INTRODUCTION:

The identification of animal proteins as to species and to type of tissue is important in fresh and comminuted meat where adulteration is suspected. The adulteration of ground or comminuted meat is not a common occurrence but is known to occur. The presence of horsemeat in ground beef is no longer prevalent but recently the presence of lean mutton in imported beef has been observed. Physical inspection of the frozen boxes is time-consuming and most likely inconclusive when only small quantities are present. The addition of pork to ground beef could occur when the supply of pork is plentiful. The detection of this type of adulteration is difficult by physical or classical chemical techniques.

Analysis of the fat has been used particularly for horsemeat since horse fatty tissue has a high linolenic acid content (Crowell, 1944; Hynds, 1951 and Crouse and Teffier, 1953). A gas chromatographic technique for the identification of horse, beef and pork fat was reported by Cook and Sturgeon (1966). This method reported only that there were differences observed between individual species but no attempt was made to identify species in a mixture. In addition, the quantity of fat contributed by the species used for adulteration may be only a small percentage of the total fat, therefore, making detection difficult. Starch-gel electrophoresis has been used to demonstrate species differences in skeletal muscle. However, no results were presented on mixtures of different species (Thompson, 1961).

The serological identification of proteins was reported as early as 1931 by Bolin. Other workers (Proom, 1943; Tanner, 1944; Kaplan and Buck, 1951; Oswald, 1953; Weinstock, 1953; Brandly, 1954 and Milgrom et al., 1964) have reported the use of serological techniques in the identification of animal tissue. However, cross-reactions were observed between closely related species such as bovine and ovine skeletal tissue. Development of antibodies against tissue extracts was difficult and many times the titers were low or nonexistent.

In any serological reaction there are two main components. The antigen which is the foreign protein is injected into the animal's tissue fluid. This will result in the production of blood soluble substances known

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as antibodies. These circulating antibodies in the globulin fraction of the blood serum will react specifically with the specific foreign protein (antigen) either in vivo or in vitro.

**EXPERIMENTAL METHODS:**

The following antigenic protein materials were isolated and evaluated:

2. Serum (Campbell et al., 1963).
3. Serum alum precipitate (Oswald, 1953).
4. Muscle extract alum precipitate (Modified Technique of Oswald, 1953).

Rabbits weighing approximately 2 kg were injected intravenously, intraperitoneally or intramuscularly with quantities and frequency as shown in Table 1, using methods described in serological text (Campbell et al., 1963). The lyophilized water extracts of tissue were rehydrated in 2 ml of saline and emulsified in 5 ml of Freund complete adjuvant prior to intramuscular injections.

Blood was removed from the rabbits by the cardiac puncture technique and allowed to clot. After holding overnight at 4°C, the antiserum was decanted and centrifuged at 1000 x G for 30 minutes to remove any remaining blood cells. The antiserum was transferred to test tubes and merthiolate added (1:10,000) and stored at 4°C. The absorbed antiserum was prepared by adding approximately 8 mg/ml of the lyophilized extracts of the cross-reacting species. The antiserum was agitated at room temperature for four hours and held overnight at 4°C. The antigen-antibody complex formed was removed by centrifugation at 2000 x G for 30 minutes at 4°C.

The animal tissue or ground meat mixture to be tested was blended with saline at low speed for 30 minutes in an Osterizer. The slurry was centrifuged at 10,000 x G for 15 minutes and supernatant filtered through No. 4 Whatman filter paper. For tests using isolated gamma globulin, the saline extracts were heated for 15 minutes at 50°C and the precipitate removed by filtering through No. 4 Whatman filter paper.

