EFFECT OF THE REMOVAL OF LIPIDS ON SPECIFIC PRECIPITATION*

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(Received for publication, July 1, 1950)

The widespread use of alcohol in the fractionation of serum or plasma, with the resulting partial or complete removal of lipids from many of the fractions, has made it advisable to reinvestigate, this time by quantitative methods, the effect of extraction of lipids on the characteristics of the antibodies in immune sera.

There has been considerable study of immune reactions in sera deprived of lipids. Hartley (1) extracted antisera with 10 volumes of alcohol-ether at −10°C. according to the method of Hardy and Gardiner (2). The precipitin reaction of a rabbit serum was thereby abolished and a diphtheria toxin–horse antitoxin system failed to flocculate and showed only faint opalescence in 24 hours. Neutralization tests in animals demonstrated that the toxin and antitoxin had actually combined. Similar results were obtained by Linderstrøm-Lang and Schmidt (3). Horsfall and Goodner reexamined the role of lipids in immunological reactions (4). Their extraction procedures involved precipitation by alcohol at −10°C. followed by washing with alcohol and ether or petroleum ether. The serum analyzed showed 92 per cent loss of lipids. Extracted antipneumococcus Type I horse serum did not agglutinate Type I pneumococci nor precipitate Type I polysaccharide although there was slight opalescence after 2 hours at 37° and 18 hours at 0°C. The extracted sera protected mice against Type I pneumococci, so that combination of antigen and antibody was again demonstrated. Tayeau, Faure, Neuzil, and Pautrizel (5) reported that with sheep ψ-globulin and extracted rabbit antiserum there was complete inhibition of precipitation, while in antisera to egg albumin and horse serum albumin the flocculation time was merely lengthened. Delsal, Perez, and Laporte (6) observed a similar modification of the flocculation rate after extraction of antiserum with ether-alcohol. Removal of cholesterol by ether alone did not affect the precipitin reaction. The report of Oikawa (7) was received after the present studies were finished. Only he, outside of this laboratory, appears to have centrifuged and analyzed reaction mixtures with sera deprived of lipids. Thus he arrived at conclusions partly at variance with those of previous workers and more

* These studies were carried out in part under a grant from The Rockefeller Foundation, and in part under the Harkness Research Fund of the Presbyterian Hospital, New York. Although the scope was of necessity broadened, the work began as a part of the study of Antiproteins in horse sera, and the paper may, therefore, be considered as Number V in this series.

like our own. Oikawa found that while antistaphylococcus and antipneumococcus sera extracted according to the method described in reference 4 did not agglutinate the cocci in 2 hours at 37°C., quantitative agglutinin analyses (8) showed that almost as much antibody nitrogen combined with the cells as in the unextracted sera. This contrasts with Horsfall and Goodner's statement that Type I pneumococci removed "only a fraction of the antibody" from an extracted antipneumococcus Type I horse serum based on mouse protection tests on the absorbed serum. The tests, however, show the fraction to have been a very large one.

In the present work use has been made of quantitative methods developed in these laboratories (9). Contrary to general belief based on visual observations of reaction mixtures allowed to stand only a relatively short period, it could readily be shown that removal of lipids does not eliminate precipitin or flocculation reactions and indeed often results in the deposition of even more precipitate than in the presence of lipids.

EXPERIMENTAL

1. Sera.—Horse serum 999, obtained after subcutaneous injection of rabbit serum albumin; bleeding July 2, 1941. Merthiolate as preservative. See reference 10 for schedule of injections and bleedings.

Horse serum 1046, obtained after intravenous injection of rabbit serum globulins; bleeding July 10, 1940. Merthiolate as preservative. See reference 11 for schedule of injections and bleedings.

Antipneumococcus Type III horse serum 792; bleeding February 1, 1937. Contained ether, phenol, and merthiolate.

2. Antigens.—Serum albumin was separated from normal rabbit serum in the electrophoresis laboratory of the College of Physicians and Surgeons, Columbia University, under the direction of Dr. Dan H. Moore. γ-Globulin was similarly separated from normal and anti-egg albumin rabbit sera. Concurrent studies (12) have shown that these normal and immune γ-globulins were identical as antigens. The electrophoretic homogeneity was checked by a separate analytical run. Extracted albumin and γ-globulin were obtained in the same manner from a normal rabbit serum extracted with 10 volumes of alcohol-ether (2:1) as described below.

The specific polysaccharide of Type III pneumococcus, S III, was prepared according to the procedure given in reference 13. "Extracted" S III was precipitated from saline by 10 volumes of alcohol-ether (2:1) and was washed with alcohol-ether and with ether.

