IMMUNIZATION EXPERIMENTS WITH LECITHIN.

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(Received for publication, March 30, 1927.)

In the case of Forssman's heterogenetic antigen it was found possible to incite the production of antibodies by the injection of its specific component along with proteins. In a similar manner immune bodies were obtained with alcoholic extracts of blood corpuscles (1) and of organs (2). Since it is assumed generally though not proven that the specific part of the heterogenetic antigen is of lipoid nature it was natural to test the immunizing properties of lipoids of known chemical constitution. This was undertaken by Sachs and Klopstock (3) with lecithin and cholesterol. Indeed these authors state that they obtained antibodies by injecting into rabbits emulsions of lecithin, or cholesterol, containing pig serum. The tests were carried out mainly by means of complement fixation. A confirmatory paper was published by Ornstein (4) who also reported on successful immunizations with cephalin and with cerebrosides.

There are some points in the observations of Sachs and Klopstock not easily understood on the basis of their assumptions. Their antilecithin serum reacted on lecithin Merck but it also reacted on cholesterol and even more intensely on the latter than on a certain lecithin preparation of higher purity than lecithin Merck. This phenomenon is ascribed by Sachs and Klopstock to the presence of some cholesterol in the injected antigen but against such a view may be pointed out that in the experiments of these authors it was rather difficult to produce sera which react upon cholesterol, by injections of this substance. Another difficulty arises from the fact that the purer of the two lecithin preparations employed was the less active. The explanations for these peculiarities offered by Sachs and Klopstock do not settle conclusively the questions at issue. Since the production of antibodies for well known lipoids would be of great

significance, it seemed desirable to us to repeat the experiments with various lecithins prepared by ourselves.

EXPERIMENTAL.

We injected three lots of five rabbits each, with ox brain lecithin, egg lecithin and hydro egg lecithin, respectively. In addition sera were prepared with a commercial egg lecithin preparation (Merck) as used by Sachs and Klopstock.

Lecithin Preparations.—Lecithin No. 1. Alcoholic extract of egg yolk was treated with a 25 per cent solution of cadmium chloride in methyl alcohol, the precipitate extracted twice with ether and decomposed with methyl alcohol containing 25 per cent of ammonia. The solution was concentrated and the residue was extracted with cold alcohol. The lecithin was again precipitated as a cadmium salt and the latter was repeatedly washed with ether. The cadmium salt was then treated with methyl alcohol containing ammonia gas; the solution was concentrated, taken up in a minimum quantity of ether and precipitated with acetone.

Analysis: C 64.6; H 10.49; N 2.17; P 3.96; amino N 0.

Lecithin No. 2. 15.0 gm. of lecithin No. 1 were dissolved in methyl alcohol, acetone was added until a sample on cooling to −5°C. showed the formation of a precipitate. The entire solution was then brought to a temperature of −5°C. and the precipitate formed was removed by centrifugalization. The mother liquor was concentrated nearly to dryness, the residue was taken up in ether and the solution was poured into an excess of acetone. The yield was 11.0 gm. This material was dissolved in 20.0 cc. of methyl alcohol, 20.0 cc. of water were added and the solution was adjusted to pH 4. Acetone was added so long as a precipitate formed. The yield was 9.0 gm.

Analysis: C 65.3; H 10.61; N 2.00; P 4.12.

Egg Lecithin No. 3. The cadmium salt, prepared as No. 1, was extracted with ether 8 times. The lecithin obtained in this manner was further purified as follows: 26.0 gm. of lecithin were dissolved in 40.0 cc. of ether, 40.0 cc. of 10 per cent acetic acid were added, the mixture was shaken for 1 hour and the lecithin precipitated with 500 cc. of acetone.

Analysis: C 66.00; H 10.59; N 2.03; P 3.90; amino N 0.

Brain Lecithin. The cadmium salt was decomposed as usual, the filtrate was concentrated, and taken up in ether; acetone was added until a small precipitate formed. This was removed by filtration. The filtrate was concentrated nearly to dryness, the residue was taken up in ether, acetone was added to the solution to incipient opalescence. The solution was chilled to approximately −8°C. A precipitate formed which was removed by centrifugalization. The mother liquor was concentrated almost to dryness and taken up in a little ether. Acetone was added to opalescence and the solution brought to about −20°C. A precipitate formed, which was separated by centrifugalization. It was then extracted with

acetone and dried under diminished pressure. It was preserved in an atmosphere of nitrogen gas.

Hydrolecithin. This was prepared from egg lecithin by reduction with hydrogen and colloidal palladium as a catalyst.

Analysis of Merck lecithin: C 65.41; H 10.55; N 1.81; P 3.41; NH_2-N 0.

Tests were made also with a number of other samples of lecithin prepared in the laboratory with various modifications of the above described methods.

Immunization.—Rabbits were selected the sera of which gave no reactions in flocculation and complement fixation tests with emulsions of cholesterolized alcoholic beef heart extract[1] and of Merck's egg lecithin. 240 mg. of lecithin were emulsified with 15 cc. of saline and 3 cc. of pig serum diluted to 15 cc. with saline were added. This emulsion was kept at room temperature for 1 hour before injection.

