LIPIDS AND IMMUNOLOGICAL REACTIONS

IV. THE LIPID PATTERNS OF SPECIFIC PRECIPITATES FROM TYPE I ANTIPNEUMOCOCCUS SERA

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It has been shown (1) that, after the extraction of lipids, antipneumococcus horse serum fails to give type-specific agglutination or precipitation, and with antipneumococcus rabbit serum these reactions are almost entirely eliminated. If these extractive procedures are carried out in such a manner as to avoid denaturation of proteins the removal of the lipids in no way interferes with the fundamental capacity of the antibody to combine with the specific antigen; lipid extraction merely affects the secondary *in vitro* phenomena of immunological reactions (1, 2).

A tentative hypothesis formulated to explain these results held that antipneumococcus antibodies might be lipo-protein complexes. This theory found some support in the fact that the original agglutinating and precipitating properties of the lipid-extracted immune sera could be restored by the addition of certain purified phosphatides, although in this restoration there was a curious selectivity dependent upon the species origin of the antibody. Thus the "essential" phosphatide for antipneumococcus horse serum is *lecithin*, while that for antipneumococcus rabbit serum is *cephalin* (1).

The more obvious approach to the establishment of this thesis would be through the study of the antibody isolated in a completely pure form. Since the absolute isolation of antibodies has not yet been accomplished, a second and somewhat indirect approach was adopted. Specific immune precipitates were obtained by adding the pneumococcus capsular polysaccharide to antipneumococcus sera. These precipitates might have been expected, from the general theories of antigen-antibody reactions, to contain only the carbohydrate and the antibody. It was found, however, that these precipitates also contained lipid, the amounts varying from 4 to 51 per cent of the total mass of the precipitate (3). This finding was

unexpected in still another sense, for it had been determined in the earlier work (1) that the amounts of phosphatide necessary to restore the *in vitro* properties of extracted antipneumococcus sera were very small (approximately 0.025 mg. per cc.) and thus could not account for the relatively large amounts of lipid in the immune precipitates. As these studies progressed it became increasingly apparent that this large quantity of lipid was, for the most part, present as a result of adsorption, since the amount in the precipitate seemed to be a function of the lipid concentration of the reacting mixture (3). In spite of this fact and even though the probable amount of the essential lipids would be too small to estimate by any of the present chemical methods, it seemed important to determine the nature and amounts of the various lipids in the immune precipitates, particularly with reference to the concentration of these lipids in the immune sera.

In this paper are presented quantitative data determined by the microchemical study of Type I antipneumococcus horse and rabbit sera and of the specific precipitates prepared from them. From these data total lipid patterns have been calculated.

Methods

Two separate lots of unconcentrated and monovalent Type I antipneumococcus rabbit serum and one lot of Type I antipneumococcus horse serum have been used as antibody source. The sera were first filtered through a Berkefeld V and were then whirled at 0°C. for 30 minutes in the angle centrifuge to remove any particulate matter. As the specific precipitant, the acetyl capsular polysaccharide of Pneumococcus Type I was used. Precipitates were prepared and washed according to the method of Heidelberger and Kendall (4). The analysis of total nitrogen in the precipitates was carried out in a manner identical to that previously described (3), and lipids were extracted from the precipitates by the technique described in the same paper. Lipid fractions were analyzed by the methods of Kirk, Page, and Van Slyke (5), each determination being made on two aliquots of the extracts. The figures given in Table I represent the mean value of duplicate determinations in each instance. The precipitates were analyzed for total nitrogen, lipid carbon, lipid nitrogen, lipid amino nitrogen, lipid phosphorus, total cholesterol, and free cholesterol. Total lipid, total phosphatide, phosphatide carbon, free cholesterol carbon, esterified cholesterol, cholesterol esters, cholesterol ester carbon, neutral fat carbon, and neutral fat have been calculated from these data according to the various equations of Page, Kirk, Lewis, Thompson, and Van Slyke (6). In Table I of the present paper, these equations are designated by the numbers (*i.e.*, 1 to 7) used by these authors.

