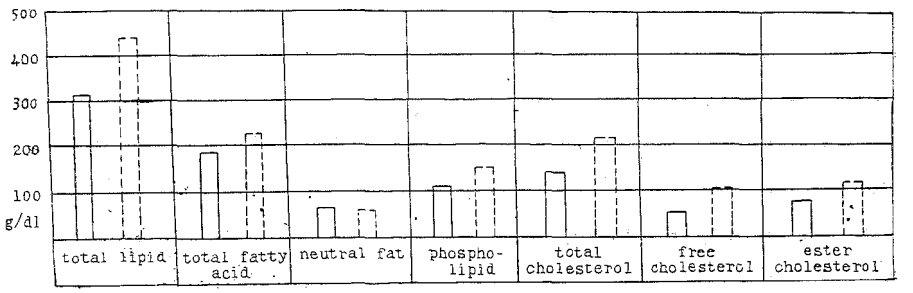
Table IV.

The lipids content of the serum of rabbits immunized with eggalbumin.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospho-lipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
9	2.4	brown/♀	557	135	93	170	294	208	86
10	2.6	white/♂	431	120	101	140	190	91	99
11	2.4	white/♂	372	298	60	129	183	60	123
12	3.0	white/♀	438	347	10	217	211	51	160
average			449.5	225.0	66.0	164.0	219.5	102.5	117.0

Fig. II.

Comparison of the lipids content of the serum of normal- and immunized-rabbits.



real line: normal serum.

blocken line: antialbumin serum.

The results indicate that the every fractions of lipids increase but only neutral fat remains almost unchanged.

III. The lipids content of the serum of rabbit immunized with the non-antigenic substance.

In the preceding experiments we know that in the immune serum, such as hemolysin or precipitin serum, various fractions of lipids increase to same extent. To know whether such increases of lipids in the immune serum are essential to the formation of antibodies or not, rabbits were immunized with gelatin solution, which is known as a protein but not possesses an antigenic character, probably for the lack of aromatic amino acids in it's molecule, or red blood corpuscles of the same species, which is of course no antigenic, and the lipids content of the serum were determined as before.

(1). The lipids content of the antigelatin serum of rabbit.

Rabbits were immunized three times with 1 ml. of 5 per cent gelatin solution each time with an five days' intervals in each injection. At the 7th day after the last injection, the blood was taken out and used for the experiment. Of course the serum indicated no production of antibody by complement fixation reaction as illustrated in Table V.

The results of determination of lipids content before and after an immunization are shown in Table VI and VII.

Table V.

Complement fixation reaction of antigelatin serum and gelatin solution.

No. of rabbit	Unit of complement				serum + complement	serum + antigen
	2	4	6	8		
13	##	##	##	##	##	##
14	##	##	##	##	##	##
15	##	##	##	##	##	##
17	##	##	##	##	##	##

In the table, cases where no hemolysis occur are indicated as (−) complete hemolysis as (##), while for immediate cases the signs (±), (+) and (+) are used.

Table VI.

The content of the serum of rabbits before an immunization.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
13	2.4	white/♂	340	192	76	113	151	56	95
14	2.6	white/♀	355	187	80	135	140	46	94
15	2.3	white/♀	373	210	86	125	162	60	102
16	2.4	white/♂	335	178	65	110	160	42	118
17	2.5	white/♂	315	205	70	120	125	50	75
average			343.6	194.4	75.4	120.6	147.6	50.8	96.8

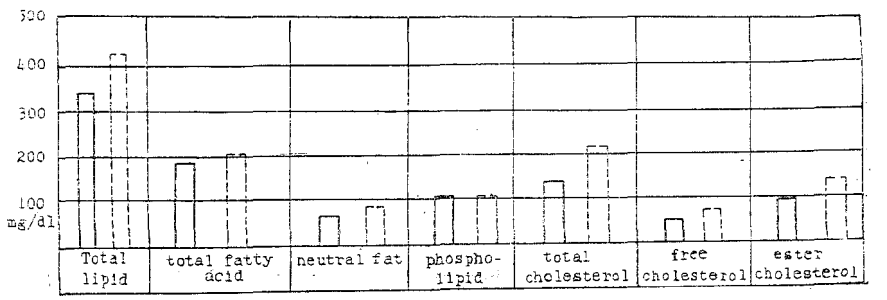
Table VII.

