STUDIES ON SEROLOGICAL DIFFERENTIATION OF HEATED ANIMAL PROTEINS.

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SUMMARY.

The technique of preparing antisera against heat-alkali-treated serum-antigens is described together with the elimination of cross-reacting "heat"-antibodies from such antisera.

The prepared antisera showed a rather low potency against normal sera but possessed a high sensitivity against serum-antigens heated from 70° to 100°C.

The absorbed (specific) antisera, however, were less satisfactory for differentiation of NaOH-extracted "insoluble" (boiled and powdered) serum-proteins than the unabsorbed (non-specific) antisera, and both failed completely to give precipitin reactions with cooked meat-proteins.

I. INTRODUCTION.

Several attempts have been made in the past to prepare antisera which could be used to identify heated animal proteins, such as horse meat, in processed goods (see below). Precipitating antisera obtained by immunizing rabbits with normal blood serum have been found of little value for identifying cooked meats that have been heated above 80° C. (Fornet and Mueller 1910, Proom 1943, Tammemagi 1954a).

Using heat-treated serum-antigens for immunization, several workers have prepared antisera which reacted either with heated only (Schmidt 1912) or with both heated and normal blood sera (Bruynoghe 1924, Meissner 1926) but not with muscle protein which had been rendered insoluble by heat (Schmidt 1912).

Apparently only Rosenberg (1926) has been able to produce antisera which would react with heat-denatured (''insoluble'') muscle protein. She immunized rabbits with ''heat-alkali'' treated antigen of Schmidt (1912) or ''heat-coagulated''-serum-antigen of Fujiwara (1922).

More recently Proom (1943) used extracts of boiled minced meat for immunization. The antibody response in the rabbit, however, was very poor, and the low titred sera obtained were non-specific.

Since no information could be found that Rosenberg's (1926) results have been reproduced, this paper describes experiments with antisera, produced by the method of Schmidt, as used by Rosenberg, for differentiating heated animal proteins.

II. MATERIAL AND METHODS.

(1) Preparation of Antisera.

(*a*) *Antigen for immunization.*—The method of Schmidt (1912) was used to prepare a "heat-alkali"-precipitogen for immunization of rabbits: 60 ml. normal saline solution was added to 60 ml. of serum and heated 30 minutes at 70°C. in a waterbath. After adding 10 ml. of N/1 NaOH, the solution was heated again 15 minutes at 70°C., by which time the previously viscous and turbid solution became clear and liquid.

(*b*) *Method of immunization.*—Six injections of 10 ml. of this antigen were given intramuscularly at weekly intervals into rabbits. The initially clear antigens obtained from pig and horse serum developed a slight deposit after a week's storage at 4°C. The ox and sheep antigens, however, developed a rather heavy deposit and became strongly opalescent. Before inoculation, the antigens were thoroughly shaken to resuspend this deposit.

Two rabbits were immunized with each of the four antigens. They were bled 2 weeks after the first inoculation, and thereafter before injecting each subsequent dose of antigen.

(*c*) *Technique of absorption.*—To eliminate cross-reacting antibodies from the antisera, the method of Weitz (1952) was used in a titration test to determine the proportion of heterologous heated antigens required to absorb the corresponding "heat"-antibodies. The antigen for the titration test was prepared by diluting normal serum 1/10 with saline solution, and then heating at 70°C. for 30 minutes, this dilution being necessary as at lower dilutions, or when undiluted, the serum became during heating either too gelatinous (dilutions 1/1—1/3), or too turbid (dilution 1/5) to be of any use.

(2) Precipitin Test.

The ring-precipitin test described in an earlier paper (Tammemagi 1954a) was used to determine the specificity and titre of the antisera against normal or heated serum and muscle proteins.

The time limit for reading a positive reaction was 2 hours at room temperature, but when negative after this time the final reading was done after incubating for 5 hours or longer at room temperature.

To determine the titres against heated serum-antigens, the homologous and heterologous normal sera were first diluted with saline to 1/10 and then heated for 30 minutes in a waterbath at various temperatures, viz. 70, 80, 90 and 100°C.

