LIPIDS AND IMMUNOLOGICAL REACTIONS

III. Lipid Content of Specific Precipitates from Type I Antipneumococcus Sera

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It has been reported (1, 2) that the presence of certain lipids is essential to the *in vitro* demonstration of type-specific agglutination and precipitation by antipneumococcus sera. It was suggested that the antibody of antipneumococcus serum might be a phosphatide-globulin complex, the nature of the lipid fraction being dependent upon the species derivation of the antibody. Because of the minute amounts of phosphatide necessary to restore the *in vitro* properties of antisera from which lipids had been extracted, it was to be expected that the amount of lipid actually associated with the antibody itself would be very small.

It is apparent that if lipid does form a portion of the antipneumococcus antibody, much the simplest means of defining both its amount and character would be by a study of the antibody in an isolated and pure condition. Since, however, technical difficulties which interfere with the isolation of pure antibodies have not been entirely surmounted, it was necessary to study the antibody in its native state. While the method adopted has the disadvantages inherent to the chemical study of a substance dispersed in a complex system, these disadvantages are to some extent offset by the fact that the antibody had not been altered by chemical manipulation. Perhaps the most convenient and satisfactory means of obtaining a separation of the antibody from the various constituents of antipneumococcus sera is by precipitation with the homologous capsular polysaccharide. Such precipitates, if carefully prepared and washed by the method of Heidelberger and Kendall (3), afford adequate and readily available material for the quantitative study of those lipids associated with type-specific

antipneumococcus reactions. It is obvious that in precipitates prepared from a solution as complex in composition as serum, one might find not only those very minute amounts of lipid assumed to be intimately associated with the antibody, but also any lipid which was adsorbed during the formation of the precipitate.

The literature on this subject is very meager. Douglas and Dudley (quoted by Hartley (4)) found that 25 per cent of a dried immune precipitate prepared from a mixture of horse serum and the corresponding rabbit antiserum was ether-soluble. Marrack and Smith reported (5) that diphtheria toxin-antitoxin floccules contained a small amount of chloroform-soluble material, but they were unable to find lipids in rabbit-anti-horse precipitates (6). Breinl and Haurowitz (7) found from 2 to 8 per cent lipid in hemoglobin-antihemoglobin precipitates, but were not able to detect cholesterol or phosphatides by the methods used. It is apparent that there is a diversity of opinion as to the presence or absence of lipid in specific precipitates.

It is the purpose of this paper to report a series of quantitative studies upon a large number of specific precipitates prepared under varying conditions from Type I antipneumococcus horse and rabbit serum.

Methods

One lot each of unconcentrated, monovalent horse and rabbit Type I antipneumococcus sera have been used as an antibody source. The sera were passed through Berkefeld V filters just prior to each experiment, and were then whirled for 30 minutes in the angle centrifuge at 0°C. in order to eliminate any particulate matter. The acetyl capsular polysaccharide of Type I Pneumococcus was used as the specific precipitating agent. This polysaccharide contained 4.4 per cent of acetyl and 5 per cent of nitrogen.

Experiments were carried out by two methods: (*a*) the addition of increasing amounts of polysaccharide to constant amounts of serum, and (*b*) the addition of constant amounts of polysaccharide to increasing amounts of serum. In the majority of experiments the total volume of each reacting mixture, *i.e.*, serum, saline, and polysaccharide, was 5.0 cc.

Precipitates were prepared and washed according to the method of Heidelberger and Kendall (3). The sera, accurately diluted with 0.9 per cent NaCl, were measured from a calibrated burette into chemically clean test tubes with an inside diameter of 1.2 cm. The serum-saline solutions were cooled to 0°C. in an ice bath, and cold polysaccharide solution in 0.9 per cent NaCl was then added.

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The two solutions were immediately and thoroughly mixed by twirling. After standing at 4°C. for 24 hours, the precipitates were firmly packed by whirling in the angle centrifuge in the cold, and were washed twice with ice cold saline. Care was taken to break up the precipitates thoroughly during each washing in order to facilitate the removal of extraneous substances. In each instance precipitates were prepared in quadruplicate in order that one set of duplicates could be analyzed for total nitrogen and the other for both lipid carbon and lipid nitrogen.

For total nitrogen studies, duplicate precipitates were dissolved in small amounts of N/10 NaOH and the resulting solutions were then washed quantitatively into pyrex digestion tubes. The digestions and gasometric micro Kjeldahl analyses were carried out by the method of Van Slyke (8). Selenium catalyst was used in the digestion mixture as recommended by Kirk, Page, and Van Slyke (9). This method is accurate to 0.002 mg. of nitrogen. Duplicate analyses were made on each single precipitate by using aliquots of the neutralized digest. In the various tables below, the figures given for total nitrogen, unless otherwise indicated, are the mean values of four analyses (*viz.*, two aliquots on each of two duplicate precipitates). The mean variation between the values for determined nitrogen on aliquots from the same precipitates was 0.002 mg., while the mean variation of the determined nitrogen on duplicate precipitates was 0.005 mg. For the calculation of protein the corrected protein nitrogen was multiplied by the factor 6.25. The calculation of corrected protein nitrogen will be described below.