The lipids content of the serum of rabbits immunized with gelatin.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
13	2.5	white/♂	408	210	92	121	195	78	117
14	2.7	white/♀	487	190	97	130	262	98	164
15	2.5	white/♀	423	230	88	126	209	67	142
16	2.6	white/♂	477	211	102	115	260	92	168
17	2.6	white/♂	369	181	72	125	172	73	99
average			432.8	204.4	90.2	123.4	219.6	81.6	138.0

Fig. III.

Comparison of the lipids content of the serum of normal- and immunized rabbits.



real line: normal serum.
blocken line: antigelatin serum.

(2). The lipids content of the serum of rabbits immunized with red corpuscles of the same species.

Rabbits were immunized three times with 1 ml. of 5 per cent suspension of corpuscles of the other rabbit each times with intervals of five days between each injection. On the 7th day after the last injection blood was taken out. No hemolysin were produced as indicated in table VIII.

The results of analysis of lipids content before and after an immunization are as follows.

Table VIII.

Hemolysing reaction of the serum of rabbits immunized with red corpuscles of the other rabbit.

No. of rabbit \ dilution of serum	25 times	50	100	500	1000	2000
18	—	—	—	—	—	—
19	—	—	—	—	—	—
20	—	—	—	—	—	—

Table IX.

The lipids contents of the serum of rabbits before an immunization.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
18	2.0	white/♂	405	172	108	92	204	61	143
19	1.9	white/♀	341	225	89	114	138	84	54
20	2.1	white/♂	461	99	142	126	193	91	102
average			402.3	165.5	113.0	110.6	178.3	78.6	99.6

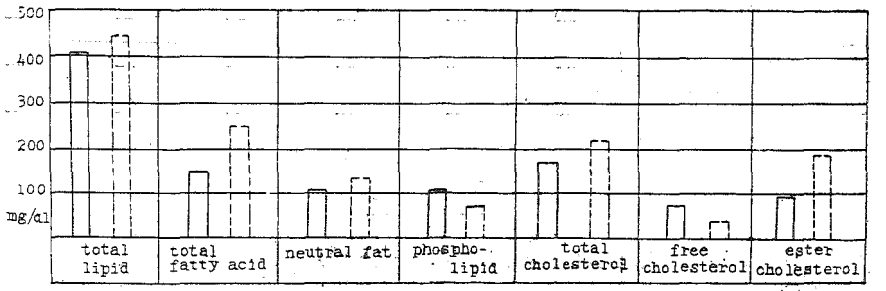
Table X.

The lipids contents of the serum of rabbits immunized with red corpuscles of the other rabbit.

No. of rabbit	body weight (Kg.)	color and sex	total lipid mg/dl	total fatty acid mg/dl	neutral fat mg/dl	phospholipid mg/dl	total cholesterol mg/dl	free cholesterol mg/dl	ester cholesterol mg/dl
18	2.1	white/♂	522	228	193	88	241	51	190
19	2.1	white/♀	370	187	119	85	166	30	136
20	2.1	white/♂	452	300	108	77	272	30	242
average			449.6	255.0	140.0	83.3	229.5	37.0	189.3

Fig. IV.

Comparison of the lipids contents of the serum of normal- and immunized rabbits.



real line: normal serum

blocken line: immunized serum

As can be seen from these results the lipids in the serum increase remarkably not only by the injection of antigenic substances, but also of non-antigenic substances such as gelatin or corpuscles of the same species. Therefore the increase of the lipid in the immune serum can not be essential to the formation of the antibodies.

IV. Isolation of the hemolysin fraction from the immune serum.

Rabbits were immunized three times with sheep red corpuscles and the hemolysin serum was obtained. The serum was fractionated into three proteins adding sodium sulphate solution in a manner indicated in the next table.

Table XI.

The method of fractionation of the hemolysin serum by Na_2SO_4

	concentration of Na_2 SO_4	protein precipitated	No. of supernatant
10 ml. of serum+8.2 ml. of 30% Na_2SO_4	13.5%	Euglobulin	I
10 ml. of 1+9.4 ml. of 30% Na_2SO_4	21.5%	Pseudo-globulin	II
10 ml. of II+excess of Na_2SO_4 crystal	satulation	Albumin	III

30 per cent solution of sodium sulphate was restored in 37°C, and the mixtures of the solution and the serum or supernatant liquid were incubated for 30 minutes at 37°C and then centrifuged to separate the precipitates. The each precipitate were dialyzed in collodium membrans against running water until the SO_4 reaction disappeared completely. The content of the membrans were dissolved in a same amount of physiological saline solution as the original serum and the hemolysing power were tested about each fraction. The results are as follows.

Table XII.