The ring test involves the use of 4 x 50 mm precipitation tubes filled approximately half-full with antiserum using a syringe and needle. The antigen was carefully layered over the antiserum to form a distinct interface. After 15 to 30 minutes, the tubes were examined against a black background under artificial light. If an antigen-antibody reaction was formed, a white ring is observed at the interface. The density of the precipitation ring was rated from + to ++++. 
The double gel diffusion method consisted of Oxoid Ionager No. 2 (0.85%) dissolved in borate buffered saline at a pH of 8.4 with merthiolate (1:10,000). After autoclaving, agar was poured to a depth of 9 mm in 15 x 50 mm petri dishes. Wells were cut in agar 2 cm apart and the bottoms sealed with a drop of hot agar. From 0.1 to 0.3 ml of the reactants were placed in the wells and the plates were stored in a moist desiccator for three days at room temperature. The precipitation bands were observed and recorded or photographed. The tube gel diffusion tests consisted of the same agar with antiserum mixed with agar just prior to solidification (1 to 1). After solidification, more agar was added to obtain a plug of approximately 3 mm. After solidification, the test extracts were added (2 ml) and held at room temperature. The precipitation bands were formed as distinct rings in the agar plug.

In the tube precipitation method using isolated gamma globulin, the gamma globulin was isolated using ammonium sulfate as described by Campbell et al., 1963. The isolated gamma globulin (1 ml) and heated test extracts (3 ml) were mixed in a small test tube. The tubes were held at room temperature for two hours and centrifuged at 2000 x G for 15 minutes. Sufficient control tubes were included to insure an accurate test. If an antigen-antibody complex was formed, a pellet is observed in the bottom of the tube which is rated from a + to ++++.

RESULTS AND DISCUSSION:

The development of specific antibodies against animal protein antigens was found to be more difficult to accomplish than the development of antibodies against bacterial or serum antigens. The quantity of antigenic protein material, the route of injection and type of antigenic protein material were of importance in developing specific animal tissue antibodies.

Many different antigenic proteins, quantities and routes of injection were evaluated as to titer and specificity. Intraperitoneal injections of 2 ml of a saline extract were administered over a 35 day period. The protein content of these extracts was 10-15 mg/ml. A slight positive reaction was observed by the ring test after 10 and 30 days for homologous antigen. However, gel diffusion tests were negative for both homologous and heterologous antigens as were ring tests after 60 days. However, when the rabbits were injected intravenously with 5 ml of homologous saline extracts of skeletal muscle after 120 days, the rabbits went into anaphylaxis shock and died within four hours. This would indicate that antibodies were present but the titer was not high enough to be detected by ring or gel diffusion tests.

Actomyosin was isolated from skeletal muscle using the method of Szent-Gyorgyi (1951) and evaluated as one of the antigenic materials using different quantities, frequency and routes of injection. The results of the various methods are shown in Table 1. A slight positive ring test was observed with injections of 150 mg of actomyosin. However, strong cross-reactions between species were observed which could not be removed by absorption. Also, the titer was not sufficiently high enough to be of use in detecting species differences.
Serum alum extract as described by Oswald (1953) and serum from different species were used as antigens. A strong positive ring test was observed with the serum with which the rabbit had previously been injected.

Table 1. Summary of Immunizing Methods

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of injections</th>
<th>Quantity/Route</th>
<th>Gel Ring diffu-</th>
<th>Gross After reaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actomyosin</td>
<td>1</td>
<td>10 mg IM</td>
<td>0 0</td>
<td>--</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>2</td>
<td>10 mg IM</td>
<td>0 0</td>
<td>--</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>2</td>
<td>10 mg SC</td>
<td>0 0</td>
<td>--</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>1</td>
<td>15 mg IP</td>
<td>+ 0</td>
<td>+++ 0</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>6</td>
<td>150 mg IP</td>
<td>+ +</td>
<td>+++ 0</td>
</tr>
<tr>
<td>Serum</td>
<td>9</td>
<td>0.25 ml IV</td>
<td>0 0</td>
<td>--</td>
</tr>
<tr>
<td>Serum</td>
<td>6</td>
<td>10 ml IM</td>
<td>++ ++</td>
<td>+ 0</td>
</tr>
<tr>
<td>Serum Alum Extract</td>
<td>1</td>
<td>10 ml IM</td>
<td>++ ++</td>
<td>+ --</td>
</tr>
<tr>
<td>Muscle Alum Extract</td>
<td>4</td>
<td>10 ml IM</td>
<td>+++ ++</td>
<td>++ --</td>
</tr>
<tr>
<td>Muscle Alum Extract</td>
<td>6</td>
<td>10 ml IM</td>
<td>++ ++</td>
<td>++ 0</td>
</tr>
<tr>
<td>Saline Extract</td>
<td>15</td>
<td>2 ml IP</td>
<td>+ --</td>
<td>+ --</td>
</tr>
<tr>
<td>Freeze-Dried Extract</td>
<td>6</td>
<td>150 mg IP</td>
<td>+++ +++</td>
<td>+ ++</td>
</tr>
<tr>
<td>Freeze-Dried Extract</td>
<td>6</td>
<td>150 mg IM</td>
<td>+++ +++</td>
<td>+ +++</td>
</tr>
</tbody>
</table>