3. Solvents.—Anhydrous ether and absolute ethanol were redistilled before use.

4. Extraction Procedures.—Usually the serum was dropped into 3 to 10 volumes of a mixture of 1 or 2 parts of alcohol and 1 of ether. Most of the operations were carried out at dry ice–acetone or dry-ice–box temperatures. The precipitate was washed several times with the solvent used for precipitation and finally with ether. Centrifugation was in a refrigerated PR-1 International centrifuge at -10 to -14°C. In some experiments the alcohol-ether extraction was preceded by exhaustion with ether alone by freezing and thawing according to the method of McFarlane (14).

A typical run was as follows: 104 ml. of horse serum 1046 was shaken with an equal volume of ether at 0°C. in heavy 250 ml. centrifuge bottles. The resulting emulsion was quickly frozen in a dry ice–acetone bath. After thawing at 0–4°C. for 16 hours, the ether layer was removed with a syringe and the serum shaken and frozen with more ether. This was repeated until the

extracts gave a negligible deposit upon evaporation. After the last ether layer was removed most of the remainder was evaporated on the water pump. 25 ml. of the serum was removed and lyophilized to take out the last traces of ether. The remaining serum was added dropwise to about 3 volumes of a 1:1 mixture of alcohol and ether cooled in a bath of dry ice and acetone.

The mixture was shaken vigorously, set in the dry-ice–box for 48 hours with occasional brief shaking, and centrifuged at −10 to −14°C. The clear supernatant was poured off and the precipitate mixed and shaken with 3 more volumes of solvent. Soaking for 6 to 16 hours, shaking, and centrifugation were carried out as in the first extraction and were repeated 5 times. The 6th extract deposited 13 mg. on evaporation, a negligible amount compared with the total, 556 mg.

The term "reextraction" in the text and figure legends is used to indicate a repetition of the entire process.

The precipitated proteins were shaken with several portions of ether at dry-ice–box temperatures and finally dried with a water pump followed by an oil pump. The powder was taken

TABLE I

Removal of Lipids by Successive Extractions with Ether and Alcohol-Ether at Low Temperatures

Serum	Extraction with ether				Extraction with alcohol-ether				Totals extracted			
	Total lipids	Cholesterol	Lipid P	Lipid N	Total lipids	Cholesterol	Lipid P	Lipid N	Total lipids	Cholesterol	Lipid P	Lipid N
999*	66	49	0	4	31	51			97	100		
1046‡	70	92	1	1	25	8	99	96	95	100	100	97
792	38*				59*				97*	100§	100§	

* Values expressed in per cent of totals obtained by extraction with hot alcohol-ether.

‡ Percentages based on totals obtained by successive extraction with cold alcohol-ether.

§ From analysis of extracted serum for residual amounts.

up in a volume of chilled saline equal approximately to that of the original serum. When the total N in the extracted serum, as determined by the micro Kjeldahl method, was higher than in the unextracted serum, saline was added to equalize the concentrations, account being taken of the amount of N extracted during the experiment, about 2 per cent of the total N in the serum. In other cases, the precipitin curves were corrected so that all were on the same basis of total N. The pH was adjusted to 6.8–7.8 with a few drops of 0.1 N NaOH.

The extracted sera were clear, but on centrifugation gave small amounts of precipitates. The extent of denaturation indicated by the N in these precipitates after washing, was of the order of 0.1 per cent. For sera put through the long extraction process a second time, the amount of N deposited was about 0.5 per cent.

5. Analysis of the Lipids.—Total lipids were determined by extraction with 10 to 20 volumes of hot alcohol-ether (2:1). The extracts were evaporated to dryness either at 10°C. or *in vacuo* below 60°C. The residues were extracted with either petroleum-ether or chloroform and these extracts evaporated to dryness and brought to constant weight in a desiccator. These "total lipids" were taken up in ether and aliquot portions used for estimation of lipid N, lipid P, and cholesterol.

Similar analyses were run on the low temperature extracts as well. N was determined by the Markham modification (15) of the micro Kjeldahl method.

After digestion with H_2SO_4 and a few drops of 30 per cent H_2O_2, P was estimated by a modification of the method of Fiske and Subbarow (16). Liebermann's color reaction (17)

was modified for the estimation of cholesterol as follows: Aliquot portions of the ether solution of the lipids were evaporated to dryness and taken up in chloroform. Acetic anhydride containing 10 per cent H_2SO_4 by volume was added, and the green color read at maximum intensity in a Coleman spectrophotometer at 650 mμ. The readings were compared with a cholesterol standard run at the same time.