For the determination of total lipid carbon and lipid nitrogen, duplicate precipitates were thoroughly broken up in alcohol-ether 3:1 (both redistilled). The granular suspensions of the specific precipitates thus formed were transferred quantitatively to 100 cc. reflux flasks. This was made possible by means of ten separate washings with alcohol-ether. The remainder of the extraction method followed that of Kirk, Page, and Van Slyke (9). Because of the relatively small amounts of lipid in the precipitates, the secondary extraction of the dried alcohol-ether residue by petrol-ether was slightly altered, so that the final volume after filtration was only 50 cc. Two 10 cc. aliquots of the petrol-ether extract were transferred into combustion tubes for lipid carbon analyses, and a 20 cc. sample was pipetted into a pyrex digestion tube for lipid nitrogen analysis. For lipid carbon determinations the manometric micro method of Van Slyke, Page, and Kirk (10) was followed. This method is accurate to 0.003 mg. of carbon. The values for lipid carbon in the various tables below represent the mean quantity found in four analyses (*viz.*, two aliquots on each of two duplicate precipitates). The mean variation between the determined lipid carbon of aliquots from the same precipitate was 0.003 mg., and the mean variation of determined lipid carbon on duplicate precipitates was 0.004 mg. For the calculation of total lipid from lipid carbon the latter figure has been multiplied by the factor 1.3. This factor implies a mixture of lipids having an average carbon content of 77 per cent, and is an approximation for serum lipids.

Lipid nitrogen was determined by the method of Kirk, Page, and Van Slyke (9). In the various tables below lipid nitrogen figures represent the mean of separate

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analyses upon duplicate precipitates. The mean variation between the determined lipid nitrogen of duplicate precipitates was 0.002 mg.

All determinations were carried out in the Van Slyke-Neill manometric apparatus.

Comparative Lipid Content of Specific Precipitates Prepared from Constant Amounts of Immune Serum and Varying Amounts of Capsular Polysaccharide

In order to determine the relation between the lipid and protein content of precipitates, analyses were carried out upon a large number

TABLE I

Total Nitrogen, Lipid Carbon, and Lipid Nitrogen of Specific Precipitates Formed from Constant Amounts of Immune Serum and Varying Amounts of Capsular Polysaccharide

Type I antipneumococcus serum		Type I capsular polysaccharide		Specific precipitate				
Species	Amount	Mg. per cc. of antiserum	N_2 (calculated)	Total N_2	Lipid N_2	Protein N_2 (calculated)	Lipid C	Lipid content of precipitate
	cc.		*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*per cent*
Horse	1.50	0.010	0.0005	0.157	0.021	0.136	0.405	38.2
"	1.50	0.040	0.002	0.350	0.015	0.333	0.392	19.7
"	1.50	0.080	0.004	0.518	0.007	0.507	0.411	14.4
"	1.50	0.120	0.006	0.594	0.018	0.572	0.390	12.4
"	1.50	0.160	0.008	0.628	0.013	0.611	0.417	12.4
"	1.50	0.200	0.010	0.645	0.017	0.624	0.398	11.7
Rabbit	1.50	0.017	0.0008	0.079				
"	1.50	0.067	0.003	0.281	0.012	0.266	0.385	23.1
"	1.50	0.133	0.006	0.500	0.008	0.486	0.392	14.4
"	1.50	0.200	0.010	0.636	0.012	0.618	0.380	11.3
"	1.50	0.267	0.013	0.673	0.011	0.656	0.388	10.9
"	1.50	0.333	0.016	0.656				

formed from constant amounts of horse and rabbit Type I antipneumococcus sera to which varying amounts of polysaccharide had been added. The results of experiments of this character are shown in Table I.

It will be noted that under the conditions of these experiments the lipid carbon content in each series of precipitates remained practically constant over the range studied, and bore no relationship to the total nitrogen. Furthermore, it will be

