EFFECT OF LIPID-EXTRACTION ON PRECIPITATING ANTISERA.

By L. TAMMEMAGI, B.V.Sc., Dr.med.vet., Animal Research Institute, Yeerongpilly.

SUMMARY.

The technique for removal of the ether fraction which remains dissolved in the serum after the extraction of lipids with ether is described.

Lipid-extraction, though it does not affect the antibody (serum-antigen) titre, is not recommended for antisera which are intended mainly for meat investigation work. Lipid-extraction substantially lowers the sensitivity of an antiserum against homologous meat and it is then less reliable for detection of small amounts of foreign meat in a meat mixture.

Such unextracted antisera, however, cannot always be freeze-dried or stored in the cold, as about 75% of rabbit sera will develop an opalescence, sometimes very pronounced, when exposed to temperatures below −25°C. for longer than a few days. They have to be kept therefore in liquid state (4°C.), though some deterioration in the antibody titre is likely to follow after storage for longer than 6 months.

In lipid-extracted antisera low temperatures do not cause any adverse effect in the physical properties or antibody titre. Storage in the frozen state is therefore recommended for such antisera, as it makes the time-consuming freeze-drying process unnecessary.

I. INTRODUCTION.

Weitz (1952) regarded freeze-drying of lipid-extracted sera as the ideal method of storage, as no deterioration in quality occurred in any of the dried sera kept by him at room temperature for over two years. Lipid-extraction was considered by McFarlane (1942) as a prerequisite procedure in freeze-drying to prevent opalescence when reconstituting later with water. This opalescence, which he regarded as due to the breaking-up of lipid complexes at the low temperatures (below —23°C.) during the freeze-drying process, may obscure or simulate a weak precipitation ring.

This paper deals with experiments to determine the effect of lipid-extraction on antisera prepared for the detection of horse meat.

II. MATERIAL AND METHODS.

The lipids were extracted from an anti-horse rabbit serum, prepared by repeated intramuscular inoculation of rabbits with alum-precipitated serum (Tammemagi 1954) by McFarlane's method. As he did not describe how the dissolved ether, which remains in the serum, was removed after the extraction of the lipids, we used vacuum suction, employing a flask provided with a sidearm. To admit air, a fine calibre pipette was passed through

the stopper and reached almost to the bottom of the flask. Incubation in a waterbath at 40°C. for about one hour was found necessary, as previous attempts to remove the ether at room temperature or 37°C. had been unsatisfactory. Some of the serum—about half—was left untreated (i.e., the ether was not removed).

Both serum portions, one containing and the other freed from ether, were sterilized by filtration, and were perfectly clear when dispensed into small vials for storage at various temperatures—room, 4°C., —10°C. and below —25°C.—or submitted to freeze-drying.

The precipitin ring-test, the technique of which is fully described in another paper (Tammemagi 1954) was used in experiments with meat extracts.

Several samples of decomposed horse meat were also examined, but with these it was found necessary to increase the usual ratio of saline to meat from 1 : 1 to 3 : 2, as otherwise the slimy extract would not pass through ordinary filter paper. For clarifying, bacteria-retaining filter pads were necessary.

III. RESULTS.

(1) Effect of Lipid-extraction on the Homologous Serum-antigen Titre.

It was found that the rabbit serum fully retained its original titre of 1 : 512,000 against horse serum-antigen after the lipids were extracted with ether, and regardless of whether the dissolved ether was removed or not.

(2) Effect of Lipid-extraction on the Homologous Meat-antigen Titre.

In a previous paper (Tammemagi 1954) the unextracted anti-horse rabbit serum was shown to have an endpoint of 1 : 8,000 with a fresh horse meat-antigen, but after the extraction of lipids the endpoint was only 1 : 800. Other fresh horse meats gave an endpoint of between 1 : 500 and 1 : 200. These titres were not influenced by the removal of the dissolved ether.

Extracts from frozen horse meat (stored below —25°C.), or from grossly decomposed horse meat, gave results comparable with those obtained with fresh meat.

Using meat mixtures containing variable amounts of horse meat, the endpoints moved *pro rata* with the content of horse meat in the mixture (Table 1).

With fresh beef-horse-meat mixture containing only 1% of horse meat, the reaction with undiluted extract was negative after the usual time limit

Table 1.

ENDPOINTS OF PRECIPITIN REACTIONS WHEN EXTRACTS OF MEAT MIXTURES CONTAINING VARIOUS PROPORTIONS OF HORSE MEAT WERE TESTED WITH LIPID-EXTRACTED ANTI-HORSE RABBIT SERUM.