The rabbits received intravenous injections of 5 cc. of the lecithin emulsion generally at intervals of 3 to 5, sometimes 7 days. The sera were tested several times during the course of the experiments, with both the lecithin used for injection and Merck's egg lecithin.

Tests. The complement fixation tests with the egg, brain and hydrolecithins were carried out as follows: To 0.25 cc. of progressively doubled dilutions of the inactivated serum starting with a dilution 1:5 were added 0.25 cc. of an emulsion of lecithin (prepared by fairly rapid addition of 24 cc. of saline to 1 cc. of a ½ per cent solution of lecithin in alcohol) and 0.25 cc. of guinea pig serum diluted 1:10. After incubation at 37°C. for 1 hour 0.25 cc. of sheep blood immune serum (2½–3 units) and 1 drop of a 50 per cent sheep blood suspension were added.

For the flocculation tests 1 part of a ½ per cent alcoholic solution of the egg lecithin was emulsified by fairly rapid addition of 5 parts of saline. 0.2 cc. of the emulsion was added to 0.2 cc. of the inactivated serum diluted 1:2 and the readings were taken after 20 hours standing at room temperature. For the flocculation tests with the brain lecithin and hydrolecithin a different procedure was adopted since emulsions prepared by the method described for egg lecithin were very unstable and were flocculated by most normal rabbit sera. 1 part of a ½ per cent alcoholic solution of brain lecithin was added rapidly to 5 parts of distilled water and 0.2 cc. of this liquid was mixed with 0.2 cc. of the inactivated serum diluted twice with a 2.7 per cent salt solution. In the case of the hydrolecithin 1 part of a ½ per cent solution of the substance was added by drops to 5 parts of boiling distilled water. The emulsion was filtered hot. For the test the serum was diluted with distilled water instead of saline.

The strength of the reactions in the tests is indicated as follows: Complement fixation tests—0 = no hemolysis, tr = trace, w = weak, d = distinct, str = strong, vstr = very strong, ac = almost complete, c = complete hemolysis. Flocculation tests—0 = no flocculation, ftr = faint trace, tr = trace; ±, +, ++, +++, etc.

[1] For the technique see: *J. Exp. Med.*, 1927, xlv, 467.

By immunizing with egg lecithin Merck we obtained four strongly and two weakly active sera among six rabbits after six injections.

The sera obtained with brain lecithin and hydrolecithin gave no distinct flocculation or complement fixation with these preparations

TABLE I.

Number of sera	Injections made with:	Flocculation with emulsions of:			
		Merck egg lecithin	Merck egg lecithin freed from cholesterol	Lecithin No. 1	Lecithin No. 1 with addition of 12 per cent cholestorol
966	Egg lecithin No. 1	0	0	0	tr
967	" " " 1	+±	0	0	+
968	" " " 1	0	0	0	tr
969	" " " 1	0	0	0	0
970	" " " 1	0	0	0	0
810	Merck's egg lecithin	+++	++±	0	±
Normal rabbit No. 1		0	0	0	tr
Normal rabbit No. 2		0	0	0	±

TABLE II.

Numbers of sera	Injections made with:	Complement fixation with an emulsion of egg lecithin No. 1	Complement fixation with an emulsion of Merck egg lecithin
966	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c
967	" " " 1	c,c,c,c,c	0,tr,c,c,c
968	" " " 1	c,c,c,c,c	c,c,c,c,c
969	" " " 1	c,c,c,c,c	c,c,c,c,c
970	" " " 1	c,c,c,c,c	c,c,c,c,c
809	Merck's egg lecithin	c,c,c,c,c	0,0,0,0,ac,c
810	" " "	vstr,ac,c,c,c	0,0,0,0,0,ac,c
Normal rabbit		c,c,c,c,c	c,c,c,c,c

or with Merck's egg lecithin after twelve injections. Occasionally weak flocculations were noticed but after further injections the reactions disappeared.

The tests with the sera taken after twelve injections of egg lecithin No. 1 are presented in Tables I and II. Complement fixation tests

TABLE III.

Emulsions used for the tests:

A. 24 cc. of saline were added fairly rapidly to 1 cc. of a ½ per cent alcoholic solution of egg lecithin No. 1. This preparation gave no Liebermann reaction for cholesterol.

B. This emulsion was made as A, with 1 cc. of a ½ per cent alcoholic solution of egg lecithin No. 1 to which had been added 0.06 cc. of a 0.25 per cent alcoholic solution of cholesterol, corresponding to 3 per cent of the weight of lecithin.

C. Emulsion made as in A, with 1 cc. of egg lecithin No. 1 to which had been added 0.06 cc. of a 1 per cent alcoholic solution of cholesterol, corresponding to 12 per cent of the weight of lecithin.

D. As A, with Merck's egg lecithin. This preparation was found by the Liebermann test to contain 1.5 per cent of cholesterol or less.