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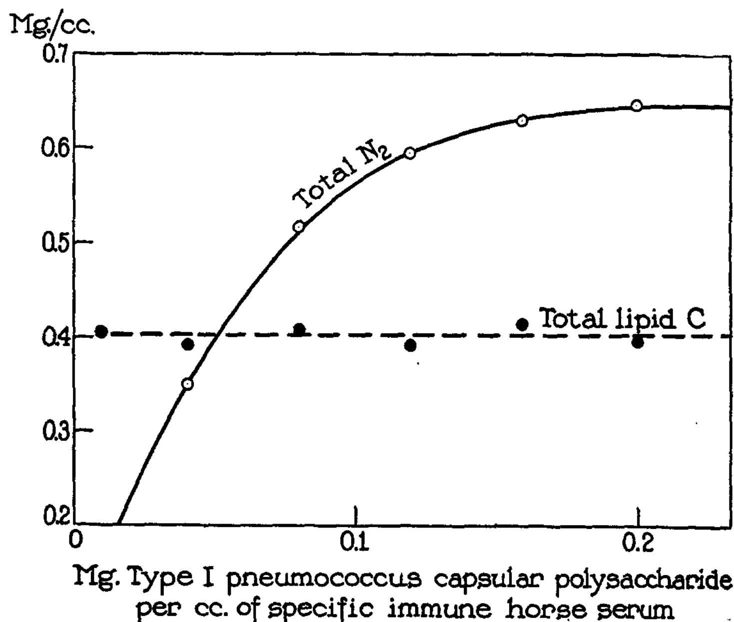
observed that the lipid nitrogen content of the precipitates remained almost constant and was not related to the total nitrogen content. As the amount of polysaccharide was increased the total nitrogen of the precipitates increased until a zone of maximum protein precipitation was reached. Upon the addition of still greater amounts of polysaccharide a gradual decrease in the total nitrogen occurred. These latter findings have been previously demonstrated by Heidelberger and Kendall (11) in a large number of careful experiments.



TEXT-FIG. 1. Total nitrogen and total lipid carbon of specific precipitates prepared from constant amounts of immune horse serum and varying amounts of capsular polysaccharide.

By plotting both the total nitrogen and the lipid carbon of these precipitates against the amount of polysaccharide per unit volume of serum, curves can be obtained which demonstrate graphically the constancy of the lipid content of precipitates prepared from constant amounts of antiserum. These curves, for both antipneumococcus horse and rabbit sera, are shown in Text-figs. 1 and 2, respectively. The absence of a direct relationship between the amounts of lipid carbon and the amounts of total nitrogen in these precipitates is clearly shown.

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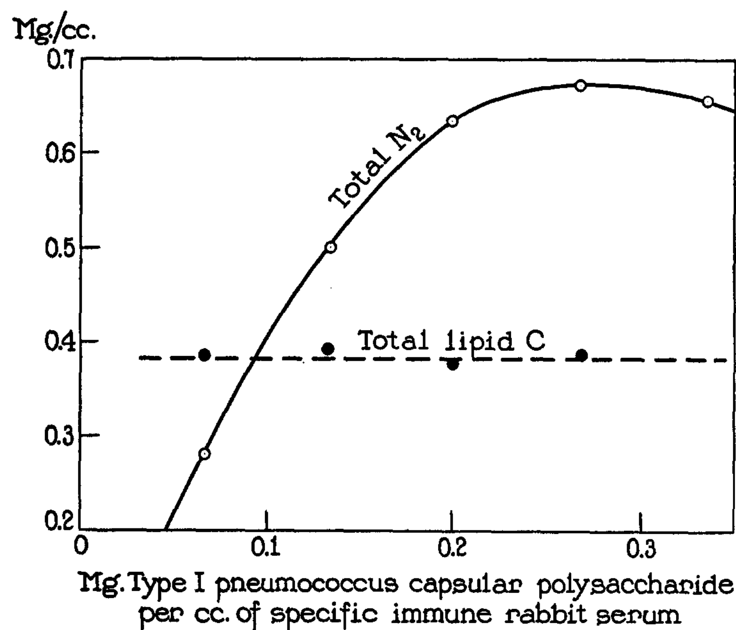
It must be pointed out that the total nitrogen of specific precipitates from Type I antipneumococcus horse or rabbit serum represents the sum of at least three separate nitrogen values. It is true that much the greater part of the total nitrogen is protein nitrogen. However, since the Type I polysaccharide contains 5 per cent of nitrogen, and a certain amount of lipid nitrogen has been shown to be present in the precipitates, these small quantities must be subtracted from the total nitrogen if an exact estimation of protein nitrogen is to be obtained.



TEXT-FIG. 2. Total nitrogen and total lipid carbon of specific precipitates prepared from constant amounts of immune rabbit serum and varying amounts of capsular polysaccharide.

It is possible in the region of antibody excess to calculate the polysaccharide nitrogen in the precipitates with considerable accuracy. In the region of antigen excess the amount of polysaccharide nitrogen can only be estimated, since the antigen content of the supernatants was not determined quantitatively. The figures for the calculated total polysaccharide nitrogen are shown in Table I. Corrected protein nitrogen was calculated by subtracting lipid nitrogen and polysaccharide nitrogen from the total nitrogen. The protein content of the precipitates was calculated by multiplying the corrected protein nitrogen by the factor

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6.25. By the addition of the figures for protein and for lipid, that part of the total mass of the precipitate derived from the immune serum was obtained. From these figures the relative content of lipid was calculated.