The results of the lipid analyses are summarized in Table I. Ether extraction followed by one with alcohol-ether removed 95 to 97 per cent of the total lipids. A second alcohol-ether extraction undoubtedly removes the remainder of the lipids although no effort was made to ascertain this by analysis.

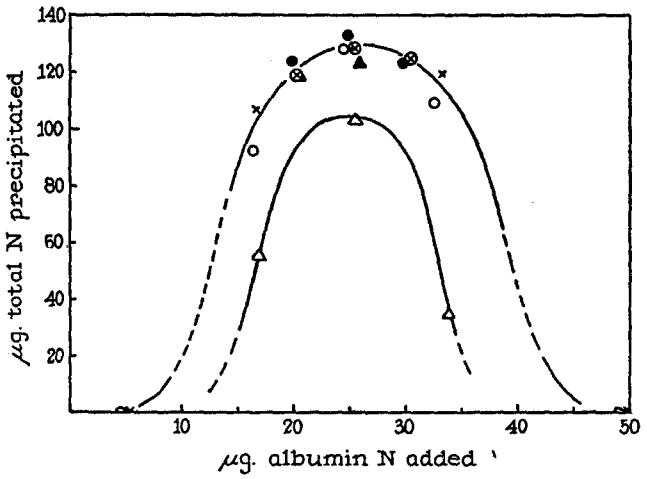

FIG. 1. Flocculation of antialbumin horse serum 999 by means of albumin procured from extracted rabbit serum by electrophoresis.

×—× Unextracted serum; flocculation in 2 days. The same curve was obtained with ether-extracted serum and unextracted albumin.

○—○ Serum extracted with ether and then with 4 volumes of alcohol-ether (1:1) in the initial precipitation. The antigen-antibody precipitate did not settle in 2 days.

△—△ Reextraction of above serum with 10 volumes. of alcohol-ether. Supernatants from tubes at 16.9 and 33.9 μg. albumin N were slightly cloudy.

Above data obtained after centrifugation at 2300 R.P.M. At 3000 R.P.M. results were as follows: ⊗—⊗ control; ●—● extracted; ▲—▲ twice extracted.

6. Precipitin Reactions of Control and Extracted Sera.—In all the experiments, regardless of lipid content of the sera, the mixtures became cloudy immediately upon the addition of antigen. While the control and ether-extracted sera flocculated and settled overnight, the alcohol-ether–extracted antiprotein sera formed fine precipitates which began to settle only after 2 to 4 days. The reextracted sera formed such aggregates in 5 to 10 days, and in such cases centrifugation at 3000 R.P.M. in the International No. 2 centrifuge instead of the customary 2000 to 2300 R.P.M. was necessary to yield clear supernatants.

Experiments with the antialbumin horse serum 999 are plotted in Fig. 1. Centrifugation at 2300 R.P.M. sufficed for complete separation of the precipitate except in the case of the twice extracted serum, for which 3000 R.P.M. were necessary. In instances in which the same extracted or unextracted serum was tested with both unextracted and extracted albumin no differences could be detected beyond the limits of accuracy of the method.

The data obtained with horse serum 1046 are shown in Fig. 2. Except in the case of the reextracted serum, centrifugation at 2200 R.P.M. yielded clear supernatants.

Results with the antipneumococcus Type III horse serum 792 are shown in Fig. 3. The finely divided precipitates in the mixtures containing the alcohol-ether-extracted serum began to settle only after 9 to 14 days and it was necessary to centrifuge at 3000 R.P.M. in

