RESEARCHES ON THE DETECTION OF MEAT BY SEROLOGICAL TEST

By

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Introduction

The serological precipitin test has been previously known as the method to detect substitution of one type of meat for another, usually, though not always, a cheaper meat for a more expensive one.

It is based on the fact that antibodies appear in the blood serum of the animals (especially at the part of globulin[5),16)]) which receive either intraperitoneal[6)], intravenous[25)] or intramuscular[11),28)] injections of a foreign protein such as blood serum of another animal or an extract of the tissues of another animal.

A grayish-white precipitate appears only when the prepared rabbit blood serum is stratified with the substance with which the rabbit has been injected.

This method has been widely used in countries where a large amount of sausages are eaten and flesh of certain animals, such as the horse, is used for food.

It is desireble in some cases to know whether ingredients contain beef or horse meat.

In our country, horse meat is widely used either in fresh style, in various meat products, or in canned meat, being mixed with another animal meat or single one. This has been the case among us, not recognized by the consumers, and so, there is need to establish the method to detect the meat of certain animals.

The purpose of this report is to search the limitation of the possibility of precipitin test in which its specificity depends on various conditions of antigen used.

The portion of soluble protein in meat used for smoked meat

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[Journ. Facul. Agr., Hokkaido Univ., Sapporo, Vol. 50, Pt. 3, 1957]

products or canned meat products are usually coagulated by heat.

In other words, it is denatured or partially denatured by heat and comes to be insoluble in water or in dilute salt solution.

Then, in the precipitin test the loss of the species specificity and the masking-effect or prevention according to heat denaturation must be considered.

To detect one animal meat from another when submitted to heat, several works have been reported till now, for instance, of qualitative or quantitative estimations of linolenic acid by using T. B. A. reagent[9] or by spectrophotometric determination[7] in ultraviolet region: and these estimations especially applicable to the detection of horse meat products, since linolenic acid content in horse meat shows much higher value than in others.

In the serological methods, a method by means of the hemolysis test using FORSSMAN's antigen[19),23),24)]— this is not considered as protein but as heat stable substance like lipoid—has been reported to be adoptable in the detection of horse meat from beef in the canned meat products called "yamatoni" in Japan.

As to the precipitin test, several efforts[21),26),28)] have been made for its application to the meat products but their results seem to have been wanting in definiteness.

In this experiment, serological methods for the detection of meat have been studied in relation to these points described above and the changes which take place then have been followed by regular and full analyses.

Experimental

Antisera

Antisera for the precipitin test were prepared by intravenous or intramuscular injection against rabbits (white landrace), making at intervals of 3–4 days for the former and of 4 days for the latter respectively.

The dose for the former was in the order of 0.5 cc. for the first; 1.0 cc. for the second; 2.0 cc. for the third;—, and this was repeated until the fifth or sixth was done. As for the latter, the total doses were about 30 cc. and every 10 cc., mixed with alumina cream[27),28)], was injected. In 10 days after the latest dose was given, the whole blood was drawn out by bleeding and was allowed to run into a sterile beaker.

After a clot had formed, the serum was separated from erythrocytes by centrifugalization, and devided into sterile small testtubes and thus was stored either in refrigerator (0-4°C) adding 10% of NaCl, or frozen at −20°C.

Antisera for the hemolysis test were prepared by injecting rabbits with that tissue emulsion of guinea-pig liver or kidney as the FORSSMAN's antigen, which had been prepared by OGATA's method.

These injections were made intraperitoneally and the dose of 10 or 20 cc. of antigen was once injected in an attempt to avoid the appearance of WASSERMANN's ambocepter.

These antisera thus prepared were inactivated at 56°C for 30 minutes before use.

Precipitin test

This was made according to the stratifying method given by OGATA[23].

Hemolysis test by FORSSMAN's heterophile antigen (F-antigen)

Using 3% suspension of red blood cells of the sheep and mixed guinea-pig serum (from 5 individuals) as the source of complement, we made this test following the method described by SHIGA *et al*[24].

Antigen

As antigens for the precipitin test, were used fresh blood sera of horse and dog, saline extract of dog meat, cooked horse blood serum, fiuid of canned horse meat, myoglobin (horse) and alkali solution of boiled horse meat solid.

Blood sera were prepared by the same method as described in the case of antisera.

The meat extract was prepared by adding equal volume of physiological NaCl solution to the ground fresh meat and by seperating clear supernatant fluid by centrifugalization after standing over night in the refrigerator.

Filtration was followed to obtain more clear solution.

Preparation of cooked blood serum was made by heating diluted fresh serum (1:2 or 1:3 dilution with distilled water) directly on the flame of gas-burner, boiling several times with continuous shaking.

It decreases its transparency but no precipitate appears.

Fluid of canned meat was the released liquid product from fresh meat which had been canned and sterilized at 110°C for 60 minutes.

Myoglobin was isolated from horse heart by means of the method reported by YASUI[30] and was served as an antigen following UENO's description[27].

To prepare the solution of boiled meat solid, the powder of boiled meat (dried) was dissolved in 1N NaOH solution and pH of the solution was adjusted at 7.8 by the addition of 1N HCl.

Nitrogen determination

KJELDAHL's method was adopted for nitrogen determination.

Electrophoresis

Electrophoretic analyses on various antisera and antigens were made. As an apparatus, the HITACHI TISELIUS elctrophoretic apparatus was used, adopting a microcell of $2 \times 15 \times 50$ mm.

Veronal buffer, pH 8.4 and ionic strength, 0.1 or 0.2 was used for these analyses.

In searching the denaturation of animal meat protein by heat, the determination of free aminoacid by means of paper partition chromatography was made accomanying with the analysis above mentioned.

Technique of this determination was described in the report of HASHIMOTO *et al*[12].

RESULTS

I. Precipitin test

Effects upon antisera by the method of injection

The intravenous injection has been well-known to obtain antisera but it requires great deal of skill in operators and in some cases, invites death in animals.

The intramuscular injection by mixing antigen with alumina cream has recently been reported to strengthen the reaction of antisera to much more degree if prepared than otherwise, and to keep animal in safety.

Our results of this problem have shown that the intramuscular method is superior to the other in safty of animal lives and in precipitin titers of antisera (Table 1).

Protein concentration of antigens is 7–8g/dl. for fresh sera, 2.5–3.8g/dl. for cooked sera and about 3g/dl. for a saline extract of meat.

TABLE 1.

(A) Antisera by intraveneous injection.

No.	Total dose cc.	Antigen used	Dilution of antigens (25×2^n)									
			$n=0$	1	2	3	4	5	6	7	8	9
1	—	horse-blood-serum	++++	++++	++++	+++	++	—	—	—	—	—
2	18	horse-blood-serum	++++	++++	+++	+++	++	++	++	++	—	—
3	—	cooked horse-blood-serum	++++	+++	+++	++	++	—	—	—	—	—
4	30	cooked horse-blood-serum	++++	++++	++++	+++	+++	++	++	++	++	++
5	6.6	cooked horse-blood-serum	++++	++++	+++	+++	++	++	++	++	++	±
6*	3.5	salin extract of horse meat	These animals injected by saline extract of horse meat failed to survive for a few minutes after 1st injection									
7*	2.0	salin extract of horse meat										

(B) Antisera by intramuscular injection.