From these results it will be noted that lipid may constitute a very considerable portion of the precipitate mass. In certain other instances, not indicated in the tables, lipid has been found to form as much as 51.2 per cent or as little as 3.8 per cent of the total precipitate mass. Heidelberger and Kendall (11) have reported a slight discrepancy between the observed values for total nitrogen of specific Type III precipitates prepared from unconcentrated sera and the calculated values as determined by their formulae. It may be pointed out that lipid nitrogen, although constituting a very small proportion of the total nitrogen, does introduce a slight correction factor with respect to protein nitrogen. This correction would tend to reduce the above noted discrepancy between observed and calculated values for total nitrogen, if one assumes that the precipitates studied contained amounts of lipid similar to those here reported.

Comparative Lipid Content of Specific Precipitates Prepared from Varying Amounts of Immune Serum and Constant Amounts of Capsular Polysaccharide

In order to extend and confirm the foregoing observations upon the lipid content of specific precipitates, additional experiments were carried out in which a large number of precipitates were formed by the addition of constant amounts of polysaccharide to increasing amounts of immune serum. The results of these experiments are shown in Table II.

It will be noted that the lipid carbon content of the precipitates expressed in terms of unit serum volume progressively and rapidly increased as the amount of antiserum was decreased. As in the previous experiments, there was no direct relation between the lipid carbon and the total nitrogen content of these precipitates. The lipid nitrogen content of the precipitates, calculated on the same basis, increased in a manner similar to the increase in lipid carbon, and was not related to the total nitrogen content of the precipitates. The quantity of total nitrogen in the precipitates in terms of unit serum volume increased to a maximum as the proportion of polysaccharide became greater, in a manner exactly comparable to that of the previous experiments.

Polysaccharide nitrogen, corrected protein nitrogen, and the relative lipid content of each precipitate have been calculated in the manner described above.

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By plotting the total nitrogen and the lipid carbon values against the amounts of polysaccharide in milligrams per cubic centimeter of antiserum, curves are developed as shown in Text-figs. 3 and 4. These figures clearly demonstrate the absence of any direct relationship between the protein and lipid content of specific precipitates as prepared by this method. They also indicate that, in terms of unit serum volume, the lipid content of these precipitates is quite different from that of precipitates prepared by the previous method.

TABLE II

Total Nitrogen, Lipid Carbon, and Lipid Nitrogen of Specific Precipitates Formed from Varying Amounts of Immune Serum and Constant Amounts of Capsular Polysaccharide

Type I antipneumococcus serum		Type I capsular polysaccharide		Specific precipitate				
Species	Amount	Mg. per cc. of antiserum	N_2 (calculated)	Total N_2	Lipid N_2	Protein N_2 (calculated)	Lipid C	Lipid content precipitate
	cc.		*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*per cent*
Horse	3.0	0.040	0.002	0.348	0.008	0.338	0.238	12.8
"	2.5	0.048	0.002	0.371	0.010	0.359	0.266	13.4
"	2.0	0.060	0.003	0.466	0.012	0.451	0.311	12.5
"	1.5	0.080	0.004	0.558	0.017	0.537	0.404	13.5
"	1.0	0.120	0.006	0.607	0.025	0.578	0.580	17.3
"	0.5	0.240	0.012	0.619	0.009	0.606	1.035	26.2
Rabbit	3.0	0.080	0.004	0.338*	0.005	0.329	0.213	11.9
"	2.0	0.120	0.006	0.460*	0.008	0.446	0.294	12.0
"	1.0	0.240	0.012	0.660*	0.015	0.639	0.518	14.4

* Interpolated from Text-fig. 2.

Factors Determining the Lipid Content of Immune Precipitates

From the foregoing results it is apparent that the amount of lipid in the immune precipitate bears no relationship to the amount of precipitated protein. This fact suggested the possibility that much of the lipid may be present in the precipitate as the result of adsorption. In order to explore this possibility, certain calculations have been applied to the observed data to determine if the amount of lipid in the precipitate bore any relationship to the concentration of lipid in the reacting mixture.