* as determined by gel diffusion

Level of Reaction

- **0** = Negative
- **+++** = Strong
- **+** = Slight
- **++++** = Very Strong
- **++** = Moderate
- **--** = No Data

However, ring tests showed only slight to moderate reactions with saline extracts of skeletal muscle, as did the gel diffusion plates over a 90-day period. When cross-reacting antibodies were removed by absorption, no reaction was observed by gel diffusion tests.

Water extracts of skeletal muscle were precipitated using alum by a method similar to that described by Oswald (1953) for serum. Multiple intramuscular injections of muscle extract alum in two series resulted in an antiserum which showed slight to moderate ring test and precipitation bands for the injected species as well as cross-reactions with other species. When cross-reacting antibodies were removed by absorption, no reaction was observed for the specific species injected.

Since previous tests had shown the necessity for injection of relatively large quantities of protein material, skeletal muscle was extracted with water and lyophilized. Multiple intramuscular and intraperitoneal injections of the lyophilized extract rehydrated in 2 ml saline were made as shown in Table 1. For the intramuscular injections, the rehydrated muscle extracts were emulsified in 5 ml of Freund complete adjuvant. After
30 days, strong to very strong reactions were observed by both the ring and
gel diffusion tests. However, a slight cross-reaction was observed among
beef and lamb. These cross-reactions could be removed by the addition of
lyophilized extracts of the cross-reacting species. The titer of the ab-
sorbed antiserum was similar to the unabsorbed antiserum. The intramus-
cular route of injection gave more consistent results and higher titers
than the intraperitoneal injections. This method was therefore selected as
the method of choice to produce antiserum against tissue extracts.

Further investigation of this method using more rabbits and dif-
ferent species showed that four intramuscular injections of 150 mg in two
series approximately 22 days apart gave consistently high titers for ex-
tended periods of time. However, 30 days were required before a species
antiserum was observed for each species after absorption. The maximum
titer of the antiserum was observed after 60 to 90 days and continued for up
to 270 days. The titer for each of the species specific antiserum and
protein level detected for skeletal muscle are shown in Table 2.

Table 2. Titer and protein concentration detected using species specific
antiserum.

<table>
<thead>
<tr>
<th>Tissue (skeletal muscle)</th>
<th>Highest dilution detected(^a)</th>
<th>Protein concentrations(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>1:256</td>
<td>0.20</td>
</tr>
<tr>
<td>Beef</td>
<td>1:192</td>
<td>0.20</td>
</tr>
<tr>
<td>Lamb</td>
<td>1:256</td>
<td>0.24</td>
</tr>
<tr>
<td>Pork</td>
<td>1:256</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\(^a\)as determined by gel diffusion

\(^b\)as determined by Kjeldahl nitrogen

Initially, horse and pork antiserum were both species specific
while some cross-reactions were observed between beef and lamb. The reac-
tion of unabsorbed beef antiserum with saline extracts of skeletal muscle
120 days after the last injection are shown in Fig. 1. In addition to a
precipitation band with beef, a strong cross-reaction with lamb and a
moderate cross-reaction with pork is observed. Cross-reactions were more
evident for all species as the time after the last injection increased but
could be removed by absorption as shown in Fig. 2.