E. As A, with Merck's egg lecithin from which the cholesterol was removed by dissolving 2 gm. in 15 cc. of ether and reprecipitation with 30 cc. of acetone. This purification was repeated twice. The product gave no Liebermann reaction.

F. 1 cc. of a ½ per cent alcoholic solution of cholesterol was slowly added to 24 cc. of boiling distilled water and the emulsion was filtered hot (method of Keeser (5)). The dilution of the sera was made with 1.8 per cent salt solution.

Number of sera	Injections made with:	Complement fixation with emulsions of:					
		Egg lecithin No. 1	Egg lecithin No. 1 + cholesterol	Egg lecithin No. 1 + cholesterol	Merck's egg lecithin	Merck's egg lecithin after removal of cholesterol	Cholesterol
		A	B	C	D	E	F
967	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c	ac,c,c,c,c	0,0,ac,c,c	0,0,ac,c,c	0,0,d,ac,c
809	Merck's egg lecithin	c,c,c,c,c	c,c,c,c,c	str,c,c,c,c,c	0,0,0,0,str,c	0,0,0,w,c,c	0,0,0,0,d,ac,c
810	" "	vstr,ac,c,c,c	ac,c,c,c,c	d,vstr,c,c,c,c	0,0,0,0,0,str,ac	0,0,0,0,w,c,c	0,0,0,0,0,str,ac
Normal rabbit No. 1		c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
Normal rabbit No. 2		c,c,c,c,c	ac,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

performed after five injections were negative as well as flocculation tests at various other times.

The serum No. 967 which gave moderate reactions, as can be seen from Tables I and II, was tested against emulsions differing in their cholesterol content in comparison with sera prepared with Merck's egg lecithin (Table III).

The highly active sera resulting from the injection with lecithin Merck gave uniformly negative tests by the method of complement

TABLE IV.

Immune sera prepared by injections with Merck's egg lecithin	Complement fixations with emulsions prepared by addition of 24 parts of saline to a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	0,0,0,0,d,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 810	0,0,0,0,0,tr,c	vstr,c,c,c,c	ac,ac,c,c,c	ac,c,c,c,c
" 811	0,0,0,0,ac,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 812	0,0,ac,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

Immune sera prepared by injections with Merck's egg lecithin	Flocculations of emulsions made by addition of 5 parts of saline to 1 part of a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	+++	±	0	0
" 810	+++	tr	ftr	ftr
" 811	++±	±	tr	0
" 812	+±	+	0	tr
Saline control	0	0	0	0

fixation with our egg lecithin preparations 1, 2 and 3. In the flocculation tests preparations 2 and 3 reacted faintly, No. 1 somewhat better, but considerably weaker than lecithin Merck (Table IV). The results with brain lecithin and hydrolecithin were similar. Some other of our preparations gave more distinct flocculation with the Merck lecithin immune sera but practically negative complement fixation as far as they were examined.

SUMMARY.

In testing several egg lecithin preparations prepared by ourselves it was found that they did not react in complement fixation tests with immune sera made by injections with commercial egg lecithin Merck. With the flocculation method two of the preparations reacted only faintly. Also the brain lecithin and hydrolecithin gave no distinct reactions.

The immunization experiments of Sachs and Klopstock could easily be confirmed when commercial egg lecithin Merck was used for the injections. Immunizations with brain lecithin and hydrolecithin yielded no active sera. With an egg lecithin (No. 1) prepared by us the results were not satisfactory though a great number of injections was made. Only one serum gave reactions of medium strength by complement fixation and in flocculation tests with emulsions of Merck lecithin. It did not react however with the lecithin preparation No. 1 itself. In this respect the results resemble to a certain degree those of Sachs and Klopstock with their lecithin Böhringer immune serum. While the reactions of Merck lecithin were slightly diminished by the removal of cholesterol, addition of cholesterol to the lecithin No. 1 had no marked effect on the complement fixation tests, even when a larger amount was added than that present in the Merck preparation. The cholesterol content of this lecithin therefore does not suffice to account for the difference in the results. It is noteworthy that our lecithin immune serum No. 967 gave complement fixation with emulsions of cholesterol although this substance was not present in the injected material.

There are several plausible explanations for our results. According to one, the production of antibodies for lecithin would depend on certain physicochemical conditions of the emulsion injected or upon the presence of auxiliary substances in the lecithin preparation (*cf.* Sachs and Klopstock). Another possibility is that the active agent inducing the formation of antibodies is not lecithin itself but some other substance present in the active lecithin preparations. With regard to the latter assumption it may be mentioned that we obtained definite immunization effects from several injections of quantities

as little as 0.2 mg. of purified preparations of Forssman's heterogenetic haptene mixed with pig serum.[2]

To decide between the alternative explanations, further studies are necessary.

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[2] It may be stated that while in these experiments small quantities (1 mg.) of certain purified preparations of Forssman's heterogenetic haptene were active, no or a very slight effect was obtained on using larger amounts such as 100 mg. for each injection. These experiments will be fully described in a later communication.