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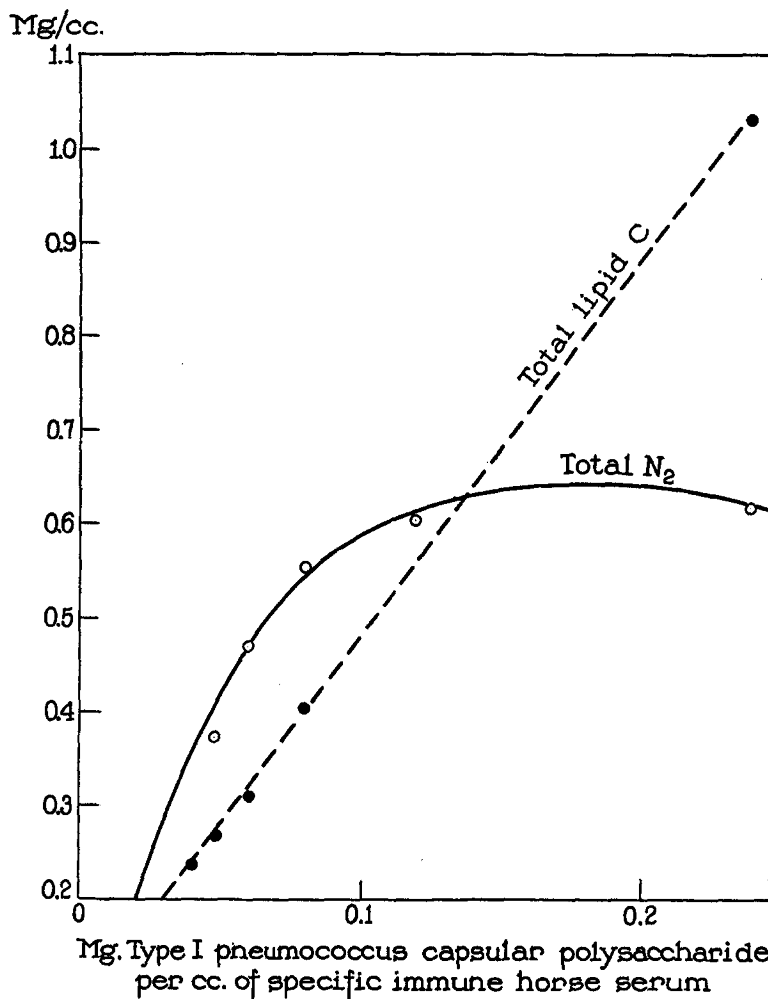
In Tables I and II, lipid carbon has been expressed in terms of unit serum volume. If, however, the total lipid carbon of each precipitate



TEXT FIG. 3. Total nitrogen and total lipid carbon of specific precipitates prepared from varying amounts of immune horse serum and constant amounts of capsular polysaccharide.

is expressed in its absolute value, irrespective of the amount of serum used, it becomes apparent that this value is definitely related to the amount of serum in those instances in which the total volume of the

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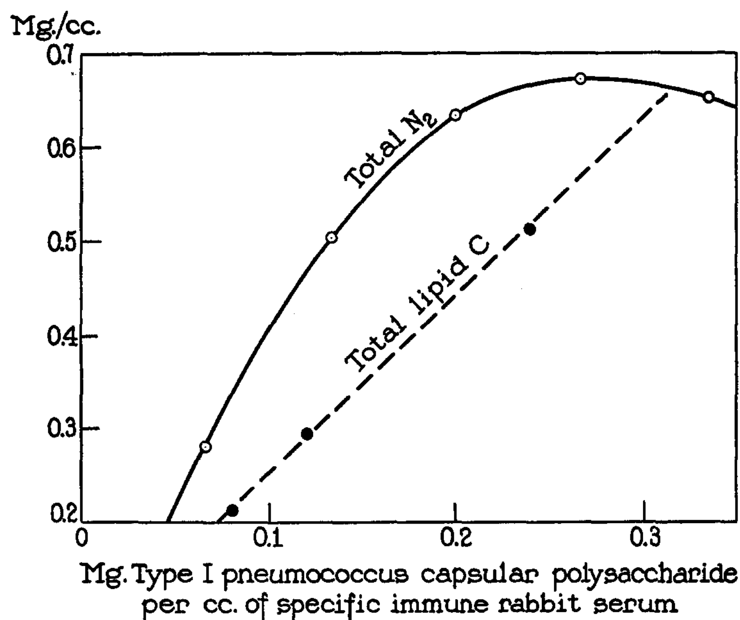
reacting mixture has been kept constant. These relationships are shown in Table III. It will be noted that with a constant amount of serum the lipid carbon of the precipitates is constant, but that with progressively increasing serum volume the lipid carbon of the precipitates also progressively increases. This is true in precipitates formed both from rabbit and from horse immune serum.



TEXT-FIG. 4. Total nitrogen and total lipid carbon of specific precipitates prepared from varying amounts of immune rabbit serum and constant amounts of capsular polysaccharide.

In the majority of instances listed in Table III, the total volume of the reacting mixture was 5.0 cc., irrespective of the amount of antiserum or of polysaccharide used. In these experiments, then, it can be taken that the concentration of lipid in the reacting mixture is directly related to the volume of antiserum. The total lipid carbon content of the horse antiserum was 1.942 mg. per cc., and of the rabbit antiserum 1.737 mg. per cc. From these values and those given in Tables I and II, it is possible to derive the values given in Table III for the concentration of lipid carbon in the reacting mixture and the absolute lipid carbon content of the precipitates.