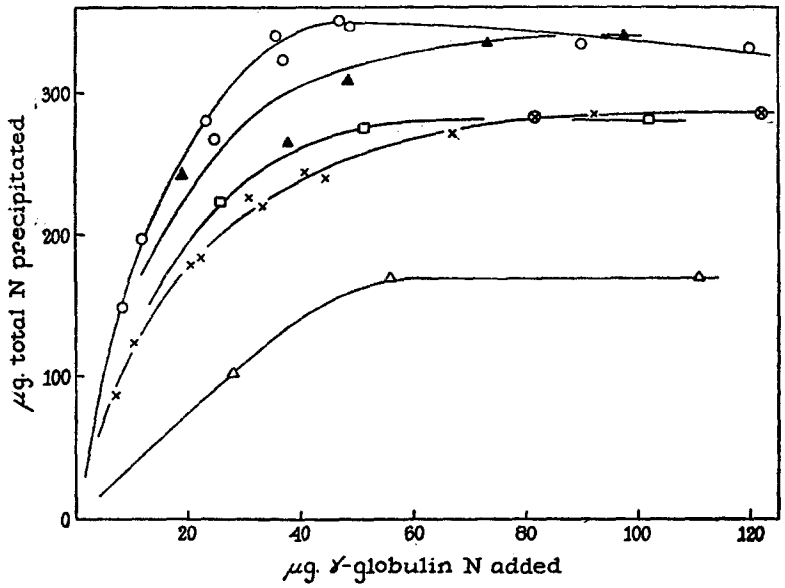

FIG. 2. Precipitin reaction of horse serum 1046 and electrophoretic rabbit γ-globulin.

×—× Control, unextracted; flocculation and settling overnight; centrifuged at 2200 R.P.M.; ⊗—⊗ centrifuged at 2600 R.P.M.

□—□ Ether extraction only; flocculation and settling overnight.

○—○ Ether extraction followed by 3 volumes of alcohol-ether (1:1); aggregation in 2 days, settling in 3 to 6 days. Points represent two independent preparations. Serum extracted directly with alcohol-ether gave a curve which almost coincided.

△—△ Reextracted with 5 volumes alcohol-ether; fine precipitate in 6 days, no settling. Tubes centrifuged after 4 weeks at 2800 R.P.M.; supernatants slightly cloudy.

▲—▲ Serum 1046 extracted with 5 volumes alcohol-ether, reconstituted and reextracted with 10 volumes alcohol-ether. Set up with extracted γ-globulin. Fine aggregates, no settling in 10 days; centrifuged at 3000 R.P.M. after 10 to 20 days.

order to obtain clear supernatants. Alcohol-ether extraction of Type III polysaccharide did not influence the results.

DISCUSSION

Since Hartley (1) studied the effect of removal of lipids from antisera on the precipitin, toxin-antitoxin, and agglutination reactions, and demonstrated that antigens and antibodies combined in the absence of lipids but did not necessarily show effects visible to the naked eye, rigorous quantitative methods for the

analysis of antigens, antibodies, and the products of their interaction have been introduced, with the result that the mechanisms of these reactions have been elucidated (9, 18). Except in the very recent work of Oikawa (7) which came to the attention of the writers after completion of the present studies, quanti-

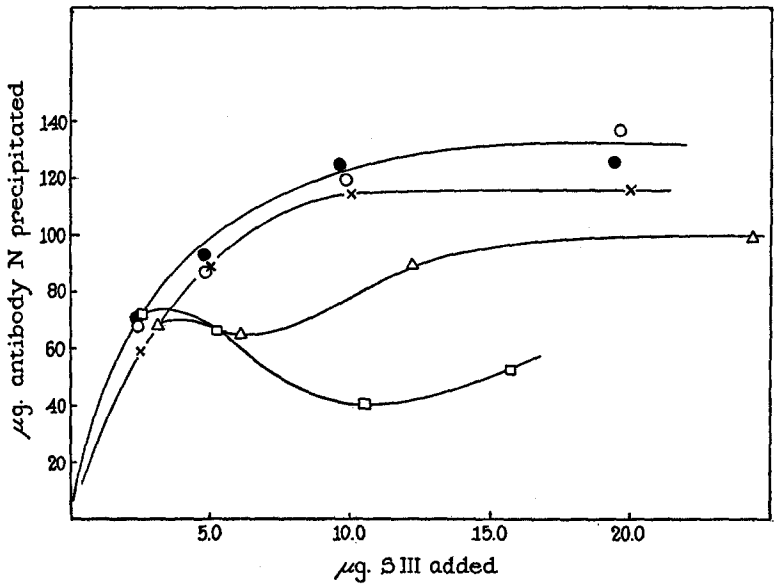

FIG. 3. Precipitin reaction of horse serum 792 and Type III pneumococcus polysaccharide.

×—× Control, unextracted; flocculation and settling overnight. Tubes centrifuged at 2100 or 3000 R.P.M. gave identical values.

○—○ Serum extracted with 2.5 volumes alcohol-ether at dry ice box temperatures; fine precipitation in 9 days. Centrifugation in 16 days at 3000 R.P.M.; supernatants slightly opalescent.

□—□ Same as ○—○ except initial centrifugation at 2100 R.P.M., and those for washing the precipitates at 3000 R.P.M.

●—● Serum extracted according to the method described in reference 4. Centrifugation in 16 days at 3000 R.P.M. for 3 hours; supernatants clear.