(3) Preparation of Heated (Insoluble) Proteins for Testing.

(*a*) *Heated serum-proteins.*—The method of Schmidt (1912) was used to prepare heated (insoluble in indifferent solvents, i.e. water, saline etc.) serum-proteins for identification tests:

Undiluted blood serum was heated for 3 hours in a boiling waterbath. To remove all traces of soluble protein particles, the coagulum was thoroughly washed with saline, dried overnight at 37°C. in an open petri dish and pulverised. A heaped knife-point-full of the powder was shaken with 10 ml. of N/10 NaOH, and heated for 10 minutes in a waterbath at 70°C. Some of the water-insoluble protein dissolves in the NaOH solution. To minimize the destructive effect of the warm NaOH, the solution was then poured into a flask containing 10 ml. of cooled saline solution. After filtering through paper, it was neutralized with N/20 HCl.

In Schmidt's original technique the neutralization point was a slight pinkish coloration of the solution with phenolphthalein as indicator. In these experiments, however, pH indicator-paper-slips were used to reach a slight alkaline reaction (about pH 7·5). Over-neutralization with HCl to neutral or acid reaction will cause a sudden turbidity. Addition of NaOH reverses this, and brings the proteins back into solution. According to Schmidt (1912) this manipulation does not interfere with the ultimate results.

(*b*) *Heated meat-proteins.*—Finely cut raw meat samples from various animal species were placed in screw-capped jars and heated in a boiling water-bath for from 30 minutes up to several hours. The juice that appeared during the boiling was collected and tested separately.

Portion of the meat residue was extracted with an equal weight of saline for one to five days, and the extracts, after being clarified through Whatman No. 1 filter papers or Zeiss coarse filter pads, examined serologically.

Another portion of the meat residue was treated in the same way as serum—i.e., dried at 37° C., powdered, extracted with NaOH and neutralized with HCl. However, treatment with NaOH produced very turbid filtrates, apparently due to fats being taken into solution by NaOH. Fat was eliminated eventually from the heated meat with chloroform which was changed several times during the day, and when necessary left to act over-night until no greasy flecks were left on filter paper.

III. RESULTS.

(1) Antibody Response to "Heat-alkali"-antigen.

(*a*) *Antibody titres against normal sera.*—From Table 1 it is evident that antisera produced by the use of "heat-alkali"-antigens gave positive reactions with normal sera, but the titres were surprisingly low compared with earlier results (Tammemagi 1954a) when normal or alum-precipitated antigens were used for immunization.

(*b*) *Antibody titres against heated sera.*—Compared with results obtained against normal sera (Table 1), the "heat-alkali"-antisera showed much higher titres against heated sera (Table 2). In some instances the highest homologous

Table 1.

PRECIPITIN TITRES OF ANTISERA, PREPARED BY INOCULATION OF RABBITS WITH SIX INTRAMUSCULAR INJECTIONS OF "HEAT-ALKALI"-TREATED ANTIGENS, WHEN TESTED WITH HOMOLOGOUS AND HETEROLOGOUS NORMAL SERA.

Antiserum.	Titres *Against Normal Sera of —			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse	**100**	5	N	20
Anti-ox	N	**2,000**	200	20
Anti-sheep	10	500	**500**	50
Anti-pig	N	500	100	**500**

* Titres are expressed as reciprocals of dilution. Homologous titres are shown in black.

N indicates that a positive reaction appeared with undiluted ("Neat") normal serum only.

Table 2.

PRECIPITIN TITRES OF ANTISERA AGAINST HOMOLOGOUS AND HETEROLOGOUS SERA HEATED AT VARIOUS TEMPERATURES.