Hemolysing power of the fractions of the hemolysinserum.

dilution of solution / fraction	50 times	100	200	500	1000	2000
Albumin	—	—	—	—	—	—
Pseudoglobulin	##	##	##	##	##	+
Euglobulin	—	—	—	—	—	—
Albumin+ Pseudoglobulin	##	##	##	##	##	+
Albumin Euglobulin	—	—	—	—	—	—
Pseudoglobulin+ Euglobulin	##	##	##	##	##	+
Pseudoglobulin+ Euglobulin+Albumin	##	##	##	##	##	+
Original serum	##	##	##	##	##	##

The result indicates that by the fractionation of the hemolysin serum with sodium sulphate, antibodies are collected completely only in the pseudo-globulin fraction and other fractions can not act even auxiliary.

As we know that the hemolysins are contained in the pseudoglobulin fraction completely, the experiment was performed to determine whether

the lipid increase in the pseudoglobulin fraction of the immune serum in comparison with that of the normal serum or not. As the hemolysin serum, only the serum which hemolysin titer rised over 1000 times was used. The pseudoglobulin fractions, prepared from normal- and immunized rabbits were dried in vacuum.

The contents of the total lipids of the both fractions were determined by extraction with ether in soxlet's apparatus and following results were obtained.

Table XIII.

Content of total lipid in the pseudoglobulin fractions.

Pseudoglobulin fraction from normal rabbit serum	Pseudoglobulin fraction from hemolysin serum
3.87%	4.93%
4.11%	4.48%
3.44%	4.59%

The content of total lipids increases in the pseudoglobulin fraction from the hemolysin serum compared with in that from normal serum.

V. The effect of removing of lipid from pseudoglobulin fraction.

The pseudoglobulin fraction prepared from the hemolysin serum was dialyzed in a collodium membran and the precipitate was treated with alcohol and ether successively at a temperature not exceeding -10°C and dried in vacuum as rapidly as possible. The dried matter was extracted in Soxlet's apparatus with ether completely, and then dissolved in a same amount of saline solution as original serum. The hemolysing power of this solution was tested and obtained a following result.

Table XIV.

Hemolysing reaction of the defatted pseudoglobulin fraction of the hemolysin serum.

dilution of solution	25 times	50	100	200	500	1000
	##	##	##	##	+	—

VI. The effect of digestion of the pseudoglobulin fraction with trypsin.

From the preceding experiment we know that the removal of the lipid from the pseudoglobulin fraction of the hemolysin serum has no remarkable effect on it's hemolysing power. In this section I attempted to know the effect of a digestion of the protein portion of the pseudoglobulin fraction.

For the digestion of protein Merck's trypsin was used after purified by Willstatter's method to remove a trace of lipase. The mixture of the pseudoglobulin fraction and the purified trypsin solution was incubated at PH 7.8 at 37°C until the biuret reaction disappeared. The mixture was heated at 56°C for 30 minutes to inactivate the trypsin and the hemolysing powers was tested. The control test was performed using the trypsin solution, inactivated by heating at 56°C for 30 minutes, instead of the native enzyme solution.

The result is indicated in the next table.

Table XV.

The effect of digestion of the protein portion of the pseudoglobulin fraction.

dilution of the serum	25 times	50	100	200	500	1000	2000
Test I	++	—	—	—	—	—	—
control test	+++	+++	+++	+++	+++	++	—
Test II	+	+	—	—	—	—	—
control test	+++	+++	+++	+++	++	+	—

Conclusion

(1). The content of total lipid in rabbit serum increases remarkably by an immunization, but the increase is also found in the serum of rabbits immunized with non-antigenic substance such as gelatin or corpuscles of the same species. Consequently the increase of the lipids in the immune serum seems to be not essential to the formation of antibodies.

(2). The increases of various fractions of the lipid by the immunization are as follows.

(a). Phospholipids increase in the antigelatin- and antieggalbumin-rabbit serum and somewhat decrease in the serum of the rabbit immunized with sheep red corpuscles or other rabbit red corpuscles.

(b). Free cholesterol increases in every cases except in the serum of rabbit immunized with the rabbit red corpuscles.

(c). Total cholesterol, cholesterol ester, neutral fat and total fatty acids increase in all cases.

(3). In the anti-sheep corpuscles serum of rabbit hemolysins are collected completely in the pseudoglobulin fraction prepared with sodium sulfate. The lipid content of the pseudoglobulin fraction from the immune serum increases compared with that from the normal serum.

(4). The removal of the lipid from the pseudoglobulin fraction exerts no remarkable effect on the hemolysing power, but by the digestion of the protein part of the fraction by trypsin the hemolysin power disappears almost completely.

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