No.	Total dose cc.	Antigen used	Dilution of antigens (25×2^n)									
			$n=0$	1	2	3	4	5	6	7	8	9
1*	30	cooked horse-blood-serum	++++	++++	+++	++	++	++	++	++	++	±
2⊙	30	cooked horse-blood-serum	+++	+++	+++	+++	++	++	++	++	++	++
3*	30	horse-blood-serum	+++	+++	+++	++	++	++	++	++	±	—
4⊙	30	horse-blood-serum	+++	+++	+++	+++	++	++	++	++	++	++
5⊙	24	saline-extract of dog meat	+++	+++	+++	++	++	++	—	—	—	—
6⊙	25	dog-blood-serum	++++	+++	+++	++	++	++	++	++	++	±

++++ +++ ++ ++ ± decreasing amount of ring formation from complete to partial.
— no ring formation.
* Volume ratio, antigen : alumina cream, 1 : 4.
⊙ Volume ratio, antigen : alumina cream, 1 : 1.

Detection of fresh meat by means of precipitin test

Using immunized serum by blood serum, saline extract of meat and myoglobin, precipitin tests are examined to distinguish each species of different fresh meat.

The precipitin reaction with original antigen is shown in Table 2 and the reaction with meat extracts is shown in Table 3.

With regard to the titer, in these groups of antisera, the anti-myoglobin-serum is the most effective one, the anti-meat extract-serum the next and the anti-serum-serum the lowest.

On the other hand, each group reaction with beef extract demonstrates that the anti-myoglobin-serum and the anti-serum-serum are

TABLE 2. The precipitin reaction with original antigens.

Dilution of antisera (2^n)	Dilution of antigens (25×2^n) $n=0$	1	2	3	4	5	6	7	8	9		
$n=0$	++	++	++	++	++	+	+	+	+	+	A	As an antigen horse-blood-serum has been used
1	++	++	++	++	++	+	+	+	+	±		
2	++	++	++	+	+	+	+	±	±	−		
3	++	+	+	+	+	+	±	±	±	−		
4	+	+	+	±	−	−	−	−	−	−		
5	+	−	−	−	−	−	−	−	−	−		
$n=0$	++	++	++	++	+	+	+	+	+	±	B	As an antigen dog-blood-serum has been used
1	++	++	++	++	+	+	+	+	+	±		
2	++	++	+	+	+	+	+	+	+	−		
3	++	++	+	+	+	+	+	±	−	−		
4	++	+	+	+	+	+	±	±	−	−		
5	+	+	+	+	+	±	±	−	−	−		
$n=0$	++	++	++	++	+	+	+	−	−	−	C	As an antigen dog-meat-extract has been used
1	++	+	+	+	+	+	±	−	−	−		
2	+	+	+	+	+	+	−	−	−	−		
3	+	+	+	+	+	±	−	−	−	−		
4	+	+	+	+	+	−	−	−	−	−		
5	+	+	+	+	±	−	−	−	−	−		
$n=0$	++	+	+	+	+	+	+	+	+	+	D*	As an antigen horse-myoglobin has been used
1	++	+	+	+	+	+	+	+	+	±		
2	+	+	+	+	+	+	+	+	±	±		
3	+	+	+	+	+	+	+	±	±	±		
4	−	−	−	−	−	−	−	−	−	−		

* Protein concentration of myoglobin, 0.5~1.0 g/dl.

TABLE 3. The precipitin reaction of antisera A and D (in Table 2) with the saline extract of horse meat and of antisera B (in Table 2) with the saline extract* of dog meat.

Dilution of antisera (2^n)			Dilution of meat extract (25×2^n)						
			$n=0$	1	2	3	4	5	6
	A	$n=0$	+	+	+	−	−	−	−
		1	+	+	±	−	−	−	−
		2	+	+	±	−	−	−	−
		3	+	±	−	−	−	−	−
		4	−	−	−	−	−	−	−
	B	$n=0$	+	+	+	+	−	−	−
		1	+	+	+	+	−	−	−
		2	+	+	+	+	−	−	−
		3	+	+	±	±	−	−	−
		4	−	−	−	−	−	−	−
	D	$n=0$	+	+	+	+	+	+	+
		1	+	+	+	+	+	±	±
		2	+	+	+	+	±	±	±
		3	+	+	+	±	±	±	±
		4	+	+	+	+	±	±	±

* The extraction has been made by adding an equal volume of saline to meat.

TABLE 4. The group reaction of antisera A, B, C and D (in Table 2) with the saline extract* of beef.

Dilution of antisera (2^n)			Dliution of beef extract (25×2^n)							Dilution of beef extract (25×2^n)				
			$n=0$	1	2	3	4			$n=0$	1	2	3	4
	A	$n=0$	−	−	−	−	−	C	$n=0$	+	+	±	±	−
		1	−	−	−	−	−		1	+	+	±	±	−
		2	−	−	−	−	−		2	+	+	±	−	−
	B	$n=0$	±	−	−	−	−	D	$n=0$	±	−	−	−	−
		1	±	−	−	−	−		1	−	−	−	−	−
		2	±	−	−	−	−		2	−	−	−	−	−

* The extraction has been made by adding an equal volume of saline to beef.

TABLE 5. The precipitin reaction with original antigens.

Dilution of antisera (2^n)	Dilution of antigens (25×2^n) n=0	1	2	3	4	5	6	7	8	9		
n=0	++	++	+	+	+	+	+	+	+	+	A′	As an antigen cooked horse-blood-serum has been used
1	++	++	+	+	+	+	+	+	+	+		
2	++	+	+	+	+	+	+	±	±	+		
3	++	+	+	+	+	+	±	±	±	±		
4	+	+	±	±	±	−	−	−	−	−		
5	+	+	+	±	±	−	−	−	−	−		
6	+	+	±	−	−	−	−	−	−	−		
n=0	++	+	+	+	±	±	−	−	−	−	B′△	As an antigen fluid of canned horse meat has been used.
1	+	+	+	+	±	±	−	−	−	−		
2	+	+	±	±	−	−	−	−	−	−		
3	+	+	±	−	−	−	−	−	−	−		
4	+	±	±	−	−	−	−	−	−	−		
n=0	++	++	++	++	+	+	+	±	−	−	C′*	As an antigen alkali solution of boiled horse meat solid has been used
1	++	++	+	+	+	+	+	±	−	−		
2	+	+	+	+	±	±	±	−	−	−		
3	+	+	+	+	±	±	±	−	−	−		
4	+	+	+	±	±	±	−	−	−	−		
5	−	−	−	−	−	−	−	−	−	−		

* Protein concentration of alkali solution of boiled horse meat solid, 15.3 g/dl.
△ Protein concentration of fluid of canned horse meat, 5.0 g/dl.