Heated Antigen.	Titres* of Antisera.			
	Anti-horse.	Anti-ox.	Anti-sheep.	Anti-pig.
Horse serum heated at 70°C.	**32,000**	8,000	8,000	16,000
Horse serum heated at 80°C.	**64,000**	8,000	4,000	32,000
Horse serum heated at 90°C.	**126,000**	8,000	4,000	—(4,000)
Horse serum heated at 100°C.	**32,000**	8,000	4,000	—(2,000)
Ox serum heated at 70°C.	8,000	**16,000**	4,000	16,000
Ox serum heated at 80°C.	8,000	**16,000**	4,000	16,000
Ox serum heated at 90°C.	16,000	**16,000**	8,000	?
Ox serum heated at 100°C.	16,000	8,000	4,000	?
Sheep serum heated at 70°C.	16,000	8,000	**32,000**	8,000
Sheep serum heated at 80°C.	16,000	8,000	**16,000**	16,000
Sheep serum heated at 90°C.	16,000	16,000	**16,000**	16,000
Sheep serum heated at 100°C.	16,000	8,000	**8,000**	4,000
Pig serum heated at 70°C.	16,000	4,000	4,000	**32,000**
Pig serum heated at 80°C.	16,000	4,000	4,000	**64,000**
Pig serum heated at 90°C.	16,000	4,000	—(4,000)	**32,000**
Pig serum heated at 100°C.	16,000	4,000	—(2,000)	**8,000**

* Titres are expressed as reciprocals of dilution. Homologous titres are shown in black.

? No determination possible because of interfering turbidity of the heated antigen dilution.

— No reaction after 2 hours of incubation at room temperature.

Figures in brackets indicate results after at least 4 hours of incubation.

or heterologous titres appeared with sera heated at 90°C., but usually the "heat"-antibody titres were about the same regardless of whether the antigens tested were heated at 70°, 80° or 90°C. Heating at 100°C, however, was generally followed by a decrease in the antibody reaction, and in some instances a ring-precipitation could be observed several hours after the usual time limit of 2 hours.

Increasing the temperature was usually followed by a progressively stronger opacity of the antigen solution. The reading of an homologous reaction was not greatly affected, as a ring-precipitation was distinct from the highest dilution (whatever the titre) down to a concentration of at least 1:100, or even to 1:10 of heated antigen. In heterologous reactions, however, where the ring formation was always weaker, the opacity of the antigen (especially when heated at 90° or 100°C.) frequently prevented detection of a positive reaction at a dilution lower than 1:1,000.

The ox serum when heated produced least opacity, followed in increasing order by horse, sheep and pig serum, where the turbidity was the greatest at any temperature of heating. Centrifugation or filtration through Whatman No. 1 filter paper did not clarify the turbid antigen (1/10) solutions. Filtration through Seitz bacteria-retaining filter pads produced perfectly clear filtrates, but apparently the antigen proteins were also removed from the filtrates as no precipitation reactions followed even in homologous reaction which was strongly positive prior to filtration.

(2) Absorption of Non-specific Antibodies.

From Table 2 it is seen that all four "heat-alkali"-antisera were non-specific, and except for anti-horse and anti-pig sera, there was no essential difference between the homologous and heterologous titres.

In attempts to obtain specific antisera against heated serum-antigens, a preliminary titration was done to determine whether heated heterologous antigens would absorb the corresponding "heat-alkali-precipitins" from the antiserum. For this purpose horse serum, diluted 1/10 and heated for 30 minutes at 70°C., was tested against horse antibodies in the anti-ox serum. The presence of any "normal" precipitins—that is, antibodies reacting only with normal, unheated serum-antigens—was regarded as unimportant.

Using Weitz (1952) technique for the titrations, it was observed that at the antigen (1:10)/antiserum ratio of 1/20 all horse antibodies ("Heat-alkali"-precipitins) apparently were removed as indicated by a negative precipitin reaction of the supernatant fluid in the corresponding tube when tested with 1:100 dilution of heated horse serum. Antigen solution 1:10 was unsatisfactory for testing because of a strong interfering turbidity at this dilution. The supernatant fluid still gave with sheep and pig antigens (heated) positive reactions even up to a dilution of 1:10,000 of these antigens, indicating that sheep and pig "heat-alkali-precipitins" were still present in the anti-ox serum after the absorption of the horse antibodies.

Since the heterologous antibody titres of all four antisera were about the same, it was considered that when increasing the ratio of the heterologous antigens in actual absorption from 1/20 to 1/10, it would be possible to absorb all cross-reacting antibodes (''heat-alkali-precipitins'') regardless of their titre.