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From a consideration of these data, it appears that the lipid carbon content of the specific precipitates is a function of the concentration of

TABLE III

The Relation of the Lipid Content of Specific Precipitates to the Concentration of Lipids in the Reacting Mixtures

Type I antipneumococcus serum		Capsular polysaccharide	Total volume of reacting mixture	Total lipid C in reacting mixture	Concentration of lipid C in reacting mixture	Specific precipitate		Calculated lipid C of precipitate (formula 1)	Deviation of found from calculated
Species	Amount					Total N_2 of precipitate	Total lipid C of precipitate		
	cc.	*mg.*	*cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg.*	*mg.*	*mg.*
Horse	1.5	0.015	5.0	2.914	0.583	0.235	0.608	0.606	0.002
"	1.5	0.060	5.0	2.914	0.583	0.525	0.588	0.606	0.018
"	1.5	0.120	5.0	2.914	0.583	0.777	0.616	0.606	0.010
"	1.5	0.180	5.0	2.914	0.583	0.892	0.585	0.606	0.021
"	1.5	0.240	5.0	2.914	0.583	0.942	0.628	0.606	0.022
"	1.5	0.300	5.0	2.914	0.583	0.967	0.597	0.606	0.009
"	3.0	0.120	5.0	5.826	1.165	1.044	0.715	0.702	0.013
"	2.5	0.120	5.0	4.856	0.971	0.927	0.664	0.676	0.012
"	2.0	0.120	5.0	3.884	0.777	0.932	0.623	0.639	0.016
"	1.5	0.120	5.0	2.914	0.583	0.837	0.607	0.606	0.001
"	1.0	0.120	5.0	1.942	0.388	0.607	0.580	0.562	0.018
"	0.5	0.120	5.0	0.971	0.194	0.309	0.518	0.490	0.028
"	1.5	0.093	6.0	2.914	0.486		0.593	0.588	0.005
"	1.5	0.093	6.0	2.914	0.486		0.590	0.588	0.002
"	1.5	0.093	6.0	2.914	0.486		0.589	0.588	0.001
"	1.5	0.093	6.0	2.914	0.486		0.593	0.588	0.005
"	1.5	0.090	10.0	2.914	0.291		0.543	0.544	0.001
"	3.0	0.046	12.0	5.826	0.485	0.756	0.584	0.588	0.004
Rabbit	1.5	0.10	5.0	2.606	0.521	0.421	0.578	0.566	0.012
"	1.5	0.20	5.0	2.606	0.521	0.750	0.588	0.566	0.022
"	1.5	0.30	5.0	2.606	0.521	0.954	0.570	0.566	0.004
"	1.5	0.40	5.0	2.606	0.521	1.009	0.583	0.566	0.017
"	3.0	0.24	5.0	5.211	1.041	1.014*	0.639	0.643	0.004
"	2.0	0.24	5.0	3.474	0.695	0.920*	0.589	0.597	0.008
"	1.0	0.24	5.0	1.737	0.347	0.660*	0.518	0.522	0.004
"	3.0	0.375	12.0	5.211	0.434	1.551	0.535	0.545	0.010

* Interpolated from Text-fig. 2.

lipid in the reacting mixture. To test the validity of this relationship, other precipitates were prepared in the equivalence zone, using various

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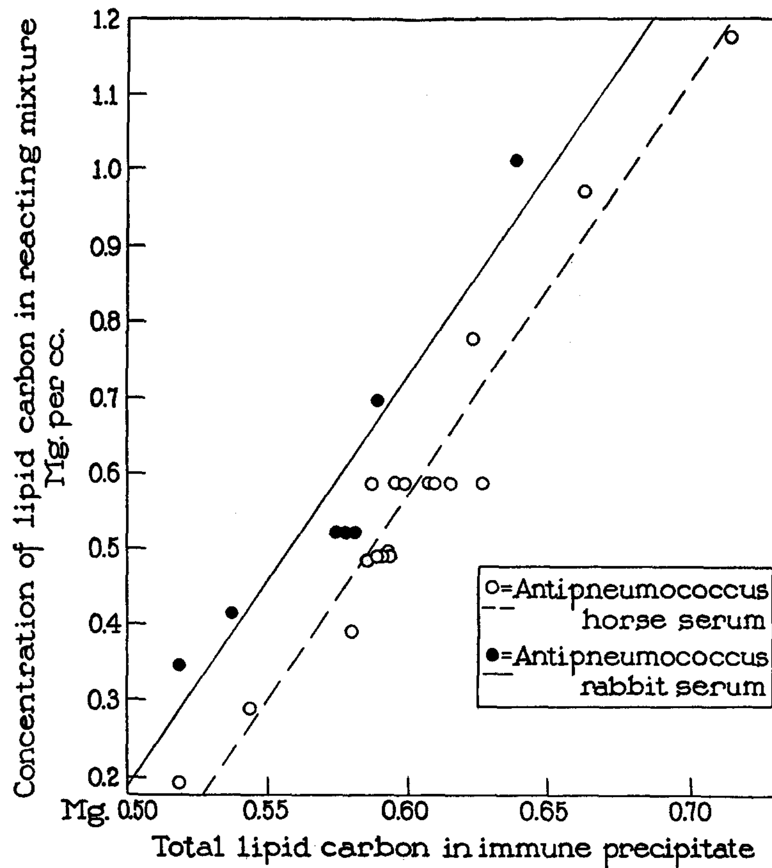
amounts of serum and total volumes of reacting mixtures which ranged from 5 to 12 cc. The values for lipid carbon found in these precipitates, as well as the total amount and concentration of lipid carbon in the reacting mixtures, are also given in Table III.