The detection of adulteration is based upon the quantity of pro-
tein extracted in a saline extract. Saline extracts (1:4 dilution) had a
protein concentration of approximately 15 mg/ml depending on the species and
fat content. Based upon a similar amount of protein extracted for each
species, a specific antiserum which reacted at a saline protein extract
dilution of 1:128 should react with a particular species if the total meat was as low as 3% in a meat mixture. If a reaction was observed at a dilution of 1:256 with a specific antiserum, a reaction at a meat level of 1.5% should be observed.

Samples of ground beef were adulterated with horsemeat at levels of 0.5, 1, 2, 3, 4 and 6%. These samples were extracted with saline (1:12 dilution). The average protein concentration was 15 mg/ml which showed a definite precipitation band on gel diffusion plates when horsemeat was present at the 3% level. The saline extracts were concentrated by pre-evaporation to a protein concentration of 24.5 mg/ml. A weak but definite precipitation band was observed at the 1% level. Controls with beef, lamb and pork saline extracts were negative when tested against horse antiserum. These results are superior to those reported by Pike and Sulkin (1957) who observed a faint precipitation band at the 5% level.

The effect of fat level on the quantity of protein extracted was studied. Saline extracts (1:3) of ground beef containing 10, 15, 25, 35, 45 and 55% fat were made. The quantity of protein extracted was linearly related to the percent fat as shown in Fig. 3. However, the minimum quantity of protein extracted was 14 mg/ml for ground beef containing 55% fat, which showed a precipitation band at a dilution of 1:192. Based on the ground meat mixture containing this quantity of fat, a reaction for adulterated meat could be observed at the 2-3% level.

The identification of species in comminuted meats which have been heat processed is more difficult than in fresh tissue. Saline extracts of skeletal muscle were heated at 550, 650, 750 and 850°C. for 5, 10, 15, 20 and 30 minutes with agitation in a water bath. A precipitation band was observed for the extracts of beef, horse, lamb and pork heated for 15 minutes at 750°C. (167°F.). The protein content of these extracts which exhibited a reaction ranged from 2.4 to 3.5 mg/ml. A few additional reactions were observed at higher temperatures and times, particularly for the lamb and pork. Based upon these reactions, it should be possible to detect adulteration in processed meats which have not been heated to a temperature higher than approximately 750°C. (167°F.).

Saline extracts of commercial frankfurters composed of beef and pork and pure beef were tested against specific antisera of beef and pork. Though the percentage of beef and pork was not known, a precipitation band was observed with the saline extracts of frankfurters known to be heated to approximately 650°C. (156°F.). However, no reaction was observed with the pure beef frankfurters heated to 80°C. (176°F.). Further work is necessary to determine the level of detection possible at different temperatures and mixtures of meat from different species.

Preliminary work on the identification of organ tissues by serological techniques showed that antibodies were developed against lyophilized water extracts of the organ meats. Cross-reactions were observed between organ meats of the same species such as beef lung and beef spleen as well as the same organ meats of other species. These cross-reactions could be removed by selective absorption. A species specific antiserum for beef lung and beef spleen was obtained in this study. However, more difficulty was encountered in obtaining a species specific antiserum for organ meats.
The specific antisera for beef, pork, horse and lamb skeletal muscle were tested against saline extracts of various organ meats from different species. A definite precipitation band was observed between the species antiserum and the organ meat of that species. However, no precipitation bands were observed between the specific antiserum and the organ meats of the other species. These results are in agreement with Witebsky (1962) who reported that there is tissue specificity attributed to the presence of an antigen characteristic of a particular organ in a single species and a second order specificity attributed to an antigen characteristic of the same organ in many species.

From this initial work, it was found that antibodies are developed against organ tissue using lyophilized water extracts of the tissue as antigens. Further work is necessary to determine the nature of cross-reacting antibodies and whether a species specific antiserum for additional organ meats can be obtained and the level of detection possible in a meat mixture.

The detection of antigen-antibody reactions by the ring and gel diffusion tests was evaluated for species identification. Previous research (Oswald, 1953; Pike and Sulkin, 1957 and Hay, 1962) reported the use of both techniques for the identification of animal species, but no comparison was made between the two methods. The ring test can be carried out in less than two hours while the gel diffusion tests required from 24 to 72 hours; however, there is a low variation between duplicate gel diffusion tests. A comparison of the ring and gel diffusion tests is shown in Table 3. The average percent disagreement between ring and gel diffusion tests was 23%. This would indicate that the ring test has serious limitations for critical evaluation. This would be particularly true because the ring test gave 20.8% positive results when the gel diffusion test showed negative results.