TABLE 6. The precipitin reaction of antiserum A′ (in Table 5).

Dilution of antiserum (2^n)	Dilution of meat extract (25×2^n) n=0	1	2	3	4	5	6	
n=0	++	++	+	+	+	+	±	Reaction with saline extract of horse meat (1:1)
1	+	+	+	±	±	−	−	
2	+	+	±	±	±	−	−	
3	+	±	±	−	−	−	−	
4	±	±	±	−	−	−	−	
n=0	+	+	+	±	±	−	−	Reaction with saline extract of beef (1:1)
1	+	±	−	−	−	−	−	
2	−	−	−	−	−	−	−	
3	−	−	−	−	−	−	−	
4	−	−	−	−	−	−	−	

TABLE 7. The precipitin reaction of antiserum B′ (in Table 5).

Reaction with	Dilution (25×2^n)						
	$n=0$	1	2	3	4	5	6
horse blood-serum	−	−	−	−	−	−	−
cooked horse blood-serum	±	±	−	−	−	−	−
fluid of canned beef	+	+	±	±	±	−	−

TABLE 8. (1) The precipitin reaction of antiserum C′ (in Table 5) with alkali solution of boiled beef solid.

Dilution of antiserum (2^n)	Dilution of alkali solution of boiled beef solid (25×2^n)								
	$n=0$	1	2	3	4	5	6	7	8
$n=0$	++	++	+	+	+	+	±	−	−
1	++	+	+	+	+	±	±	−	−
2	+	+	+	+	+	±	−	−	−
3	+	+	+	+	±	−	−	−	−
4	−	−	−	−	−	−	−	−	−

(2) The precipitin reaction of antiserum C′ (in Table 5) with alkali solution of canned meat*.

antigenic substance	Alkali sol. of canned horse meat								Alkali sol. of canned beef							
dilution of antigen (25×2^n)	$n=0$	1	2	3	4	5	6	7	$n=0$	1	2	3	4	5	6	7
$n=0$	+	+	+	+	+	±	−	−	+	+	+	+	+	±	−	−
1	+	±	±	±	±	−	−	−	+	+	+	+	±	−	−	−
2	+	+	+	+	+	±	−	−	+	+	+	+	±	±	−	−
3	±	+	+	+	+	±	−	−	+	+	+	+	+	+	±	−
4	+	+	+	+	+	±	−	−	±	+	+	+	+	±	±	−
5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Dilution of antiserum (2^n)

* This has been prepared in our laboratory by adding Japanese sauce called "shōyu", sugar and a small amount of spices to meat.
Cooking temperature and cooking period was at 110°C and for 60 minutes.
Ingredients of this canned product have heen thoroughly washed by water and ground through chopper, and after boiling 3 times with water, they have been dried at 85°C and served as the antigenic substance through the same process for the alkali solution of boiled meat solid.
The precipitin reaction with saline extract of fresh meat by this antiserum has shown negative result.

far more effective than the antiserum immunized by meat extract (Table 4).

These results show that the antiserum immunized by saline extract of dog meat indicates, to a considerable degree, group reaction, while the antiserum immunized by dog blood-serum proves to be possessed of far less group reaction than the anti-meat extract-serum, although its group reaction is considerably greater than that of anti-horse serum-serum.

Detection of cooked meat by means of precipitin test.

We have prepared antisera against cooked blood serum, fluid of canned horsmeat and alkali solution of boiled horse meat solid.

Titer and proup reaction are shown in the following tables (Table 5–8).

Antisera A′ and C′ show their state specificity (Table 5) but not species specificity (Table 7, 8).

As for the antiserum A′, it possesses high species specificity as well as state specificity, while its group reaction increases slightly (Table 5, 6).

Then we have made some experiments using this antiserum and the other two antisera (A and D in Table 2) in order to investigate the limitation of the biological precipitin test and the applicability to cooked meat. The test method employed by WEINSTOCK[28] has been used partially modified by us to perform a series of tests in which 50 g. of small cut lump of horse meat (about $2.5 \times 2.0 \times 1.5$ cm) is immersed in water baths of constant temperature for 30 minutes. The samples are then taken out, ground, extracted with saline, centrifuged and filtered. As shown in Table 9., meat cooked at 70°C gives a negative precipitin ring to the antisera A, giving, however, a possitive one to the antiser D and A′.

Samples heated above 80°C give complete negative results. Results thus obtained point out that the serological precipitin test is adoptable to the detection of horse meat adulteration under 70°C in heating. Precooked meat products such as minced meat and sausages are genereally processed by immersion in water bath at bout 70°C but the temperature of the center of products hardly attains to 70°C.

From these facts, although meat products processed mildly can be detectable by the precipitin test, products prepared by severe heat process such as canned products, can not be detected by this method.

TABLE 9. The effect of heating in water bath.

Samples	Horse meat			Cured horse meat			Beef		
temp.	antisera								
	A	D	A′	A	D	A′	A	D	A′
Fresh meat	++	++	++	+	+	+	−	−	−
50°C	++	++	++	+	+	+	−	−	−
60°C	+	++	++	+	±	±	−	−	−
70°C	+	−	±	±	−	−	−	−	−
80°C	−	−	−	−	−	−	−	−	−
Boiling point	−	−	−	−	−	−	−	−	−
*110°C	−	−	−	−	−	−	−	−	−

Antisera A, D and A′ (shown in Table 2 and Table 5) have been diluted 1:2 for A and D, 1:4 for A′ to avoid the group reaction.

* Samples have been packed in can and cooked at 110°C for 60 minutes. Extractions have been made by adding 2 fold volume of saline to meat.

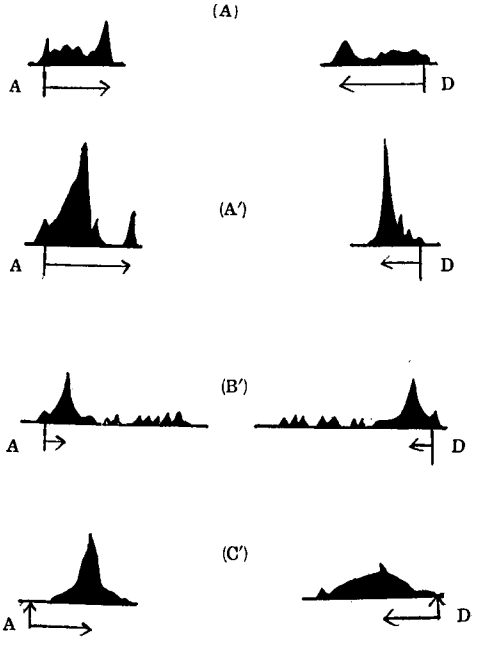

Fig. 1. Electrophoretic patterns of antigens

A : Horse blood-serum.
A′ : Cooked horse blood-serum.
B′ : Fluid of canned horse meat.
C′ : Alkali solution of boiled horse meat solid.