△—△ Reextracted serum; initial precipitation for the second extraction with 10 volumes alcohol-ether. Centrifuged at 3000 R.P.M.; supernatants clear.

tative analytical estimations of the quantities of antigen-antibody complexes precipitated have not been used to supplement the unaided human eye in the study of immune reactions with sera deprived of lipids. This gap has now been filled, and the result has been the upset of current beliefs, based as they are on methods of observation that were the best available at the time (1, 3) but are no longer adequate.

Removal of lipids from antisera does not prevent precipitation of the antigen-antibody complex, a fact abundantly illustrated in the figures. As noted

by the earlier workers, however, there is a delay in the appearance of the precipitate, which is finely divided and remains partially suspended even after prolonged standing in the refrigerator. It is also frequently necessary to centrifuge mixtures deprived of lipids at higher speeds in order to obtain clear supernatants.

As a check on the efficiency of the methods of extraction used, it will be seen from Table I that in all instances 95 to 97 per cent of the lipids were removed. This compares well with the 92 per cent reported in reference 4 and with the extent of extraction indicated in reference 1.

It is clear from Fig. 1 that the water-soluble antibody in the antitoxic type serum, 999, showed no changes in the proportions in which it combined with antigen after extraction of the lipids. Most of the antibodies in sera 792 and

TABLE II

Comparison of Initial Combining Ratios of Antibody/Antigen in Control and Extracted Sera

Serum	Treatment	Antigen	Initial combining ratio
1046	None	γ-Globulin	16*
1046	Alcohol-ether	γ-Globulin	22*
792	None	S III	36‡
792	Ether	S III	36‡
792	Alcohol-ether	S III	47‡

Ratios were plotted against $\sqrt{\gamma\text{-globulin N}}$ or $\sqrt{\text{S III}}$.

A plot of ratios *versus* S III was also linear for the untreated serum.

* μg. antibody N precipitated/μg. γ-globulin N precipitated, extrapolated to 0 antigen N.

‡ μg. antibody N precipitated/μg. S III precipitated extrapolated to 0 S III.

1046 which give reactions of the precipitin type, are in the water-insoluble fractions of the globulins. The behavior of these antibodies after removal of the lipids is different in that there is an increase in the amount of total nitrogen precipitated and an increase in the initial combining ratios of antibody/antigen. This is illustrated in Figs. 2 and 3, and in Table II.

In an attempt to account for the higher total nitrogen levels obtained, especially with the alcohol-ether–extracted serum 1046, the possibility was considered that the lipids in the unextracted serum might inhibit the precipitation of part of the antibody. 100 ml. of the serum was accordingly treated with 1 ml. of γ-globulin (1 mg. N/ml.) at 0°C. This amount was calculated to precipitate all the antibody, and supernatant tests after 48 hours showed the absence of γ-globulin and antibody. The clear supernatant was extracted with alcohol-ether in the usual way. However, the extracted supernatant did not precipitate when mixed with appropriate amounts of γ-globulin nor did it add nitrogen to a specific precipitate formed in its presence from known amounts of γ-globulin and extracted 1046 serum.

In the experiments presented in another paper (19), in which 84 per cent of the lipids were removed in the course of fractionation with alcohol, there was a greater change in the combining properties of the antibody than was produced when all the lipids were removed. It appears that the changes are due more to the aggregating effects of the alcohol-ether treatment upon the water-insoluble globulins (19–21) than to the absence of lipids.

From the data presented it is evident that lipids are not necessary for complete precipitation of the antibodies in sera by antigens if time is allowed for the reaction to go to completion and if the centrifugal speeds used are roughly 50 per cent higher than those ordinarily employed.

The role of lipids appears to be the mechanical or physical one of providing nuclei for the formation of particles as proposed in reference 9*a* in analogy with the accelerating action of dust particles on crystallization. Possibly, also, lipids assist in the deposition of the smaller aggregates formed in their absence by acting as a kind of cementing material which may bind the smaller particles into larger and heavier aggregates.

SUMMARY

Horse antisera to rabbit albumin and globulins and to Type III pneumococci were extracted at low temperatures with alcohol-ether.

The precipitin reactions given by these sera deprived of lipids were studied by quantitative analytical methods.

With longer periods of standing and slightly higher speeds of centrifugation than those usually required for lipid-containing sera, precipitation may be made complete in immune sera from which the lipids have been removed.

The function of lipids in immune reactions is discussed, as well as the effects on antibody globulin of the procedures used in their removal.

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