TEXT-FIG. 5. Relation of absolute lipid content of specific precipitates to initial lipid carbon concentration of reacting mixture.

The absolute lipid carbon content of the various precipitates from horse and rabbit antiserum, irrespective of the method of preparation or the total volume of the reacting mixture, have been plotted in Text-fig. 5 against the initial concentration of lipid carbon. Two straight

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lines have been empirically inserted in Text-fig. 5 to indicate possible linear relationships between the lipid carbon content of precipitates and the concentration of lipid carbon in the reacting mixtures. Within the relatively narrow range covered by these experiments, these two lines appear to agree with the experimentally determined points fairly well. However, no extension of the lines shown is justified, for should they be extended it will be observed that they would intercept the zero concentration axis at 0.46 and 0.49 mg. respectively, an obviously impossible circumstance.

Since the lipid carbon in a specific precipitate is independent of the amount of the precipitate within the limits studied, but is a function of the initial concentration of lipid carbon in the reacting mixture, it seems reasonable to assume that much the greater part of the lipid present in a specific precipitate may be carried down as a result of adsorption.

It has been found that these data agree well with the Freundlich adsorption equation $\frac{y}{a} = kx^n$, in which $\frac{y}{a}$ = amount adsorbed per unit of surface, and x^n = a function of the concentration. Quite obviously, no calculation of the surface area of the precipitating particles is permissible, particularly since this surface progressively and rapidly decreases from the moment of the initial reaction between antigen and antibody to the time when the almost solid precipitate has settled out. Because of this difficulty it has been necessary to disregard the actual surface area of the forming precipitate, and to make the not inconsiderable assumption that it had in each instance a unit value. It has been found that the equation, under these circumstances, has the form:

$$y = k\,x^{0.2} \tag{1}$$

For the horse antiserum studied, k has been found to be 0.680, while for the rabbit antiserum k = 0.645. It is possible, by using this equation, to calculate the expected lipid carbon content of a single precipitate when the concentration of lipid carbon in the reacting mixture is known. These calculations have been carried out, and the results are shown in Table III. The deviation of the calculated from the observed values for the lipid carbon content of the precipitates has also been determined. The mean deviation of these values for precipitates from either horse or rabbit antiserum is ± 0.010 mg. In the case of immune horse serum, this amounts to ± 1.6 per cent, and to ± 1.7 per cent in the case of rabbit antiserum.

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Lipid Content of Successive Precipitates from the Same Portion of Antiserum

In the preceding sections it has been demonstrated that the lipid content of any single specific precipitate from antipneumococcus horse or rabbit serum is a function of the concentration of lipid in the reacting mixture before precipitation occurs. In these instances only a single precipitate was produced with each portion of antiserum. It is possible, however, to produce a number of precipitates from any given amount of antiserum by the consecutive addition of small amounts of

TABLE IV

Lipid Content of Specific Precipitates Prepared Successively from the Same Portion of Antiserum

Experiment	Precipitate	Type I antipneumococcus serum		Capsular polysaccharide	Total volume of reacting mixture	Total lipid C in reacting mixture	Concentration of lipid C in reacting mixture	Specific precipitate		Calculated lipid C of precipitate (formula 1)	Deviation of found from calculated
		Species	Amount					Total N_2 of precipitate	Total lipid C of precipitate		
			cc.	*mg.*	*cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg.*	*mg.*	*mg.*
A	1	Horse	2.5	0.120	5.0	4.856	0.971	0.927	0.664	0.676	0.012
	2	"	1.5	0.015	4.66	2.517	0.540	0.134	0.431	0.601	0.170
	3	"	0.965	0.008	5.0	1.343	0.269	0.043	0.035	0.523	0.488
B	1	"	3.0	0.120	5.0	5.826	1.165	1.044	0.715	0.702	0.013
	2	"	2.0	0.020	5.0	3.063	0.612	0.196	0.475	0.616	0.141
	3	"	1.0	0.008	5.0	1.551	0.310	0.046	0.434	0.538	0.104

polysaccharide. It was important, therefore, to determine the lipid content of consecutively formed precipitates. The results of two experiments, in each of which three separate precipitates were formed from the same portion of antiserum, are presented in Table IV.

It will be noted that in each experiment the lipid carbon content of the second and third consecutively formed precipitates was considerably less than that of the first precipitate. This reduction in lipid in the precipitates was considerably greater than the diminution in the concentration of lipid in the reacting mixtures. The extent of this reduction becomes more apparent when the observed lipid carbon values for the second and third precipitates are compared with values calculated by formula 1. Although, as shown in Table III, there is a close cor-

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relation between the observed and calculated lipid carbon values for an initial precipitate, this relationship is considerably altered in the second and third consecutively formed precipitates. In the latter there was at least 0.10 mg. less lipid carbon than would be anticipated by application of the formula used for initial precipitates. This result was to be expected, since it is likely that those lipids most readily adsorbed would be carried down by the first precipitate, thus leaving somewhat less easily adsorbed lipids in the supernatant. Under these circumstances the second and the third precipitates would be formed in the presence of lipid which was less and less readily adsorbable, and consequently should contain amounts of lipid considerably smaller than those found in initial precipitates. These considerations are borne out by the experimental observations.