Veronal buffer, pH 8.4, μ=0.2. Temp., 10°C

	A	A′	B′	C′
Electrical field, V/cm.	—	3.0	2.9	5.7
Prot. conc., g/dl.	—	1.96	1.26	2.08
Duration of electrophoresis, sec.	—	14,100	7,800	12,600

Electrophoretic observations on antisera and antigens

It has been found out that antibodies[5] are formed by injection of some antigens at the part of globulin in serm.

The object of this observation by electrophortic analysis is to investigate the amount and the position of antibodies formed from various antigens. Elctrophoretic patterns for antigens are shown in Fig. 1. The patterns of the saline extract of dog meat (C in Table 2) are not demonstrated here, since they are substituted by those of horse meat in Fig. 5 and Fig. 6 because of their similarity in the protein destribution in tissue proteins of mammals. On the same account, antigen B (in Table 2), is substituted by A (in Table 2). As the antigen D (in Table 2), horse myoglobin, was once reported in detail by one of the writers[30], here it is omitted. Antisera immunized by these distinctly different proteins (Fig. 1) are shown in Fig. 2–4.

In contrast to the normal serum N in Fig. 2, every serum immunized with other protein increases its amount of γ-gloculin, β-globulin or both of them. Antisera illustrated in Fig. 2 have been injected with antigens which are mixed with alumina cream at the volume

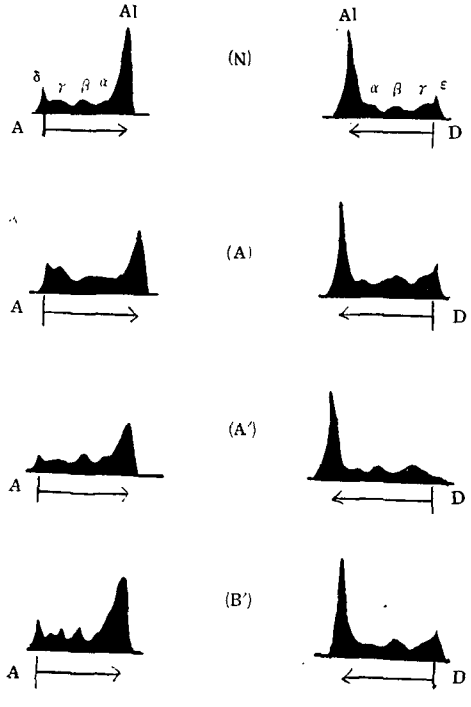

Fig. 2. Electrophoretic patterns of normal serum and antisera.

N: Normal serum.
A: Immunized by horse blood-serum.
A′: Immunized by cooked horse blood-serum.
B′: Immunized by fluid of canned horse meat.

Veronal buffer, pH 8.4, $\mu=0.2$. Temp., 10°C.
Duration of electrophoresis, 7,200 sec.
Protein conc., 2.0~2.2 g/dl.
Electrical field, 2.5~3.0 V/cm.

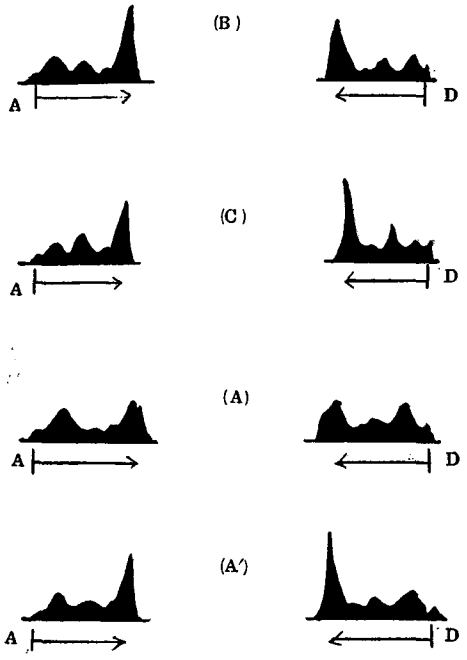

Fig. 3. Electrophoretic patterns of various antisera.

B: Immunized by dog blood-serum.
C: Immunized by saline extract of dog meat.
A: Immunized by horse blood-serum.
A′: Immunized by cooked horse blood-serum.
Veronal buffer, pH 8.4, $\mu=0.2$. Temp., 10°C.
Duration of electrophoresis, 7,200 sec.
Protein conc. 2.5~3.0 g/dl.
Electrical field, 5.0~5.3 V/cm.

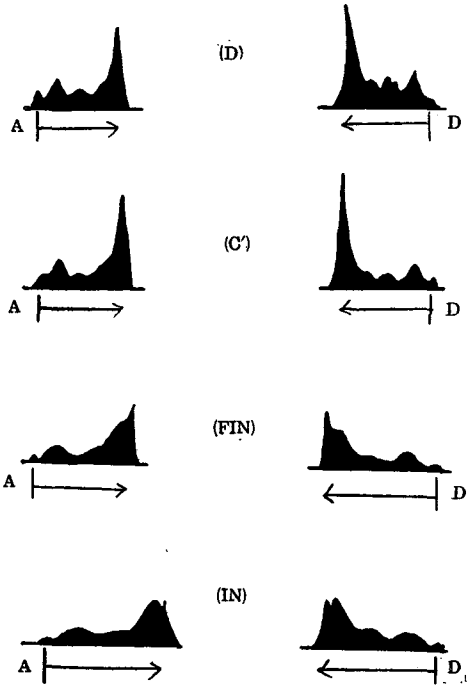

Fig. 4. Electrophoretic patterns of antisera

D: Immunized by horse myoglobin.
C′: Immunized by alkali solution of boiled horse meat solid.
FIN: Inactivated antiserum immunized by F-antigen.
IN: Inactivated normal serum.
Veronal buffer, pH 8.4, $\mu=0.2$. Temp., 10°C.
Duration of electrophoresis, 7,200 sec.
Prot. conc., 2.3~3.0 g/dl.
Electrical field, 4.7~5.7 V/cm.

ratio of 1:4. The other antisera illustrated in Fig. 3–4 have been done with that at the mixing ratio 1:1.

From these data, it can be said that in the case of the antisera preparation by the intramuscular injection at the mixing ratio of alumina cream and antigen 1:1, good results are achieved in formation of antibodies. The reason of this fact seems to be probably an effect caused by protein concentration in the mixture.

Antibodies are generally formed more at the part of γ-globulin than at the part of β-globulin, but distinct contrast is seen between serum C and A (including B), the former increasing its portion of γ-globulin remarkablly, while the latter its β-globulin. This distinct difference of antibody formation probably causes different behaviors in specificity. Patterns FIN and IN (in Fig. 4) are inactivated sera at 56°C for 30 minutes, the former being the antiserum which has been injected with 20 cc. of tissue emulsion of guinea-pig kidney containing FORSSMAN's antigen (this will be disscussed later), and the latter being the normal serum inactivated.

Through the inactivating process, serum portein is denatured and aggregation occours between albumin and α-globulin.