In order to obtain precipitates with a sufficient lipid content to permit of adequate analyses, it was necessary to use a considerable quantity of antisera in their preparation. The values given in Table I were found in precipitates prepared in the equivalence zone from 3.0 cc. of antiserum. Other precipitates were pre-

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pared under the same conditions from rabbit antiserum by the addition of an equivalent amount of capsular polysaccharide to duplicate 15.0 cc. quantities of serum. In addition, a large amount (0.45 gm.) of specific precipitate[1] which had been prepared from polyvalent Type I and Type II antipneumococcus horse serum by the addition of Type II capsular polysaccharide was divided into equal portions and was analyzed in duplicate for the various lipid fractions. The figures given in Table I are, for the sake of comparison, expressed in milligrams per gram of that part of the total precipitate derived from the immune serum, that is, milligrams per gram of the sum of corrected protein nitrogen multiplied by the factor 6.25 and total lipid carbon multiplied by the factor 1.3 (3). For the antisera themselves the various values are expressed in milligrams per 100 cc. of serum.

Lipid Patterns of Specific Precipitates from Type I Antipneumococcus Horse and Rabbit Sera

Specific precipitates were prepared in the equivalence zone from both horse and rabbit antipneumococcus sera. These were analyzed by gasometric micro methods in order that complete lipid patterns could be calculated. The sera themselves were also analyzed by identical methods so that the lipid patterns both of the sera and of the precipitates could be compared. Directly determined analytical data upon these sera and the precipitates from them, as well as those values calculated from these figures, are presented in Table I.

It will be noted that there was a striking difference between the quantity of lipid amino nitrogen in the precipitate from horse antiserum as compared with that in the precipitate from rabbit antiserum. Of the total lipid nitrogen found in the former, 54.8 per cent was in the amino form, whereas no significant amount of lipid amino nitrogen was detected in the latter. This is in sharp contrast to the almost identical values for lipid phosphorus and indicates a definite qualitative difference in the phosphatides of the two precipitates. That this qualitative difference in the precipitates was not simply a reflection of a similar difference in the phosphatides of the antisera is demonstrated by the values for total lipid nitrogen, lipid amino nitrogen, and lipid phosphorus in the latter. Of the total lipid nitrogen in the horse antiserum, 35.7 per cent was in the amino form, whereas of the rabbit antiserum lipid nitrogen, 31.1 per cent was amino nitrogen. Except

[1] This precipitate was kindly prepared by Dr. M. Heidelberger.

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for this sharp qualitative difference, there was a considerable similarity between the lipid patterns not only of the precipitates but also of the sera themselves. The relationships between the various fractions comprising the total lipid patterns of these antisera and precipitates are shown graphically in Text-fig. 1.

TABLE I

Lipid Patterns of Type I Antipneumococcus Horse and Rabbit Sera and of Specific Precipitates Derived Therefrom

Substance	Method	Type I antipneumococcus serum				Specific precipitates			
		Horse		Rabbit		Horse antiserum		Rabbit antiserum	
		Mg. per 100 cc.	Per cent of total lipid	Mg. per 100 cc.	Per cent of total lipid	Mg. per gm.	Per cent of total lipid	Mg. per gm.	Per cent of total lipid
Total nitrogen.......	Determined					150.2		150.8	
Lipid carbon.........	Determined	194.2		173.7		63.0		52.1	
Total lipid...........	Equation 7	252.5		225.9		81.9		67.7	
Lipid nitrogen.......	Determined	5.41		2.48		3.08		1.54	
Protein nitrogen.....	Calculated					147.1		149.2	
Lipid amino nitrogen.	Determined	1.93		0.77		1.69		0.08*	
Lipid phosphorus....	Determined	2.42		1.15		0.82		0.85	
Phosphatide.........	Equation 1	56.9	**22.5**	27.0	**12.3**	19.4	**23.9**	20.0	**29.8**
Phosphatide carbon..	Calculated	37.5		17.8		12.8		13.2	
Total cholesterol.....	Determined	87.9		87.1		46.8		37.1	
Free cholesterol......	Determined	20.6	**8.1**	10.3	**4.7**	25.3	**31.1**	22.3	**33.1**
Free cholesterol carbon............	Calculated	17.2		8.7		21.2		18.7	
Esterified cholesterol.	Equation 2	67.3		76.7		21.6		14.8	
Cholesterol esters....	Equation 3	115.5	**45.7**	129.8	**59.1**	36.4	**44.9**	25.0	**37.2**
Cholesterol ester carbon............	Calculated	94.3		107.5		30.2		20.7	
Neutral fat carbon...	Equation 4	45.1		39.8		0.0		0.0	
Neutral fat..........	Equation 5	59.5	**23.5**	52.5	**23.9**	0.0	0.0	0.0	0.0
Total lipid...........	Equation 6	252.5		219.7		81.1		67.3	

* Quantity not significant.

In order to confirm these results complete lipid patterns have been determined on four separate precipitates from the two lots of Type I antipneumococcus rabbit sera. In these precipitates lipid amino nitrogen was found to account for an average of but 5.62 per cent of

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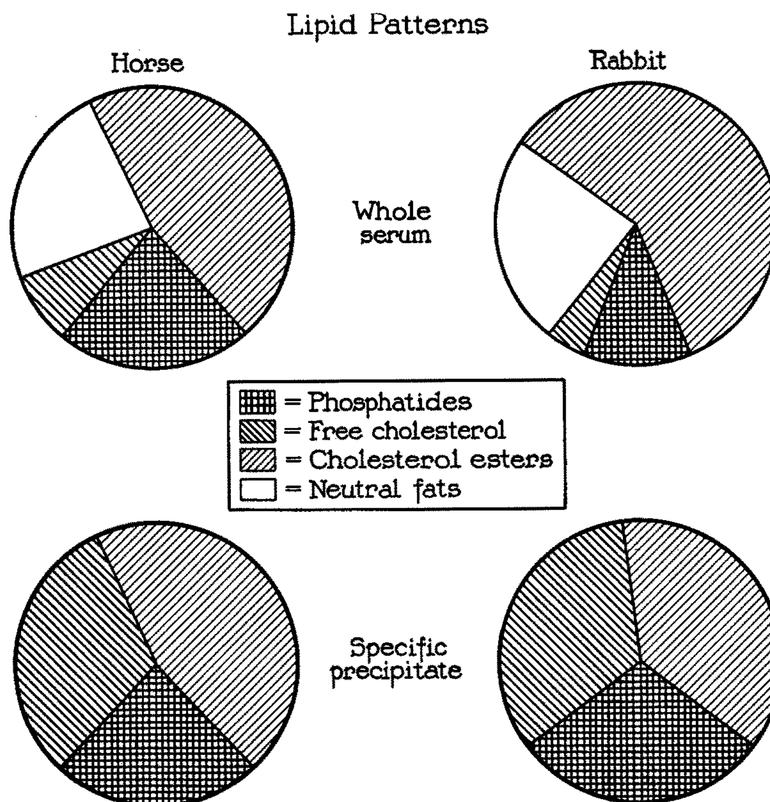
the lipid nitrogen, with a range of 4.32 to 7.44 per cent. Lipid patterns have also been determined on four separate precipitates from the two lots of antipneumococcus horse sera; two from Type I antiserum and two from a mixed Type I and Type II serum. In these instances



TEXT-FIG. 1. Diagrammatic representations of the lipid patterns of Type I antipneumococcus horse and rabbit sera and of specific precipitates derived therefrom. The differences in the phosphatide fractions are fully described in the text.

lipid amino nitrogen formed an average of 54.68 per cent of the lipid nitrogen, with a range of 54.18 to 55.40 per cent.

In the light of these observations, the difference noted in the phosphatide fraction of the lipids in specific precipitates assumes definite

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significance. Since both rabbit and horse antisera have been shown to contain amino and non-amino phosphatides in similar amounts, the very small amount of amino phosphatide in the specific precipitate from rabbit antiserum and the large amount of amino phosphatide in the specific precipitate from horse antiserum suggests the possibility that a selective adsorption of phosphatide by the forming precipitates occurs.

Certain additional studies have been made upon the effect of the addition of purified phosphatides to horse antipneumococcus serum prior to the formation of specific precipitates. The results of these studies are not entirely conclusive, since it is extremely difficult to add lipids in any state other than an unnatural colloidal suspension, but it may be stated that evidence has been obtained which indicates that the presence of added lecithin or cephalin in the reacting mixture causes a marked reduction in both the total and free cholesterol of the precipitates. This decrease in cholesterol in the precipitates is more pronounced in the presence of added lecithin than in the presence of added cephalin.

DISCUSSION

The data presented indicate that, although the specific precipitates from Type I antipneumococcus horse and rabbit sera contain relatively similar amounts of total lipid, the nature of the various separate lipids comprising this total is different for the two species. This difference in the lipid patterns of the precipitates appears to lie almost entirely in the phosphatide fraction and is only detected when lipid nitrogen and lipid amino nitrogen analyses are compared. It then becomes evident that, although 54.6 per cent of the lipid nitrogen of precipitates from horse antisera is in the amino form, only 5.6 per cent of the lipid nitrogen in precipitates from rabbit antisera is amino nitrogen.

Since it has been shown that the relationships between lipid phosphorus, lipid nitrogen, and lipid amino nitrogen are almost identical in the antisera themselves, it is not possible to explain the observed differences in the phosphatides of the precipitates simply on the basis that they are a reflection of the lipid patterns of the antisera.

Of those phosphatides known to occur in sera, lecithin and cephalin form much the greater part. The former contains no amino nitrogen, and of the nitrogen present in the latter all is in the amino form. Although accurate micro methods have not yet been developed which allow of the direct estimation of either lecithin

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or cephalin in small amounts, it is possible to gain an approximation of their quantity by indirect means. This involves calculations based upon lipid phosphorus, lipid nitrogen, and lipid amino nitrogen. When, as in the case of the lipid fraction of the specific precipitate from rabbit antisera, almost insignificant quantities of lipid amino nitrogen are found and appreciable amounts of both lipid phosphorus and lipid nitrogen are determined, it becomes obvious that cephalin, an amino nitrogen-containing phosphatide, is present in amounts so small as almost to escape detection by the methods used. In order to account for both the lipid phosphorus and lipid nitrogen found it is necessary to assume that in this instance these form parts of a non-amino nitrogen-containing phosphatide. Since lecithin fulfills these requirements and is known to constitute a large portion of the phosphatides of serum, it is logical to suppose that lecithin is present and comprises much the larger proportion of the phosphatide in the specific precipitate from rabbit antiserum.

On the other hand, when, as in the case of the specific precipitates from horse antisera, as much as 54.6 per cent of the total lipid nitrogen is in the amino form, and when this quantity of amino nitrogen is considerably in excess of the theoretical amount necessary to give an atomic ratio of 1 with the lipid phosphorus found, it becomes apparent that the larger part of the phosphatide present in the precipitate contains amino nitrogen. Since cephalin contains both amino nitrogen and phosphorus, and is known to be present in serum, it is fair to consider that the phosphatide fraction in the specific precipitates from horse antisera is largely cephalin.

Finally, it may be suggested that since the lipid patterns of specific precipitates from horse and rabbit antisera are quite different as regards the nature of the phosphatide fraction, although the lipid patterns of the antisera themselves are very similar, selective adsorption of phosphatides by the two different precipitates may account for the observed dissimilarity. It appears that from serum containing both lecithin and cephalin, a forming precipitate resulting from the union of the horse antibody and the homologous capsular polysaccharide carries down an amino nitrogen-containing phosphatide which is probably for the most part cephalin, while under the same circumstances the rabbit antibody-polysaccharide complex carries down a non-amino nitrogen-containing phosphatide which seems to be largely lecithin.

These findings present a curious paradox. It has been shown that lecithin restores the *in vitro* properties of horse antiserum extracted by

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lipid solvents, and that cephalin acts similarly in the case of rabbit antiserum, whereas cephalin will not affect extracted horse serum nor lecithin extracted rabbit serum (1). Because of this finding these two phosphatides have, in a descriptive sense, been termed essential and it has been suggested that lecithin may form a portion of the antibody complex in horse antiserum, and that cephalin may be similarly placed in rabbit antiserum. The analyses of specific precipitates from horse and rabbit immune sera have shown what appears at first glance to be exactly the opposite, for the phosphatide in the precipitate from horse antiserum is almost entirely cephalin, while that in the rabbit precipitate is very largely lecithin. It must be emphasized, however, that the amount of so called essential phosphatide is very minute, as little as 0.025 mg. per cc. of extracted serum being sufficient to restore *in vitro* properties. Of the various precipitates analyzed, the largest was prepared from 20.0 cc. of antiserum, and from theoretical considerations would therefore contain but 0.50 mg. of essential phosphatide, at most. In the methods used this quantity of either lecithin or cephalin would give but 0.00093 mg. and 0.0026 mg. of directly determinable nitrogen and phosphorus respectively, amounts which are too minute to permit of accurate estimation.

Although these findings do not contribute to the solution of the important question as to whether the antibody is a phosphatide-globulin complex, they do demonstrate that antigen-antibody aggregates possess curious and selective properties dependent upon the species derivation of the antibody. It is suggested that these properties may explain certain differences in the various immunological characteristics of these two immune sera (7, 8).

SUMMARY

1. Complete lipid patterns of specific precipitates from horse and rabbit Type I antipneumococcus sera, as well as of the sera themselves, have been determined by gasometric micro methods.

2. The lipid patterns of horse and rabbit antisera are very similar, and as regards the phosphatide fractions are relatively identical.

3. The lipid patterns of specific precipitates from horse and rabbit antisera show one outstanding qualitative difference. The specific precipitate from horse antiserum contains an amino phosphatide,

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which is probably cephalin, while that from rabbit antiserum contains a non-amino phosphatide, which is thought to be lecithin.

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