Further work was carried out using isolated gamma globulin from the specific antiserum and saline extracts of the meats heated to 50°C. in a tube precipitation method. The heated meat extract and specific isolated gamma globulin are mixed in a small test tube and allowed to stand at room temperature for two hours and centrifuged. If an antigen-antibody complex is formed, a pellet is observed in the bottom of the tube after centrifugation. This method can be carried out in less than three hours and is simple to perform. In addition, where one meat is a very small amount of the total meat mixture after extraction, the gamma globulin can be added to 4 ml of the extract. Therefore, a greater quantity of protein is available for that species to react with the gamma globulin as compared to the gel diffusion plates.

Also, the isolated specific gamma globulin for a species can be mixed with the agar in a small test tube and allowed to solidify. Approximately 3 mm of clear agar is added and allowed to solidify and then the saline extract of the meat is added. If an antigen-antibody complex is formed, distinct rings will be observed in the agar plug as a positive test. However, at least 24-72 hours are required for conclusive results to be obtained. However, these could be photographed for a permanent record.
Table 3. Comparison of ring and gel diffusion tests.

<table>
<thead>
<tr>
<th></th>
<th>0 ring and gel diffusion</th>
<th>+ ring and gel diffusion</th>
<th>++ ring and gel diffusion</th>
<th>+++ ring and gel diffusion</th>
<th>Total diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>214</td>
<td>112</td>
<td>54</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>Percent of total</td>
<td>51.3</td>
<td>26.9</td>
<td>12.9</td>
<td>5.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Number agree</td>
<td>170&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Percent agreement</td>
<td>79.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number disagree</td>
<td>44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Percent disagreement</td>
<td>20.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- a = negative ring, negative gel diffusion
- b = negative ring, positive gel diffusion
- c = positive ring, positive gel diffusion
- d = positive ring, negative gel diffusion

**SUMMARY:**

Specific antisera were developed against lyophilized water extracts of animal tissue. Antisera developed in this manner were superior to those developed from actomyosin, serum, serum alum precipitate, muscle extract alum precipitate or saline extracts of tissue. At least 30 days were required before a specific antisera could be obtained with the maximum titer occurring between 60 and 90 days and continuing for up to 270 days.

The specific antisera could detect the adulteration in fresh meat at a level of 1-3% depending on the titer, fat content of the meat mixture and protein content of the extracts using gel diffusion tests. Additional results indicated that levels lower than this may be detected using isolated gamma globulin in a tube precipitation method.

The same antisera developed against fresh tissue could be used to detect species differences in comminuted meats which were not heated to temperatures higher than 700°C. (158°F.). However, additional work is necessary to determine the level of detection possible in a meat mixture processed to different temperatures.

Antibodies were developed against organ tissue. However, the results indicated that cross-reactions were more prevalent. A specific anti-
serum is possible but more difficult to obtain since both species and organ tissue cross-react.

Gel diffusion tests are more reliable than ring tests in evaluating tissue extracts even though at least 24-72 hours are required to complete the test. The tube precipitation method using isolated gamma globulin and mildly heated extracts is at least as sensitive as the gel diffusion test and can be carried out in less than three hours.

REFERENCES


University of Georgia, College of Agriculture Experiment Stations, Journal Paper Number 95, College Station, Athens.

The authors wish to thank the American Meat Institute Foundation for their support of this investigation. The authors express their appreciation for partial support of Mr. M. O. Warnecke as a Public Health Trainee Fellow under USPH Grant TO-1-UL-1044. Data is from Ph.D. thesis of M. O. Warnecke.

Fig. 1. Reaction of unabsorbed beef antiserum with meat extracts.

1 = Beef extract; 2 = Pork extract; 3 = Lamb extract; 4 = Horse extract; 5 = Heated beef extract; 6 = Heated horse extract.
Fig. 2. