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THE PRECIPITIN TEST IN THE DETECTION OF HORSE MEAT.

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

SUMMARY.

Repeated inoculation of alum-precipitated serum by the intramuscular route has proved to be the method of choice in preparing high-level precipitating sera in rabbits.

The antiserum so produced is non-specific, but by absorption with heterologous sera the specificity can be fully restored without reducing the titre of the homologous antibody.

A high homologous titre of the antiserum is an advantage in meat identification work, as the meat-antigen titre is always many times lower than the corresponding serum-antigen titre. Sera of high antibody titre have higher sensitivity against homologous meats. Consequently such sera are more reliable than low-level antisera for detection of small amounts of adulteration with a foreign meat.

The precipitin test is of little practical value for identification of cooked meat. Positive precipitations can be obtained with meats only which have not been heated at a higher temperature than 80°C. for not longer than 10 minutes.

Unmelted fat can be used as an alternative source of material when no raw meat is available for species identification.

I. INTRODUCTION.

Fraudulent substitution of horse meat is a problem likely to be encountered in countries where scarcity leads unscrupulous dealers to attempts to deceive consumers. Advantage is taken of the difficulty in distinguishing the flesh of different animals when processed into smallgoods.

In some countries standard laboratory methods have been adopted to assist food inspectors to detect horse meat (e.g., German Fleischbeschaugesetz). In Australia, various State Acts dealing with the slaughter of animals declare illegal the sale of horse meat for human consumption.

Serological or chemical methods may be used to differentiate meats. Some of these methods (e.g., the glycogen test) are not very satisfactory, while others require specially equipped laboratories, as in the estimation of the

iodine-absorption number (Winkler's* Method in Germany and Hanus'† and Wijs'† Methods in U.S.A.) and in the hexabromide method (Crowell 1944). The refractrometer index evaluation method also requires special apparatus. These tests are not entirely satisfactory, as, apart from the glycogen test, they are dependent on the presence of horse fat in the sample, and the isolation of this for the test is not always possible.

Serological methods are generally regarded as the most reliable. The precipitin test is usually preferred to the complement fixation test, as it can be done without difficulty in a small laboratory. A third method, based on anaphylactic reaction, has value only as a supplementary test.

As antisera for the precipitin test are not readily available in Australia, we prepared our own. In this paper are described the methods used and the results obtained when applying the techniques recommended by other workers.

II. MATERIALS AND METHODS.

(1) Preparation of Antisera.

(*a*) Antigens.

(i) *Antigens in preliminary trials.*—In preliminary experiments meat extracts and normal blood sera from horse, ox, sheep and pig were used.

Extracts were prepared by mixing 100 grams of finely cut lean meat with 100 ml. of sterile saline solution for a few minutes in a Waring Blendor. After storage overnight in a refrigerator, this mixture was squeezed through several layers of gauze, made up to the original volume of 100 ml. with saline, then passed through a Seitz sterilizing filter pad.

(ii) *Alum-precipitated antigen.*—Horse, ox, sheep and pig sera were precipitated with a solution of potash alum as described by Proom (1943). Ten millilitres of the suspension was made equivalent to 2·5 ml. of serum.

(*b*) Method of Immunisation.

All antisera were produced in rabbits. In preliminary trials one rabbit was used for each antigen. With meat-antigens, seven doses ranging from 0·2 ml. to 2·0 ml. were injected intravenously at intervals of 3 or 4 days, and the rabbits bled 17 days later. When normal sera were used, the rabbits received eight injections of 0·1–2·0 ml. intravenously. The immunological

* Cited by Schroeter-Hellich in "Das Fleischbeschaugesetz" (p. 591), 6 Ed., 1943. Richar Schoetz: Berlin.

† Cited in "Official Methods of Analysis of the Association of Official Agricultural Chemists", (p. 432-3), 1950, 7 Ed. Association of Official Agricultural Chemists: Washington.

response was checked 3 days after the last injection, and on three consecutive trial bleedings at intervals of 4 days. One rabbit received a single intramuscular injection of 2·5 ml. normal horse serum, the response being checked 5 and 10 days later.

With alum-precipitated antigens, in one series, each rabbit received one or two intramuscular injections of 10 ml. of either alum-precipitated horse, ox, sheep or pig serum, 5 ml. being injected into each thigh. When two injections were given, the second was done 30 days after the first.

A large volume of anti-horse serum was produced by injecting a batch of 4 rabbits three times intramuscularly with alum-precipitated horse serum at 30-day intervals.

The rabbits were bled for maximal yield when their sera had reached a titre of at least 1:1,000, as required by Ostertag (1934) and Kaplan and Buck (1951).

(*c*) Technique of Absorption.

The technique of Weitz (1952) was used to absorb the non-specific antibodies from an anti-horse serum, prepared by repeated intramuscular inoculation of rabbits with alum-precipitated antigen.

(2) Preparation of Meat Extracts for Testing

(*a*) Raw Meats.

Twenty to thirty grams of finely cut raw horse, ox, sheep and pig meats were extracted overnight at 4°C. with an equal weight of sterile saline as recommended by Ginsberg (1948). After filtration through Whatman No. 1 filter paper, the extract was, when necessary, further clarified by passing through a Seitz clarifying filter pad. The determination of an adequate albumin concentration, such as is required in the German method (see "Das Fleischbeschaugesetz," pages 540-542), was omitted for the reasons outlined by Ginsberg (1948).

(*b*) Heated Meats.

Ten grams of finely cut raw horse meat was distributed in 1-oz. screw-capped bottles and heated 10, 20 or 30 minutes in a waterbath at various temperatures (see Table 7). The juice produced during the heating was discarded, and the residue extracted overnight at 4°C. with 10 ml. of saline. The extract was clarified by passing through a Seitz coarse clarifying pad (Horman-Ekwip filter pad No. D_0).*

* Supplied by Industrial Equipment Pty. Ltd., 171 William street, Sydney, N.S.W., Australia.

(*c*) Fats.

The technique of Wittels and Welwart (1910), modified by Ginsberg (1948), was adopted for examination of unmelted fats from horse, ox, sheep and pig. Twenty grams of finely cut fat was extracted with 40 ml. of saline for 3 hours at room temperature and then for 20–22 hours at 4°C. In order to free it from all fat particles, it was filtered through Whatman No. 1 filter paper, and then through a Seitz coarse clarifying pad (Hormann-Ekwip D_0). In case of horse and pig fats, a finer pad (D_6) was necessary to obtain a water-clear filtrate.

(3) The Precipitin Test.

All the determinations were made by the "ring-test." Dilutions of the antigen, consisting of homologous and heterologous normal sera or meat extracts, were layered over the antiserum in narrow agglutination tubes 6·0 cm. x 0·6 cm. with tapered base.

About 2 or 3 drops of the antiserum were pipetted to the bottom of the tube with a pasteur pipette. The side of the tube was moistened as the pipette was withdrawn. An equal amount of the antigen dilution was then carefully superimposed on the antiserum with another pipette, starting from the highest dilution, and keeping the test tube nearly horizontal to reduce the rate of flow of the antigen down the tube. Previous moistening with the antiserum assisted the antigen to run smoothly down the tube and form a sharp interface when the tube was again set upright. The time limit for reading a positive reaction was 20 minutes at room temperature in the preliminary trials, while in the later experiments the final reading was done after incubating for two hours at room temperature as recommended by Weitz (1952).

III. RESULTS.

(1) Preparation of Antisera.

(*a*) Antibody Response to Meat-antigen.

The results obtained in the preliminary trials are summarized in Table 1. The titres of rabbit sera were tested only against meat extracts and not against normal sera as was done in all subsequent experiments.

From Table 1 it is evident that apart from low meat-antigen titres, particularly of the anti-pig serum, considerable cross reactions occurred with heterologous meat extracts.

Table 1.

PRECIPITIN TITRES OF ANTISERA, PREPARED BY INOCULATING RABBITS INTRAVENOUSLY WITH MULTIPLE DOSES OF MEAT EXTRACTS, WHEN TESTED AGAINST HOMOLOGOUS AND HETEROLOGOUS MEAT-ANTIGENS.

Antiserum.	Titres* When Tested with Extracts of Meats from			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse ..	**200**	5	10	0
Anti-ox ..	100	**400**	100	5
Anti-sheep ..	0	100	**400**	0
Anti-pig ..	0	0	0	**10**

* Titres are expressed as reciprocals of dilution.

Homologous titres are given in black.

(*b*) Antibody Response to Normal Serum.

(i) *Multiple intravenous injections.* The results in Table 2 show a satisfactory response in rabbits to rapid immunisation with multiple intravenous doses of normal sera, but all the antisera so produced gave cross reactions with heterologous serum-antigens. The maximum titres shown in the table were reached in some rabbits by the seventh day, and in the others by the eleventh day, after the last injection.

Table 2.

PRECIPITIN TITRES OF NON-SPECIFIC ANTISERA, PREPARED BY INTRAVENOUS IMMUNISATION OF RABBITS WITH MULTIPLE DOSES OF NORMAL SERA, WHEN TESTED WITH HOMOLOGOUS AND HETEROLOGOUS SERUM-ANTIGENS.

Antiserum.	Titres* When Tested with Serum of			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse ..	**2,000**	100	100	100
Anti-ox ..	100	**10,000**	10,000	100
Anti-sheep ..	50	10,000	**10,000**	50
Anti-pig ..	100	500	500	**2,000**

* Titres are expressed as reciprocals of dilution.

Homologous titres are given in black.

(ii) *Single intramuscular injection.* Normal horse serum only was used for inoculation. The maximum homologous titre obtained was 1:4,000. It was reached on the sixth day after the inoculation. There was no evidence of further increase in the titre 4 and 8 days later. When the rabbit serum was tested with heterologous serum-antigen, no reaction occurred with the pig serum-antigen, but cross reactions appeared with ox and sheep antigens to a dilution of 1:100.

(*c*) Antibody Response to Alum-precipitated Antigen.

The response in rabbits to a single or double intramuscular inoculation of alum-precipitated antigen is shown in Table 3.

Table 3.

PRECIPITIN TITRES OF ANTISERA, PREPARED BY SINGLE OR DOUBLE INTRAMUSCULAR INOCULATION OF RABBITS WITH ALUM-PRECIPITATED SERA, WHEN TESTED WITH HOMOLOGOUS AND HETEROLOGOUS SERUM-ANTIGENS.

Antiserum.	Titre of Reaction when Tested with a Maximum Concentration of 1 : 50 of the Sera of			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse (1 injection)	**8,000**	50	50	0
Anti-ox (2 injections) ..	50	**32,000**	2,000	0
Anti-sheep (2 injections)	50	1,000	**4,000**	0
Anti-pig (1 injection) ..	0	0	0	**4,000**

Homologous titres are given in black.

The titres of anti-horse and anti-pig sera were obtained by a single inoculation, while the figures for anti-ox and anti-sheep sera were the result of two injections. The second dose was given 30 days after the first, because at trial bleedings, 15 and 20 days after the first injection, the titres did not rise higher than 1:500, which was regarded as insufficient.

When investigating for specificity, it was found that excepting the anti-pig serum, the three others gave cross reactions with some of the heterologous antigens. As a maximum concentration of 1:50 of the antigen was used, it is possible that the anti-pig serum was not specific either.

The response of rabbits to multiple intramuscular inoculations of alum-precipitated horse-serum was studied in four rabbits. The homologous titres were determined for each rabbit after each injection. After the third inoculation the sera were pooled and the heterologous titres then determined.

One injection gave homologous titres of 1: 16,000 in three and 1: 2,000 in one rabbit. After the second injection the titres had risen to 1:64,000 in three and 1:256,000 in the fourth rabbit, which had previously shown the lowest response. After the third injection, the homologous titres were at least 1:256,000 in all four animals. No higher dilutions of the horse serum-antigen were tested.

The antisera collected after the first inoculation were discarded in order to maintain a high titre in the pooled sera collected from the next two bleedings.

Table 4.

RESULTS OF THE PRELIMINARY TITRATION OF THE ANTI-HORSE SERUM, PRODUCED BY REPEATED INOCULATION OF THE RABBITS WITH ALUM-PRECIPITATED HORSE SERUM, WHEN TESTED AGAINST HETEROLOGOUS ANTIGENS.

	Number of Tube.							
	1	2	3	4	5	6	7	8
Antiserum volume (ml.)	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25
Dilution of heterologous antigens	Undiluted	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
Volume of dilution of heterologous antigen added ..	0·025	0·025	0·025	0·025	0·025	0·025	0·025	0·025
Final antigen/anti-serum ratio	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Ring test of supernatant :								
(1) Ox antigen 1/10	−	+	++	+++	+++	++++	++++	++++
Ox antigen 1/100	−	+	+	++	++	+++	+++	+++
(2) Sheep antigen 1/10	+	+	++	++	++	+++	+++	+++
Sheep antigen 1/100	+	+	+	+	+	++	++	+++
(3) Pig antigen 1/10	−	−	±	+	+	++	++	+++
Pig antigen 1/100	−	−	−	+	+	++	++	+++

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

The pooled antiserum showed a titre of 1:512,000 which was the highest dilution tested. When tested with heterologous serum-antigens, the titres were 1:1,000 against ox and sheep and 1:2,000 against pig serum.

(*d*) Results of Absorption of Non-specific Antibodies.

The anti-horse serum from four rabbits was absorbed according to the technique of Weitz (1952) in order to obtain a serum reacting only with horse serum or meat extract.

In preliminary titration the amount of antigens required for absorption of the corresponding heterologous antibodies was determined.

The results of the titration (Table 4) showed that the heterologous antibodies were absorbed entirely, as indicated by a negative precipitin reaction of the supernatant fluid, in tube 1 by the ox antigen, and in tube 2 by the pig antigen, giving the ratio of the antigens to be added to the bulk of the anti-horse serum as 1/10 and 1/20 respectively. Since with sheep antigen a weak positive reaction still appeared at the antigen/antiserum ratio of 1/10, it was assumed that by doubling the amount of the antigen to 1/5, all sheep antibodies would be absorbed.

To the initial bulk (160 ml.) of the anti-horse serum, 16 ml. of ox serum (1/10), 8 ml. of pig (1/20), and 33 ml. (1/5) of sheep serum were added, incubated for 4 hours at 37°C., allowed to precipitate overnight at 4°C. and centrifuged. The supernatant gave no precipitation with heterologous serum-antigens. After sterilization by filtration, the absorbed antiserum showed a homologous serum-antigen titre of 1:512,000.

(2) Examination of Meats.

(*a*) Raw Meats.

The unabsorbed and absorbed antisera were tested against various meat extracts. Of the unabsorbed antisera, firstly a series of non-specific antisera, prepared by immunisation of rabbits with one or two intramuscular injections of alum-precipitated horse, ox, sheep and pig sera were used, whose homologous serum-antigen titres were determined as 1: 8,000, 1: 32,000, 1: 4,000 and 1: 4,000 respectively. Secondly, an unabsorbed non-specific anti-horse serum with a homologous titre of 1:4,000, obtained by a single intramuscular injection of normal horse serum, was tested against different meats. Lastly, an absorbed (specific) anti-horse serum with a titre of 1:512,000, prepared by repeated immunisation with alum-precipitated horse serum, was used.

(i) *Results with unabsorbed (non-specific) antisera.* The results with non-specific antisera, prepared by one or two intramuscular injections of alum-precipitated serum-antigens, are shown in Table 5.

Table 5.

PRECIPITIN REACTION WITH RAW MEATS USING UNABSORBED (NON-SPECIFIC) ANTISERA PREPARED BY SINGLE OR DOUBLE INTRAMUSCULAR INJECTION OF RABBITS WITH ALUM-PRECIPITATED SERUM-ANTIGENS.

Antiserum.	Titre* of Reaction When Tested with Undiluted and Diluted Extracts of Meat from			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse ..	**500**	10	0	0
Anti-ox ..	0	**500**	100	0
Anti-sheep ..	0	50	**100**	0
Anti-pig ..	0	0	0	**10**

* The titres are expressed as reciprocals of dilutions.

Homologous titres are given in black.

The non-specificity of these antisera is evident by the various cross reactions obtained with the different meat extracts. However, the results are somewhat different when compared with the results obtained with normal sera (see Table 3). The antisera gave much lower endpoints against the homologous meat-antigens than against homologous serum-antigens. No cross reactions occurred now with the anti-ox and anti-sheep sera against horse meat-antigen. Surprisingly, the anti-pig serum showed a rather low potency against homologous meat-antigen (i.e., 1:10), but the anti-sheep serum, which had the same serum-antigen titre (1:4,000) as the anti-pig serum, gave a positive reaction up to a dilution of 1:100 of the sheep meat extract.

The results against different meats with the non-specific anti-horse serum, prepared by a single intramuscular injection of normal horse serum, are shown in Table 6. This table also shows the variation of the endpoints of the precipitin reactions when the amount of horse meat mixed into a minced beef varied.

The antiserum that had a titre of 1:4,000 against homologous serum-antigen gave a meat-antigen titre of only 1:200 against homologous meat-extract. There was also a progressive decline in the endpoints proportional to the decrease of horse meat in the mixture down to a level of 10%. Lower levels of horse meat could not be distinguished from the beef and mutton extracts, as the same endpoints were obtained with these extracts. No reaction occurred with pork extract.

(ii) *Results with absorbed (specific) anti-horse serum.* This antiserum with a serum-antigen titre of 1:512,000 was tested against different meat extracts after the heterologous (ox, sheep and pig) antibodies had been absorbed with serum-antigens. With pure meat extracts it gave a positive

Table 6.

THE ENDPOINTS OF PRECIPITIN REACTIONS WHEN MEAT EXTRACTS WERE TESTED WITH A NON-SPECIFIC ANTI-HORSE SERUM, PREPARED BY A SINGLE INTRAMUSCULAR INOCULATION OF NORMAL HORSE SERUM.

Dilution of Extracts.	Percentage of Horse Meat in Minced Beef.							Meat Extracts from		
	100	75	50	25	10	5	1	Ox.	Sheep.	Pig.
Undiluted ..	++++	++++	++++	+++	++	++	+	+	+	—
1 : 5 ..	++++	++++	++++	++	++	+	+	+	+	—
1 : 10 ..	++++	++++	+++	+	+	+	+	+	±	—
1 : 20 ..	++++	+++	++	+	±	—	—	—	—	—
1 : 50 ..	+++	++	+	±	—	—	—	—	—	—
1 : 100 ..	++	+	±	—	—	—	—	—	—	—
1 : 200 ..	+	±	—	—	—	—	—	—	—	—
1 : 400 ..	—	—	—	—	—	—	—			

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

reaction with 1:8,000 and a doubtful reaction with 1:16,000 dilution of the horse meat extract. No cross reactions appeared with extracts prepared from ox, sheep, pig and dog meats, even with undiluted extracts.

A series of minced beef samples, containing various proportions of added horse meat, were also examined to determine the endpoints of precipitin reactions at different horse meat levels. It was again found that when lowering the ratio of horse meat, the endpoints decreased correspondingly, so that with 80% of horse meat the endpoint appeared at a dilution of 1:4,000 of the mixture extract, with 60% at 1:2,000, with 40% at 1:2,000, and with 10% of horse meat at 1:500.

(*b*) Heated Meats.

The results with heated horse meats are summarized in Table 7.

It can be seen that precipitin reactions occurred with extracts of meat heated for 30 minutes at 70°C. At 80°C. a reaction was obtained when meat was not heated longer than 10 minutes.

The above results were all obtained with the specific anti-horse serum which had shown previously a serum-antigen titre of 1:512,000, and a meat-antigen titre of 1:8,000 against raw horse meat.

In control tests with ox, sheep and pig meats, heated for 10 minutes at 70°C. and 100°C., no positive reactions appeared.

Table 7.

PRECIPITIN REACTION OF EXTRACTS FROM HEATED HORSE MEAT WITH SPECIFIC ANTI-HORSE SERUM.

Dilution of Meat Extract.	Temperature and Duration of Heating (Minutes).											
	70°C			80°C			90°C			100°C		
	10	20	30	10	20	30	10	20	30	10	20	30
Undiluted ..	+++	+++	+++	+++	—	—	—	—	—	—	—	—
1 : 2 ..	+++	+++	+++	++	—	—	—	—	—	—	—	—
1 : 4 ..	+++	+++	++	+	—	—	—	—	—	—	—	—
1 : 8 ..	+++	+++	+	±	—	—	—	—	—	—	—	—
1 : 16 ..	+++	++	+	—	—	—	—	—	—	—	—	—
1 : 32 ..	++	+	±	—	—	—	—	—	—	—	—	—
1 : 64 ..	++	+	—	—	—	—	—	—	—	—	—	—
1 : 128 ..	+	±	—									
1 : 256 ..	±	—	—									
1 : 512 ..	—	—	—									

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

(*c*) Unmelted Fats.

Lipid-extracted anti-horse serum only was used in these tests. The technique of McFarlane (1942) was used for extracting the lipids from the antiserum.

Positive reactions appeared at a dilution of 1:100 to 1:200 of the extracted horse fat as compared with 1:800 in the case of horse meat. (The effect of lipid extraction on the potency of an antiserum will be discussed in detail in a subsequent paper.) Ox, sheep and pig fats all gave negative results when undiluted and diluted extracts were examined.

IV. DISCUSSION.

The results shown in this paper have largely confirmed the findings of Hektoen and Welker (1933) and Proom (1943) that immunisation of rabbits with alum-precipitated serum-antigen by the intramuscular route is vastly superior to the usual intravenous injection of normal serum for the production of high-level antisera. By the latter method multiple injections are required to obtain an antiserum of workable potency, but this invariably leads to a loss of specificity (Meissner 1926; Heidelberger and Kendall 1935; Adair and Hamilton 1939; Hooker and Boyd 1941; Leone 1952, a.o.)

Attempts have been made by Ascoli (1902), Kister and Weichardt (1902), Liepmann (1903), Obermayer and Pick (1904), Friedberger and Collier (1919), Manteufel and Beger (1921), Yu (1923), Beger (1924) and others to eliminate the interfering antibodies from such non-specific antisera by absorption with heterologous antigens or with physical adsorbents. However, as the results had not been satisfactory enough for practical application, some workers have tried to avoid cross reacting antibodies in the antiserum by using heat-coagulated antigens (Manteufel and Beger 1921; Fujiwara 1922; Beger 1924; Meissner 1926), alcohol-precipitated antigens (Tsukasaki 1922; Manteufel and Tomioka 1924), or "heat-alkali" treated antigens (Schmidt 1912; Rosenberger 1926) for immunisation of rabbits. Other workers again have attempted to prepare specific sera by reducing the number of intravenous injections to a very few or even to a single injection (Satoh 1933; Wolfe 1935, 1936), but Proom (1943) has shown that an antiserum so produced has only about one-tenth the strength of an antiserum obtained by a single intramuscular inoculation of an alum-precipitated antigen.

However, a number of workers still appear to favour the multiple intravenous inoculation method. To offset the loss of specificity by this technique, a strict time limit has been adopted by them for distinguishing non-specific reactions from the specific. According to Ostertag (1934) a specific reaction is indicated by the development of a whitish precipitate at the interface of the two reagents, the anti-horse serum and horse meat extract, at the room temperature within a few minutes, whereas Manteufel and Beger (1921) specify 10, Ginsberg (1948) 20, Tsukasaki (1922) and Kaplan and Buck (1951) 30 minutes. Thirty minutes is also the official German time limit (see "Das Fleischbeschaugesetz" by Schroeter-Hellich, 6 Ed., 1943, page 541). Any precipitation which appears after this time is regarded as non-specific.

This work has not supported the claim by Proom (1943) that a single intramuscular dose of alum-precipitated antigen will produce 100% specific antisera. Weitz (1952) already had suggested that some of Proom's sera might appear less specific if he had used a maximum concentration of 1:10 instead of 1:50 of the serum-antigen when testing for specificity, and having at the same time extended also the time of reading his results from 30 minutes to 2 hours.

This criticism appears well founded, since some of the antisera obtained in our experiments by single intramuscular injection gave no precipitin reactions with heterologous antigens within the first 30 minutes of incubation, but became positive after 2 hours. Even when assuming that exceptional rabbit sera only may show such cross reacting antibodies after a single dose, one still has to be aware of such a possibility, and has therefore to examine all rabbit sera individually to select the particular ones which are free of any interfering antibodies. Quite a number of rabbits have therefore to be immunised simultaneously, as some would have to be discarded because of

insufficient titre. Re-inoculation of such rabbits to boost the titre will invariably lead to a loss of specificity. For this reason the single-inoculation method of producing specific antisera is not practical.

The alternative method, namely the multiple intra-muscular immunisation, has proved more satisfactory because much higher titres can so be achieved. Although there is a loss of specificity, this is not of particular importance, since it is possible to eliminate the interfering cross antibodies by the absorption technique devised by Weitz (1952). No actual checks for titres or specificity of a separate rabbit serum is necessary until sera from all rabbits simultaneously inoculated have been collected and pooled. Fewer rabbits are so required, as they can be bled after each inoculation. Each heterologous antibody is eliminated by absorption with the correct amount of the appropriate heterologous antigen (normal serum) determined by titration.

The elimination of some less important heterologous antibodies, such as anti-dog, anti-cat, or other unusual mammalians, which could be present in otherwise specific antiserum after the absorption of the antibodies of the common slaughter animals, was not attempted in this work, as in meat adulteration these animals play little or no role in Australia.

Undoubtedly the interpretation of a positive precipitin reaction by the use of such specific antisera is of a much more precise nature than when having to depend on the time limit of appearing precipitation by using non-specific antisera.

The experiments have shown that in meat detection, antisera with higher antibody titres have certain advantages over low-potency sera. In horse meat investigation, the minimum strength required from an anti-horse serum is given by some workers (Ostertag 1934; Kaplan and Buck 1951) as sufficient when it has a titre of 1:1,000 against homologous serum-antigen. Other workers have given higher requirements (e.g., Wolfe (1935, 1936) 1: 6,400 and Proom (1943) 1:8,000). The German standard is 1:20,000. According to our findings still higher serum-antigen titres could be of benefit, since the corresponding meat-antigen titres were found to be many times lower than the former. It was observed that antisera with higher serum-antigen titres also manifested higher meat-antigen titres—i.e., two anti-horse sera with antibody titres of 1:4,000 and 1:512,000 showed meat-antigen titres of 1:200 and 1:8,000 respectively. It would so appear that with high-titre antisera more chances are given to discover small amounts of adulteration with a foreign meat which probably would be left undetected by low-level sera. That this could be so was indicated by the results with a meat mixture which contained 10% horse meat only. With a low-level antiserum (1: 4,000) a positive precipitin reaction was obtained only to a 1:10 dilution of the meat extract, whilst with a high-level antiserum (1: 512,000) the endpoint at the same time was 1: 500.

A high serum-anigen titre appears to be particularly important when one desires to eliminate later the lipids for the purpose of storage of antiserum in a freeze-dried state. Removal of the lipids, which according to McFarlane (1942) is a prerequisite step for such storage, is accompanied, as will be discussed in a later paper, by a decrease of sensitivity against meat-proteins, while the potency against serum-proteins remains unaffected.

This work has produced evidence that the precipitin test is of little practical value for detecting cooked horse meat when it has been heated at 80°C. longer than 10 minutes. Adulteration with horse meat is most likely to be expected in processed goods, some of which are treated at boiling temperatures for a lengthy time. A positive reaction would not be expected with such material. Proom (1943) attempted to prepare antisera against cooked meats by inoculating rabbits with meat extracts, prepared from minced muscle heated at 100°C. for an hour, but the rabbit responses were poor, and such weakly reacting sera as were obtained were quite non-specific. No attempts were made by us to use cooked meat as an antigen for immunising rabbits.

Unmelted fats were found to give specific reactions, and apparently could be used successfully as an alternative source of material for species identification when no raw meat is available. The endpoints of precipitin reaction in the case of horse fat were found to be slightly lower than with raw meat. This probably is due to the lower protein content of the fats.

No tests were undertaken with melted fats, but it is very likely that the shortcomings are the same as with heated meats. For melted fats chemical tests are useful, but evaluation of the refractrometric index by the use of Abbe-Butyro refractrometer appears for practical purposes easy enough. A certain overlapping in the index-numbers is likely to occur in the horse-pig group, but horse fat gives the higher figure. According to Nussberger (cited by Ostertag 1913) the refractometric index for horse fat varies from 53·1 to 54·1, while for beef tallow the index is never over 49, and for lard not over 51·9. By the German standard an index of 51·5 is indicative of horse fat. In a short trial the figures for different fats were found by us for horse fat 56, pig 49·5, ox 43·2 and sheep 43·8. The figures were obtained by reading at 40°C. or adjusting the results to this temperature.

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