It must be pointed out that in these experiments the nature of the various lipids present in the reacting mixtures, as well as in the precipitates, has been disregarded. It is obvious that a number of different lipids (*i.e.*, cholesterol, cholesterol esters, phosphatides, neutral fats, and fatty acids) are present in the immune sera and the distinctive properties of these various substances suggest that some may participate in adsorptive processes to a greater extent than others.

DISCUSSION

The initial purpose of this study was to attempt the quantitative estimation of those lipids which previous work (1) had suggested might be intimately associated with the antibody globulin of Type I antipneumococcus sera. Inasmuch as complete isolation of the antibody has not yet been achieved, it was impossible to approach the problem by direct analysis. It was determined, therefore, to study the antipneumococcus antibody as it occurs in specific precipitates resulting from the interaction of antisera and the homologous pneumococcus capsular polysaccharide.

The specific precipitates were carefully prepared and washed by the method of Heidelberger and Kendall (3) and it was found that they contained a relatively large amount of lipid. In some, as much as 51 per cent, in others as little as 4 per cent by weight of the total precipitate was lipid. These quantities were very much greater than the amounts which had been anticipated, particularly when it is recalled that only very small amounts of phosphatide were required to restore certain *in vitro* properties to antisera extracted by lipid solvents (1).

Not only was the total lipid content of the precipitates greater than might be expected, but there was no stoichiometric relation between the lipid and protein contents of the precipitates. Were all the lipid

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of specific precipitates present only by virtue of an assumed intimate association with antibody globulin, a constant ratio should exist between the amounts of protein and lipid occurring in the precipitates. Under these circumstances, too, quantitative curves for the protein and lipid content of variously prepared precipitates should have the same form. That they do not is manifest in each of Text-figs. 1 to 4. Within the ranges studied, the total lipid content of specific Type I precipitates was entirely independent of the protein.

The total lipid content of specific precipitates was found to be a function of the initial concentration of lipid in the reacting mixture prior to the formation of the precipitate. Since certain adsorption phenomena manifest a linear relationship between the quantity of a substance adsorbed and some exponential function of its concentration in the system, the analytical results were compared with the various calculated results. The Freundlich adsorption equation: $y = kx^n$ was applied, and it was found that if the exponent n were taken to be 0.2, the observed values for y (the lipid carbon of the precipitates) had a mean deviation from the calculated values of but 0.01 mg. Although this correlation between calculated and observed values for the lipid carbon content of specific precipitates may be considered as significant, it is thought that the experimental findings scarcely warrant a definite statement that the Freundlich equation describes the relationship over the entire course of the reaction.

The fact that considerable amounts of lipid have been regularly found in Type I specific precipitates and that the quantities appear to bear a linear relationship to an exponential function of the concentration of lipid initially in the systems, neither lends weight to, nor does it invalidate the conception that lipid may be intimately associated with the antibody globulin. If, for example, one assumes that the ratio of protein to lipid in the antibody is roughly 100:1, a not unreasonable assumption in the light of the comparative molecular weights of globulin and phosphatide, and also because of the small quantity of phosphatide necessary to restore *in vitro* properties to extracted antiserum, it is possible to approximate the amount of supposed antibody-lipid in each precipitate. When this is done it is found that even in the case of the heaviest precipitate studied (*i.e.*, 0.656 mg. protein nitrogen per cc.) the associated antibody-lipid

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carbon would have a value of but 0.031 mg. per cc. In the same precipitate the total lipid carbon content was found to be 0.388 mg. per cc. In the case of smaller precipitates this associated antibody-lipid carbon would be proportionately less. Under these circumstances, and if the necessary assumptions are in any way an indication of what actually holds, it is easily conceivable that these small though significant quantities of lipid would be masked by the relatively large amounts of additional lipid found, and therefore do not seriously affect the apparent correlation between observed lipid carbon content and that calculated on the basis of adsorption.

SUMMARY

1. Specific precipitates resulting from the interaction of the homologous capsular polysaccharide and Type I antipneumococcus horse and rabbit sera have been analyzed by gasometric micro methods for total nitrogen, lipid nitrogen, and lipid carbon.

2. Lipid may, under certain conditions, form as much as 51 per cent or as little as 4 per cent by weight of specific precipitates.

3. The total lipid content of specific precipitates, within the range studied, is entirely independent of the protein content.

4. Lipid nitrogen forms a very small but detectable portion of the total nitrogen of precipitates.

5. The absolute lipid content of precipitates is a function of the concentration of lipid in the reacting mixture prior to precipitation, and seems to be governed by the laws of adsorption phenomena.

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