In a first trial, simultaneous absorption with three different heterologous antigens caused the homologous antibodies to disappear. In further attempts, therefore, the absorption tests were done first with one single heterologous antigen, and if insufficient, two or more heterologous antigens were used simultaneously. The antigens in all these tests were heated at 70°C. (''70-degree group'').

Table 3.

RESULTS OF ABSORPTION OF ANTISERA WITH 1/10 VOLUME OF HETEROLOGOUS ANTIGENS, HEATED AT 70°C., WHEN TESTED AGAINST SERUM-ANTIGENS, HEATED AT EITHER 70°C. OR 80°C., AT DILUTIONS FROM 1/50 TO 1/1,000.

Antiserum.	Antigen.							
	Horse.		Ox.		Sheep.		Pig.	
	70°	80°	70°	80°	70°	80°	70°	80°
Anti-horse absorbed with Ox antigen	+	+	−	−	−	−	+	+
Anti-horse absorbed with Sheep antigen	+	+	−	−	−	−	+	+
Anti-horse absorbed with Pig antigen	+	+	+	+	−	−	−	−
Anti-horse absorbed with Ox and Sheep antigen	+	+	−	−	−	−	+	+
Anti-horse absorbed with Ox and Pig antigen	+	+	−	−	−	−	−	−
Anti-horse absorbed with Ox, Pig and Sheep antigen	+	+	−	−	−	−	−	−
Anti-ox absorbed with Horse antigen	−	−	+	+	+	+	+	+
Anti-ox absorbed with Sheep antigen	−	−	+	+	−	−	+	+
Anti-ox absorbed with Pig antigen	−	−	+	+	−	+	−	−
Anti-ox absorbed with Horse and Pig	−	−	+	+	−	+	−	−
Anti-ox absorbed with Sheep and Pig antigen	−	−	+	+	−	−	−	−
Anti-ox absorbed with Sheep, Pig and Horse antigen	−	O	−	O	−	O	−	O
Anti-sheep absorbed with Horse antigen	−	−	+	+	+	+	+	+
Anti-sheep absorbed with Pig antigen	+	+	−	−	+	+	+	+
Anti-sheep absorbed with Ox antigen	−	−	−	−	+	+	−	+
Anti-sheep absorbed with Horse and Pig antigen	−	−	−	−	+	+	−	+
Anti-sheep absorbed with Ox and Pig antigen	−	−	−	−	+	+	−	+
Anti-sheep absorbed with Horse, Ox and Pig antigen	−	O	−	O	+	O	−	O
Anti-pig absorbed with Horse antigen	−	+	−	+	−	−	+	+
Anti-pig absorbed with Ox antigen	+	+	−	−	−	−	+	+
Anti-pig absorbed with Sheep antigen	+	+	−	−	−	−	+	+
Anti-pig absorbed with Horse and Ox antigen	−	+	−	−	−	−	+	+
Anti-pig absorbed with Horse, Ox and Sheep antigen	−	+	−	−	−	−	+	+

\+ Positive reaction.
− Negative reaction.
O Not tested

The experiments (Table 3) showed that using any of the heated heterologous antigen alone at the ratio of 1/10, the corresponding antibody was absorbed from the antiserum, but in several instances it caused also the simultaneous disappearance of some other antibodies. Generally, however, it was necessary to use two antigens simultaneously to remove from each antiserum the three heterologous antibodies concerned. The use of three different antigens simultaneously caused the disappearance of the homologous antibodies from the anti-ox, but not from the other antisera.

In several instances, however, it was observed that antisera which apparently had become "specific" against antigens heated at 70°C. still gave cross reactions with one or other heterologous antigens heated at 80°C. To remove those antibodies which might react with antigens heated at this or higher temperature, absorption was repeated with antigens heated at 80°C. After this the antisera apparently had become truly specific, as they no longer reacted with any of the heterologous antigens heated either at 70, 80, 90 or 100°C. but would give definite homologous reactions regardless of the temperature applied to the antigen.