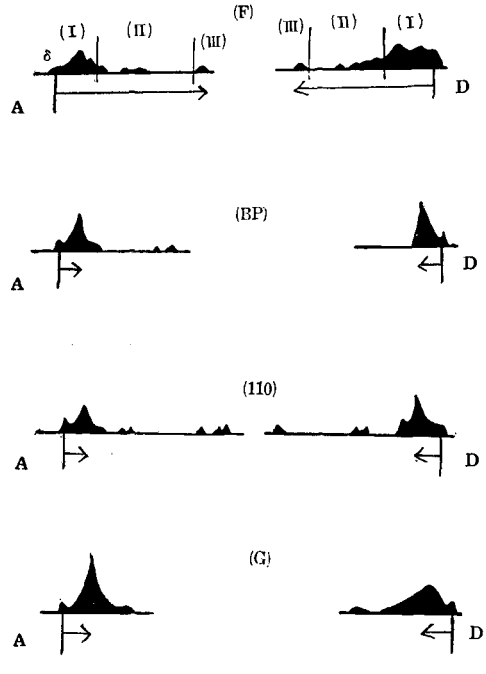

Fig. 5. Electrophoretic patterns of saline extract from horse meat

F: Fresh meat.
B.P.: Cooked at boiling point.
110: Cooked at 110°C.
G: Gelatin.
Veronal buffer, pH 8.4, μ=0.1. Temp., 10°C.

	F	BP	110	G
Prot. conc., g/dl.	1.16	0.84	0.76	1.49
Electrical field, V/cm.	4.1	4.2	3.3	4.1
Duration of electrophoresis, sec.	10,800	5,400	10,800	7,200

β and γ-globulin are not denatured tnrough this process and the antibody formation can be seen in the immunized serum at the part of γ-globulin.

Heat denaturation of proteins in meat

Many discussions have been made concerning the loss of species specificity[1),5),21)] by the denaturation[3),4),5)] of proteins which occurs under various conditions. In our experiments, the denaturation of meat proteins by heat, we think, is the most important factor which interupts the detection of meat by the precipitin test.

From this point of view, the solubility of nitrogen in the saline extraction under various heating temperatures described before, has been estimated and at the same time electrophoretic analysis has been done regarding the same saline extracts.

Changes of nitrogen solubility are illustrated in Table 10. The changes of electrophoretic patterns of saline extracts of meat which follow those of nitrogen solubility are illustrated in Fig. 5–7.

Table 10 shows that soluble nitrogen to saline decreases rapidly with the increase of temperature until at 60°C and continues to de-

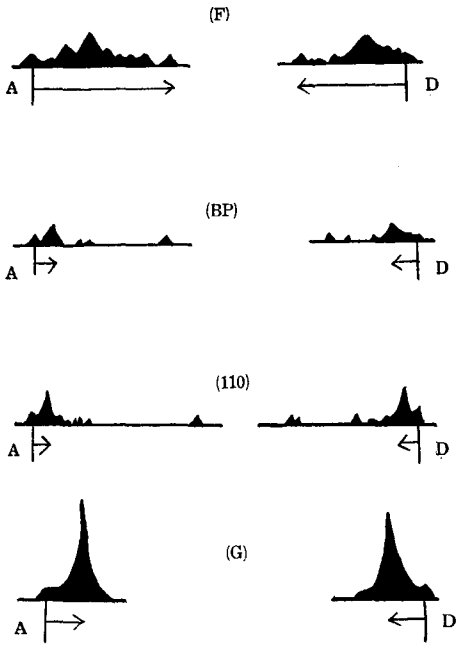

Fig. 6. Electrophoretic patterns of saline extract from horse meat

F: Fresh meat.
B.P.: Cooked at boiling point.
110: Cooked at 110°C.
G: Gelatin.
Veronal buffer, pH 8.4, μ=0.2. Temp., 10°C.

	F	BP	110	G
Prot. conc. g/dl.	1.1	0.3	1.1	1.6
Electrical field, V/cm.	2.8	2.5	2.5	—
Duration of electrophoresis, sec.	7,500	7,200	7,200	7,200

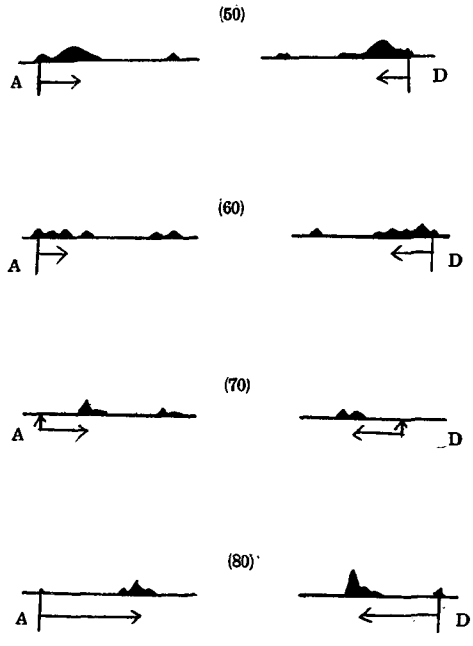

Fig. 7. Electrophoretic patterns of saline extract from horse meat

50 : Heated at 50°C.
60 : Heated at 60°C.
70 : Heated at 70°C.
80 : Heated at 80°C.

	50	60	70	80
Prot. conc., g/dl.	0.7	0.5	0.4	0.4
Electrical field, V/cm.		6.0~6.9		
Duration of electrophoresis, sec.	7,200	7,200	7,200	3,600

Veronal buffer, pH 8.4, $\mu=0.1$. Temp., 10°C.

TABLE 10. Change of soluble nitrogen in horse meat by heating.

	I			II			III*			IV**		
	Total N	Soluble N	T.N/S.N ×100	Total N	Soluble N	T.N/S.N ×100	Total N	Soluble N	T.N/S.N ×100	Total N	Soluble N	T.N/S.N ×100
	%	%		%	%		%	%		%	%	
Fresh meat	3.619	0.543	15.01	3.868	0.612	15.81	3.528	0.805	22.81	3.179	0.718	22.59
50°C	—	—	—	—	—	—	3.296	0.252	7.63	3.094	0.375	12.11
60°C	4.809	0.227	4.72	4.669	0.223	4.77	4.052	0.239	5.89	3.252	0.200	6.14
70°C	5.339	0.193	3.61	5.212	0.207	3.97	5.290	0.224	4.23	4.195	0.248	5.91
80°C	5.072	0.144	2.81	5.473	0.186	3.40	5.775	0 201	3.48	4.890	0.207	4.24
Boiling point	6.400	0.137	2.16	6.345	0.182	2.87	5.756	0.179	3.10	5.690	0.197	3.46
110°C	4.29	0.185	4.31	3.992	0.189	4.74	—	—	—	5.144	0.209	4.08

Extractions have been made by adding adequate volume of saline to meat and by cetrifugarization after stading over night in the refrigerator at 2~4°C.

* During standing period in the refrigerator, samples have been stired several times.

** After addition of saline to meat, samples have been homogenized at 12,000 r.p.m. and centrifuged immediately.

crease slightly until at boiling point, and yet there appears some increase at 110°C (for 60 minutes).

Electrophoretic patterns of saline soluble proteins in fresh meat and in cooked meat at boiling point and at 110°C are shown in Fig. 5-6 in which the same samples have been analysed under different ionic strengh, $\mu=0.1$ for Fig. 5 and $\mu=0.2$ for Fig. 6; and electrophoretic patterns of (G) in each figure are those of gelatin which has been used for the purpose of comparison with soluble proteins of heated meat under higher temperature.

In Fig. 7 patterns of saline soluble proteins under various temperatures from 50°C to 80°C ($\mu=0.1$).

From the results of many workers[8),10),13),14),18),30)], the electrophoretic patterns of saline soluble proteins in fresh meat are determined as myoalbumin group (III), globulin group (II including myosin and globulin X) and myogen group (I) in the order of migration velocity (Fig. 5). Peaks of group (I) which have formed only single boundary suggesting complex ingredients in the case of extract[8),10),30)] of distilled water, contain hemoproteins and occupy the largest area in the patterns. Peaks of group (II) are more obvious and larger than those of distilled water extracts, because globulin is more soluble in dilute salt solution.

By heating meat at 50°C, saline soluble proteins come to show only two boundaries at group (III) and group (I), in other words, globulin group (II) becomes insoluble by heat. Under the heat at 60°C, proteins almost decrease their solubility but a small amount of group (I) and group (III) are found. At 70°C, native proteins almost dissappar except slight traces of peaks of group (I) and group (III).

At temperatures above 80°C, the appearance of other unknown proteins is observed, and these proteins are shown as the peak of higher migration velocity in Fig. 7 at 80°C and as the peaks of lower migration velocity in Fig. 5 and Fig, 6 at boiling point and 110°C.

When the main peak in denatured proteins of lower migration velocity at boiling point and 110°C is compared with the electrophoretic pattern of gelatin (Fig. 5 and Fig. 6), we have noticed similarity in the migration velocity, shape and mobility among these boundaries. (Mobilities of these boundaries are all $-1.3\sim-1.4$ cm^2/v. sec.). Moreover, the same peak has been shown in the electrophoretic patterns of the fluid of canned meat (Fig. 1) and this fluid is gelatinous at room temperature.

In free amino-acid distribution determined by paper partition chromatography, it has been observed that hydroxyproline and cysteic acid appear in the extracts heated at boiling temperature and 110°C and in the fluid of canned meat (Table 11, Fig. 8).

This fact points out the modification of protein molecules and the decomposition of collagen to amino-acid by heat and also suggests the formation of gelatin as the intermedium from collagen to amino-acids.

From these results mentioned above it is confirmed that soluble proteins in meat are denatured completely by heat at 80°C and the soluble nitrogen decreases until at boiling point; on the other hand, heating above boiling temperature causes the formation of gelatin and makes the amount of soluble nitrogen increase.

TABLE 11. Distribution of free amino-acids.

Temp. / No. / Amino acids.	Saline extracts of meat						Fluid of canned meat
	Fresh meat		at boiling point		at 110°C		
	1	2	1	2	1	2	
Glu.	+		+		+	+	+
Asp.			+			+	+
Lys.	+	+	±				
Arg.	+	+	+	+	+	+	+
His.	+	+			+	+	+
Cys.							
Cys. acid.			±	+	±	+	+
Ser.	+	+	+	+	+	+	+
Thr.	+	+	+	+	+	+	+
Ala.		±		+	+	+	+
Try.					±		
Pro.							±
Hypro.			±	±	±	±	±
Tyr.							
Val./Met.						+	
Leu./Ileu.							
Ninhydrin positive			(2)		(1)		(2)
Unknowen spot			(2)		(1)		(2)

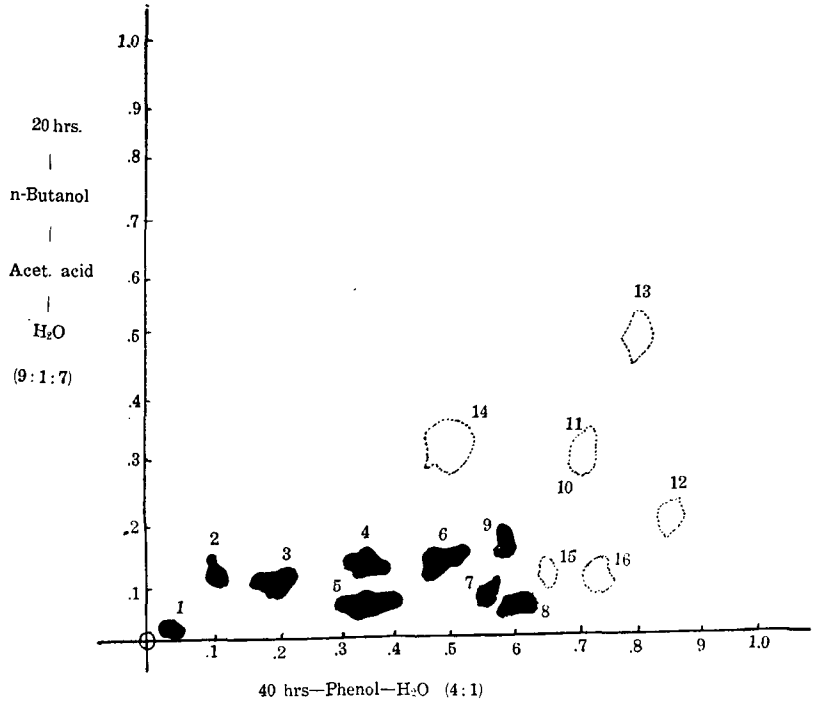

Fig. 8. Chromatogram of free amino-acids from fresh and cooked horse meat.

1. Cysteic acid.
2. Aspartic acid.
3. Glutamic acid.
4. Serine.
5. Lysine.
6. Threonine.
7. Arginine.
8. Histidine.
9. Alanine.
10. Hydroxyproline.
11. Valine/Methionine (?).
12. Proline (?).
13. Leucine/Isoleucine (?).
14. Unknown spot.
15. Ninhydrin positive.
16. Unknown spot.

II. Hemolysis reaction, using Forssman's heterophile antigen (F-antigen)

This serological hemolysis reaction used for the detection of animal meat is based upon the fact that F-antigen, unlike other tissue proteins, is lipoid-like specific antigen stable in heat and therefore, it is possible to detect the difference between two animal meat according to whether F-antigen exists or not. Moreover, we must add that F-antigen is possessed of antigen specificity, but not species specificity.

Distribution of F-antigen in animals is as follow

a. Animals with F-antigen (Guinea-pig group)

Guinea-pig. Horse, Dog, Cat, Rat, Whale, etc.

b. Animals without F-antigen (Rabbit group)
Rabbit, Cattle, Hog, Sheep, etc.

From this distribution, the detection of horse meat as an adulterant of beef is logically possible by making an examination of the presence of F-antigen in meat.

TABLE 12. The precipitin test with the alcohol extract of the original antigen.

antiserum	antigen ($25n$)					antiserum	antigen ($25n$)				
	$n=0$	1	2	3	4		$n=0$	1	2	3	4
2^n $n=0$	+	±	−	−	−	2	±	−	−	−	−
1	±	−	−	−	−	3	−	−	−	−	−

TABLE 13. The hemolysis test.

Samples**	M. H. D						
	8	4	2	1	0.5	Complement control	Antiserum control
⊙Canned horse meat	0	0	0	0	0	0	0
Fresh dog meat	0	0	0	0	0	0	0
*Boiled dog meat	0	0	0	0	0	0	0
⊙Canned beef	3	3	3′	2	1	0	0
*Boiled beef	3	3′	2	2′	1	0	0
Control (unabsorbed)	3	3	3′	2	1	0	0

The strength of the hemolytic reaction is indicated as follows:
3 complete, 3′ almost complete, 2 very marked, 2′ marked,
1 distinct, 1′ trace, 0 none.

* These meat have been immersed in boiling water for 30 minutes.

⊙ These canned products have been prepared in our laboratory.

** 10 g. of boiled (or fresh) meat samples have been ground through the chopper. Then, meat emulsion has been prepared by homogenizing at 12,000 r.p.m. for 10~20 sec. after previous boiling for 10 minutes.
After filtration of this emulsion through a coarse cloth, the filtrate has been centrifuged 5 times by washing with saline and the precipitate thus obtained has been served as the meat samples.
To 4 cc of diluted antiserum containing 8~10 M.H.D./cc has been added 1 cc of meat precipitate.
After 60 minutes' absorption at 37°C, this mixture has been centrifuged.
1 cc of this clear supernatant solution diluted in the order of 8, 4, 2, 1, 0.5 M.H.D., has been mixed with 0.5 cc of complement diluted 1:10, 0.5 cc of sheep blood cells and 0.5 cc of 0.85% saline.
The readings have been made at the end of 60 minutes' incubation at 37°C.

When the anti-F-antigen-serum is brought in to contact with meat containing F-antigen, the specific reaction occur between antigen and antibody.

In order to certify this antigen-antibody reaction, an examination is made as to whether complement is fixed or not and this reaction is visibly judged by the hemolysis reaction which occurs by adding suspension of red-blood cells of sheep.

Therefore, if there is occurrence of the hemolysis reaction due to absence of F-antigen which reacts with corresponding antibody, it can be denied that there is presence of meat belonging to the guinea-pig type.

TABLE 14. (a) The hemolysis reaction with canned meat products*.

Beef : Horse meat	M. H. D						
	8	4	2	1	0.5	Complement control	Antiserum control
100 : 0	3	3′	2	2	1	0	0
80 : 20	1′	0	0	0	0	0	0
60 : 40	0	0	0	0	0	0	0
40 : 90	0	0	0	0	0	0	0
20 : 80	0	0	0	0	0	0	0
0 : 100	0	0	0	0	0	0	0

* These products which contain various amount of horse meat from 0% to 100%, have been prepared in our laboratory.

TABLE 14. (b) The hemolysis reaction with commercial canned meat products.

Sample No.	M. H. D							
	8	4	2	1	0.5	Complement control	Antiserum control	Labeled names of samples
1	0	0	0	0	0	0	0	Beef Yamatoni
2	0	0	0	0	0	0	0	〃
3	0	0	0	0	0	0	0	〃
4	0	0	0	0	0	0	0	〃
5	0	0	0	0	0	0	0	〃
6	3	2	2	2′	1	0	0	〃
7	3	3′	2	2	1	0	0	Vienna Sausage
8	0	0	0	0	0	0	0	Beef Yamatoni
9	0	0	0	0	0	0	0	Corned Beef
10	0	0	0	0	0	0	0	Sausage Meat

Minimal hemolytic dose (M.H.D.) of antiserum has been determined by means of OGATA's description[23]. The preparation of samples and the technique for the hemolysis test have been made as given by SHIGA and coworkers[24]. Before the hemolysis test, the trials to applicate this antiserum for the precipitin test have been made with the alcohol extract of the original antigen (Table 12). But these attempts have not been successful in the practical use. The hemolysis test for meat is shown in Table 13 and results of this test for commercial canned meat products are shown in Table 14.

Results indicate as given in previous tables that the hemolysis test can be applicable for the detection of the horse meat (guinea-pig group) as an adulterant of beef (rabbit group).

Discussion

Though the precipitin test has hitherto been known of its high ability in the detection of meat, we have observed the following facts.

The specific specificity is recognizable up to the heating temperature at 70°C only in the case of the application of antisera in which heated serum and myoglobin are used as antigens to their preparation. But in the case of antisera immunised by the normal blood serum, this limitations of recongnition falls down to the tempersture at 60°C. These antisera for the preciptin test are, to keep animasl in safety, formed accurately through the intramuscular injection using mixture of antigen with alumina cream. Although it has been reported that the use of cooked serum as antigens renders the titers of antisera higher and causes some state specificity, our results have shown that is obscure so far as species specificity is concerned.

It has been found out that the blood serum produces more effective antisera than the saline extract of meat, when we use antigens for detection of fresh animal meat. This is probably due to the difference in tissue proteins and antibodies formed by them. Hemoprotein in muscle, myoglobin, has been reported to possess high immunological specificity[27),30)] as well as hemoglobin[1),27)] in blood. When this protein is used as an antigen, the antiserum which is possessed of a high precipitin titer is formed and moreover, since this is one of the most heat stable protein[25)] among muscle proteins, antiserum by this antigen expresses possitive reaction upon the saline extract of meat heated at 70°C. Examinations on the distribution of proteins in the saline ex-

tract of meat under various temperature suggest the decrease of solubilty in proteins with the increase of temperature. Solubility continues to decrease up to the boiling point, but at the temperature above boiling point some increase is noticed.

The decreasing degree of the protein solubility in saline is especially remarkable up to the temperature at 60°C.

Analysig results by the electrophoresis indicate that almost fractions of globulin (myosin, globulin X) in meat come to be insoluble by the heat at 50°C and almost fractions of albumin (myogen, myoalbumin) become also insoluble by the heat at 60–70°C. These facts agree well with the principle of the heat fractionation method[2),25)] which has been used for fractionating myosin and myogen in saline extract of meat. By heating at the temperature above 80°C, it seems, the entirely denatured proteins come to be soluble. Especially, at the higher temperature above boiling point, the protein similar to gelatin dissolves into saline.

The heat denaturation of muscle proteins in meat as described above can be recognized by existence of cystic acid in free amino-acids of cooked meat. (This amino-acid can not be seen in free amino-acids of fresh meat.)

In our experiment on determination of amino-acid, cystein and cystine are shown as a spot of cysteic acid. Therefore, an appearence of cystic acid shows the following fact that masked residues in fresh meat proteins come to be exposed by heat[4)].