Dilution of Meat Extract.	Percentage of Horse Meat in the Mixture.						
	100	75	50	25	10	5	1
Undiluted ..	++++	++++	++++	+++	++	++	− (+)*
1 : 2 ..	++++	++++	++++	+++	++	++	− (+)
1 : 5 ..	++++	++++	++++	++	++	++	− (−)
1 : 10 ..	++++	++++	+++	++	+	+	− (−)
1 : 50 ..	+++	+++	++	+	+	±	− (−)
1 : 100 ..	+++	++	+	+	+	−	
1 : 200 ..	++	+	+	±	−	−	
1 : 400 ..	+	+	±	−	−	−	
1 : 800 ..	+	−	−	−	−	−	
1 : 1000 ..	±	−	−	−	−	−	

* Results obtained after incubating for 4 hours instead of the usual time of 2 hours.

++++ Very strong reaction.
+++ Strong reaction.
++ Moderate reaction.
+ Weak but definite reaction.
± Doubtful reaction.
— Negative reaction.

of 2 hours, but a doubtful reaction appeared after 3 hours, and became definitely positive after 4 hours. At this time a doubtful reaction appeared also with a 1 : 2 dilution of this extract. Control tests with ox, sheep, pig and dog meats gave negative results, even when the time of final reading was extended to 6 hours or more.

(3) Effect of Storage on the Physical Quality of the Antiserum Samples.

No turbidity of any kind developed in any of the lipid-extracted serum samples, whether ether-free or otherwise, during storage for 6 months in liquid state at room temperature or at 4°C. Similarly, the ether-free samples which were stored at —10°C. or below —25°C. were perfectly clear when thawed out, though samples which contained the dissolved ether became slightly opalescent on thawing, but could be clarified by centrifuging for 30 minutes at 2,500 r.p.m. to a degree where they gave a staisfactory endpoint at a dilution of 1 : 128,000. At higher dilutions the haziness of the antiserum interfered with the reading.

On reconstituting freeze-dried samples with distilled water, slight opalescence again developed in those samples which contained the dissolved ether.

Control experiments with a number of unextracted horse, ox, sheep, pig and rabbit sera showed no physical deterioration when 0·5—1·0 ml. samples were submitted to temperatures below —25°C. for a few hours to several days. However, after a longer storage at this temperature, some of the sera became slightly opalescent after thawing, but, except for rabbit serum, they could be clarified again by centrifuging for from a half to one hour at 2,500 r.p.m. Of the 25 rabbit sera tested, approximately 75% developed after thawing an opalescence which ranged from a very slight cloudiness to a heavy milky appearance. This opalescence was more pronounced in sera obtained from older rabbits in good condition. Some rabbits, in spite of fasting, gave on subsequent trial bleedings sera which constantly became opalescent when submitted to cold. Centrifugation had little if any effect in clarifying such sera, but filtration through bacteria-retaining filter pads made them quite satisfactory for precipitin tests. Such samples, however, became cloudy again, though to a lesser degree, when exposed again to cold.

(4) Effect of Storage at Different Temperatures on the Potency of the Antiserum.

After two months' storage at room temperature or at 4°C. there was a slight fall in the titre from the original 1: 512,000 down to 1: 128,000. Freeze-drying apparently had no adverse effect. Storage after freezing at —10° C. or below —25°C. did not reveal any deterioration after two months. Only in the samples containing ether did the opalescence which developed on thawing interfere with the readings at dilutions higher than 1:128,000.

Re-tests after 6 months of storage at —10°C. or —25°C. showed no decline in titre. Samples kept in liquid state, however, showed a further decline down to 1: 8,000 at room temperature, and 1: 64,000 at 4°C.

IV. DISCUSSION.

In another paper (Tammemagi 1954) it was stated that the meat-antigen titre of an antiserum is much lower than the corresponding serum-antigen titre.

This work has shown that an initially high titre is particularly important when one desires later to eliminate the lipids for the purpose of storage in a freeze-dried state. Removal of lipids is accompanied, as was noticed during this work, by a decrease of the sensitivity of the antiserum against homologous meat-antigen, while the potency against homologous serum-antigen is left unaffected. Compared with the unextracted fraction, the lipid-extracted portion of the anti-horse serum retained only about one-tenth of its former potency against homologous meat-antigen. It is quite possible that low-level antisera of a potency of say only 1:1,000, such as used by some workers

(Kaplan and Buck 1951) would lose, after being deprived of lipids, so much of their potency as to become largely useless for the detection of adulterations with small amounts of horse meat. Even such a high-titre antiserum as used in this work (1:512,000) declined so much that one was unable to detect the presence of 1% horse meat in a meat mixture when the usual time limit of 2 hours was strictly observed for reading the results. A positive reaction developed only some hours later. An extension of the time for final reading appears necessary to avoid errors.

The reason for the decrease of the sensitivity of an antiserum against meat-antigens, while remaining unaltered against homologous serum, after the extraction of the lipids is not readily explained. Since about 25% of the dry precipitate in the precipitin reaction is formed by lipids, it has been stated that the presence of the ether-soluble substances (lipids, cholesterol, etc.) is essential in certain serological reactions (Hartley 1925). Fats and lipids appear to play an important part in some immune reactions (Graham 1911).

According to Hartley (1925) the complement fixing properties of a syphilitic serum are associated with the ether-soluble content, since such sera after the extraction react negatively in the Wassermann reaction. The lipids were also essential for producing flocculation but not for actual neutralization of the toxins in a toxin antitoxin reaction. Agglutination of bacteria and haemolysis of red cells by specific sera are both independent of lipids. In the precipitin reaction, however, a precipitate would not form when both the antigen and antiserum were deprived of these constituents, but would follow when present in one only of the reagents.

Since lipid-extraction of the anti-horse rabbit serum in this study had not affected the precipitin reaction with the homologous serum-antigen, the explanation for the lowered meat-antigen titre might be due to the necessary lipids being present in higher concentration in the serum than in the meat extract.

The decrease in sensitivity against meat-antigens after the removal of lipids indicates that the extraction process should be avoided when antisera are to be used mainly for meat investigation. Such unextracted sera can be kept for short periods in the liquid state at 4°C. without losing much of their titre. For longer periods freeze-drying or storage in the frozen state is required, but it is not always satisfactory, as the lipid-complexes, particularly in rabbit sera, tend to break down when exposed to low temperatures, with resultant opalescence in the sera. However, since about 25% of rabbit sera in our experiments were not affected in this way by low-temperature storage, the recommended procedure is to expose a small sample at —25°C. for at least one week. If this remains clear after thawing, the bulk of the serum can then safely be stored in the cold or freeze-dried.

It appears that storage in the cold compares favourably with freeze-drying in preserving the titre. In our experiments, no fall in potency occurred within 6 months of storage at —25°C., and Kaplan and Buck (1951) kept antisera in frozen state for over 7 years without any deterioration in potency. DeFalco (1953) stated that frozen sera can be kept indefinitely. Certainly this is much more convenient than the time consuming freeze-drying.

According to McFarlane (1942) an ether-extracted serum contains about 11% dissolved ether. Removal of this ether is included in his method for extraction of lipids, but he did not further elaborate this step.

The preliminary unsuccessful attempts in this work to remove the dissolved ether from the lipid-extracted anti-horse serum by vacuum suction at room temperature or by open evaporation in incubator at 37°C. indicate that the dissolved ether is not readily eliminated. Forssman (1921) was able to do this by vacuum only when the serum was heated up to the inactivation temperature, while elimination at 37°C. failed. He assumed that the ether is taken up by lipids from which it is released only by higher temperatures. However, the disappearance of the ether smell from our lipid-extracted antiserum by vacuum at 40°C. suggests that ether will be released at a temperature lower than that of inactivation.

Initially it was thought that the variation in the endpoints of the precipitin reactions could be used as a rough guide for estimating the approximate amount of horse meat contained in the meat mixture. A certain correlation certainly became evident, but further observations suggested caution in applying a qualitative test in a quantitative sense because the endpoints of meat extracts from different horse carcases sometimes varied quite considerably. Though the techniques in preparing extracts and performing ring-tests were in each case strictly observed, it is possible that the blood content of the samples has some relationship to these inconsistent results. That this may be so has already been suggested by Schmidt (1907) and Manteufel and Tomioka (1924), who explained the occasional failure of their even high-titre sera to give reactions with homologous meats as due to the fact that antisera are normally prepared in rabbits against serum-antigen (blood) but not against muscle protein. Any precipitin reaction which occurs with meat does so because it contains some blood, against which the antiserum reacts. Hence, meat of a very low blood content could fail to give a positive reaction.

ACKNOWLEDGEMENT.

My thanks are due to Mr. G. C. Simmons, B.Sc., Bacteriologist at the Animal Research Institute, for his helpful advice during the course of the experiments.

REFERENCES.

DeFalco, R. J. 1953. The Serological Museum, Rutgers University, New Jersey, Bull. No. 10: 6.

Forssman, J. 1921. Biochem. Z. 121: 180.

Graham, E. A. 1911. J. Infect. Dis. 8: 147.

Hartley, P. 1925. Brit. J. Exp. Path. 6: 180.

Kaplan, E., and Buck, T. C. 1951. J. Milk Tech. 14: 66.

Manteufel, P., and Tomioka, Y. 1924. Zbl. Bakt. 1 Abt. Orig. 91: 317.

McFarlane, A. S. 1942. Nature, Lond. 149: 439.

Schmidt, W. A. 1907. Biochem. Z. 5: 492. Cited by Manteufel and Tomioka (1924).

Tammemagi, L. 1954. Qd J. Agric. Sci. 83-98.

Weitz, B. 1952. J. Hyg., Camb. 50: 275.

(Received for Publication June 22, 1954.)