In the course of the experiments the antisera were rendered specific by selecting for absorption the following combinations of the heterologous antigens of the 80°C. type at the ratio of 1/10 to the antiserum:

anti-Horse serum by absorbing with Ox and Pig antigens;

anti-Ox serum by absorbing with Sheep and Pig antigens;

anti-Sheep serum by absorbing with Ox and Pig antigens;

anti-Pig serum by absorbing with Ox and Horse antigens.

After absorption the antisera were tested again with normal sera. In Table 4 it is shown that the antisera still gave cross reactions with heterologous "normal"-antigens, but a discrepancy is evident when comparing the results prior to absorption in Table 1.

Table 4.

PRECIPITIN TITRES OF ANTISERA AGAINST HOMOLOGOUS AND HETEROLOGOUS NORMAL SERA WHEN TESTED AFTER THE ABSORPTION WITH HEATED ANTIGENS.

Antiserum.	Titres* Against Normal Sera of—			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse	**100**	—	—	20
Anti-ox	N	N	N	N
Anti-sheep	N	100	**100**	50
Anti-pig	N	—	—	**500**

* Titres are expressed as reciprocals of dilution.

— Negative reaction, even with undiluted serum-antigen.

N indicates that a positive reaction appeared with undiluted ("Neat") normal serum only.

(3) Insoluble (Cooked) Serum-proteins.

Results of experiments with insoluble, pulverized serum coagulum of Schmidt, when extracted with NaOH and tested both with absorbed (specific) and unabsorbed (non-specific) antisera, are summarized in Table 5.

Table 5.

PRECIPITIN REACTIONS OF ABSORBED AND UNABSORBED "HEAT-ALKALI"-ANTISERA AGAINST NaOH-EXTRACTED INSOLUBLE (POWDERED) SERUM-PROTEINS.

Antiserum.	NaOH-extracted Serum-proteins of—			
	Horse.	Ox.	Sheep.	Pig.
Anti-horse, absorbed ..	+	—	—	—
Anti-horse, unabsorbed	+	—	—	—
Anti-ox, absorbed ..	—	—	—	—
Anti-ox, unabsorbed ..	+	+	+	—
Anti-sheep, absorbed ..	—	—	—	—
Anti-sheep, unabsorbed	—	—	+	—
Anti-pig, absorbed ..	—	—	—	+
Anti-pig, unabsorbed ..	—	—	—	+

Better results were obtained with unabsorbed than with absorbed antisera. All four unabsorbed antisera gave definite reactions with homologous antigens, but one of them, the anti-ox serum, also with horse and sheep antigens. Of the specific (absorbed) antisera only two, the anti-horse and anti-pig sera, gave homologous reactions, while the other two failed to do so.

(4) Meat Proteins.

In experiments with different meats, all the absorbed (specific) "heat-alkali"-immunsera failed to give homologous reactions with extracts prepared from minced raw or cooked meats even when the time of extraction was extended to 5 days. Similarly, negative results were obtained with juice that appeared during heating of meat samples in a boiling waterbath. Extracts, prepared by boiling minced raw meats with equal weights of saline for 1-3 hours failed also to show positive precipitin reactions.

Of the unabsorbed antisera, the anti-pig serum alone gave a positive precipitin reaction, and this only with meat juice, obtained by boiling pig meat in a waterbath for several hours.

Experiments with insoluble powdered cooked meats, when brought into solution by NaOH, all gave negative homologous reactions with both the absorbed and unabsorbed antisera.

IV. DISCUSSION.

The results of these experiments support the findings of Schmidt (1912) and Rosenberg (1926) that by using ''heat-alkali''-antigens for immunizing rabbits, antisera can be prepared which are suitable for differentiation of blood sera which have been heated from 70° to 100°C.

Treatment of the antigen with NaOH for inoculation was introduced by Schmidt (1912) in order to obtain antisera which would react also with heated, insoluble proteins when the latter, for the purpose of precipitin test, are brought into solution by the use of NaOH. If rabbits are inoculated with ''heat-precipitogen'' (not alkali treated) only, the anti-sera (''Heat-precipitin'') obtained would react with heated sera only so long as the serum-protein remains in the solution, or is soluble in indifferent solvents such as saline. When the protein has become insoluble after prolonged heating, a precipitin test is possible only when the protein is brought back into solution by a strong alkaline solution. By the action of alkaline, however, the protein undergoes such drastic changes that it would not react any more with the ''heat precipitin.'' By immunizing rabbits with an ''heat-alkali''-antigen, however, antisera are obtained which are capable of reacting strongly with such insoluble, NaOH-treated serum-proteins.

A prolonged immunization course is required, as after the first 3-5 injections of this antigen, the rabbit serum usually will react only with heated and not with heat-alkali-denatured serum-proteins, and acquires the latter property after further injections. The antisera described in this paper were the product of 6 injections, and were able to react both with heated only and with heat-alkali-denatured serum-proteins.

Our observations agree with those of Rosenberg (1926) that heat-alkali antisera are able to precipitate normal blood sera, but their titres against this antigen was in our experiments very much lower than against heated sera. Incidentally, Schmidt's (1912) heat-alkali-antisera failed to react with normal sera at all.

All the antisera produced in this work were non-specific and gave cross reactions with heterologous normal and heated serum-antigens. This evidently was the result of the multiple inoculation technique. However, it is possible to restore specificity by absorbing the cross-reacting ''heat''-antibodies with heated antigens. The antigen for this purpose must be heated at least at 80°C. to absorb the corresponding heterologous ''heat''-antibodies. There is evidence that an antiserum may contain a complex of various ''heat''-antibodies. The absorption, for example, of antibodies which react only with antigens heated at 70°C. does not eliminate those antibodies which might react with antigens heated at higher temperature. Only absorption with an antigen of the 80-degree type eliminates all ''heat''-antibodies which might react with antigens of the

70-100°C. range. Absorption with heated antigens, however, does not remove the "normal" antibodies though some changes in the normal antibody titres are expected after the absorption of "heat"-antibodies.

Absorption experiments have shown also that heated heterologous antigens can remove, in addition to the corresponding antibody, some other heterologous antibodies, and exceptionally also the homologous antibodies when three different antigens are used for absorption simultaneously. Normally, however, two heterologous antigens are required in a certain combination to absorb from each antiserum all the heterologous antibodies.

The phenomenon of disappearance during absorption of corresponding and some related heterologous normal-antibodies is lengthily discussed by Weitz (1952) on the basis of qualitative and quantitative differences in the antigenic components. The disappearance of unrelated heterologous "heat"-antibodies, however, by absorption with heated antigens might indicate that heat to a certain extent destroys or alters the species character of individual antigens, as a consequence of which an antigen is able to absorb other species antibodies.

Though absorption of cross-reacting "heat"-antibodies is certainly possible, it unfortunately renders the antiserum largely valueless against NaOH-extracted insoluble serum-proteins as only two of the four antisera retained their capacity for homologous reaction. Unabsorbed antisera appear more satisfactory in this respect, but the likelihood of cross-reactions has to be considered, though three of the four unabsorbed (non-specific) antisera surprisingly acted quite specifically.

The findings of Rosenberg (1926) that heat-alkali-antisera are suitable for differentiating cooked meats could not be sustained, since none of our antisera, absorbed or not, gave even a slight precipitin reaction with saline-extracted raw or cooked meat or with NaOH-extracted insoluble (powdered) meat-proteins. Even boiling the meat up to 3 hours, which, according to Rosenberg (1926) enhances the release of insoluble protein into the solution and consequently also the success of a precipitin reaction, failed completely. This failure of our antisera to give reactions with meats is not readily explainable, since some of our antisera seemed to have even higher "heat"-antibody titres than Rosenberg's sera. It is possible that perhaps the blood content in the meat samples (Tammemagi 1954b) has some bearing on these discrepant results. Another explanation might be that our neutralization of the NaOH-extracted protein to pH 7·5 with HCl was not the same degree as Rosenberg's, where phenolphthalein was used as an indicator. Underneutralization might leave the protein solution too alkaline and may prevent precipitation, and so lead to false results. However, ring-precipitation appear quite distinctly in our experiments with NaOH-extracted serum-protein solutions which were also neutralized to pH 7·5.

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