From this fact, it can be said that the modification of molecules of muscle proteins has occured and free SH-group has been formed, in other words, that the protein has been denatured[3),4)].

An appearence of hydroxyproline in free amino-acids at this time, certifies the existence of gelatin.

The reason is that this amino-acid has been known to be such characteristic one as used as an indicator for collagen estimation[17),29)]. Gellatin is inevitably produced as the intermedium of this reaction in the decomposition of collagen into hydroxyproline by heat.

Antisera prepared by the injection of the fluid of canned meat (proteins seem to be derived proteins[4)] containing mainly gelatin) and alkali solution of boiled meat solid (aggregated denatured protein) as antigens, have no species specificity and, therefore, not available for the purpose of this report.

The antiserum immunized by the heated serum which is deemed

as denatured protein electrophoretically, increases its precipitin titer and it is effective as well as the one done by myoglobin, but its species specificity comes to be obscure to some extent.

A necessary condition for the detection of animal meat is the presence of native proteins in meat to denote their ability of antibody formation which is possessed of species specificity.

So, the precipitin reaction appears only when there exist some coresponding native proteins in the saline extract of meat and strength of reaction decreases with the amount of native proteins in the extract. When all proteins in meat are denatured by heat, precipitin reaction does not take place any more.

When these denatured proteins are used as antigens, the antisera prepared have no species specificity, so that the precipitin test can not be adoptable to such meat as heated severely.

In this case, the heat penetration must be considered. If meat is cooked by dry heat for example in an oven, the matter is with heat penetration.

On the other hand, if meat is cooked in a water bath, denaturation occurs uniformly in all the samples (especially when lumps of meat samples are small as shown in this report).

It has been stated by many workers[15),20)] that a considerable time is required for the heat to penetrate into the center of meat product. From this point of view, the detection of meat by the precipitin test is available effectively for smoked meat products.

To other meat products which are prepared under more severe heat such as canned products, this test will not be applicable until, in future, we shall find out an adequate heat stable antigen which has high species specificity or the method to regenerate the native protein from the denatured one.

Then, another serological method, which has been not so commonly known as the precipitin test but reported by few workers[19),24)] with their good results, the hemolysis test using F-antigen, has been reaffirmed by supplemental experiments. Very decisive results of the detection has been thus obtained.

Only the existence of F-antigen can be, inevitably, determined through this test. But though beef contains no F-antigen, horse meat contains it, and it is possible to detect horse meat as an adulterant of beef with the problem of which we are much concerned in our country and upon which we have put much emphasis in our experi-

ments.

Summary

1. More effective antisera for the detection of meat by means of the precipitin test have been prepared by the intramuscular injection more safely than by the intraveneous injection.

2. The limitation for the precipitin test is up to the temperature at 70°C in the case of immunized sera by myoglobin and cooked blood serum, while it is down to the temperature at 60°C in the case of immunized serum by normal bood-serum.

3. When the entirely denatured proteins in meat are used as antigens, antisera prepared have no species specificity, so they are not available for the detection of meat.

4. Antibodies have been formed at the part of γ-globulin or β-globulin in serum by the immunization of various antigens.

5. A necessary condition for the detection of animal meat by the precipitin test is the presence of native protein in saline extract of meat.

6. Behaviors of saline soluble proteins with the change of cooking temperature has been discussed.

7. The detection of horse meat as an adulterant of beef in the products heated at severe temperature, has been made decisively by means of the hemolysis test which determines the existence of F-antigen.

References

1) ANSON, K. L. and MIRSKY, A. E. (1925): J. Gen. Phisiol., 9, 169.
2) ALLEN. (1913): ALLEN's Commercial Organic Analysis, 169.
3) AKABORI, S. (1944): Amino-acid and Protein, 502. (*In Japanese*).
4) AKABORI, A. and MIZUSHIMA, S. (1955): Protein Chemistry, II. (*In Japanese*).
5) AKABORI, S. and MIZUSHIMA, S. (1955): Protein Chemistry, III, 396. (*In Japanese*).
6) AKAO, Y., NAKANO, T. and MATSUI, T. (1955): Food Hygiene, Edited by Public Food Sanitation Section, Ministry of Public Welfare, 5, 29. (*In Japanese*).
7) CROUSE, R. H. and LEFFER, I. D. (1953): Food Research, 18, 258.
8) FISCHER, E., et al. (1949): Arch. Phys. Med., 30, 766.
9) FUJIMAKI, M. and ODAGIRI, S. (1954): Bull. Agr. Chem. Soc. Japan, 28, 963. (*In Japanese*).
10) HASHIMOTO, Y. *unpublished data.*
11) HASHIMOTO, Y., YASUI, T., FUKAZAWA, T. and MATSUMI, H. (1955): Special Bulletins of the College Experimental Farm, Hokkaido University, 11, 125.

(*In Japanese*).

12) HASHIMOTO, Y., YASUI, T. and HOSHI, K. (1956): Memoirs of the Faculty of Agriculture, Hokkaido University. 2, 213. (*In Japanese*).
13) JACOB, J. (1947): Biochem. J., 41, 83.
14) JACOB, J. (1948): Biochem. J., 42, 71.
15) JENSEN, L. B. (1948): Microbiology of Meat, 178.
16) MACKAY, I. R., VOLWILER, W. and GOLDWORTHY, P. D. (1954): J. Clin. Invest., 33, 855.
17) MILLER, M. and KATELIC, J. (1956): J. Agr. Food Chem., 4, 537.
18) MOMMAERTS, W. F. H. M. (1950): Muscular Contraction, 75, 87.
19) MORITA, H. (1932): Jap. J. Exp. Med. 16, (4). (*In Japanese*).
20) NAKAJIMA, M. (1953): Proceedings of the 4th Annual Meeting of the Japanese Meat Institute, 57. (*In Japanese*).
21) NAKAMURA, Y. (1940): Bacteriology and Immunology, 204. (*In Japanese*).
22) NELWIG, H. L. and GREENBERG, D. M. (1952): J. Biol. Chem., 198, 695.
23) OGATA, T. (1947): Methods for Serological Survey. (*In Japanese*).
24) SHIGA, I., OKAYA, T., IWAMOTO, Y. and MIYATA, M. (1953-1954): Research Bulletin of the Institute of TOYO SEIKAN Co. INC., Series 3, 44. (*In Japanese*).
25) TADOKORO, T., et al. (1928): Bull. Agr. Chem. Soc. Japan, 4, 1010. (*In Japanese*).
26) TANNER, F. W. (1944): Microbiology of Food.
27) UENO, M. (1951): Phyisiologycal, Pathological and Serologycal Studies on Myoglobin. (*In Japanese*).
28) WEINSTOCK, A. (1953): J. Milk and Food Technol., 16, 275.
29) WIERLICKI, E. and DEATHERAGE, F. E. (1954): J. Agr. Food Chem., 2, 878.
30) YASUI, T. (1956): Jap. J. Zootech. Sci., 27, 217. (*In Japanese*).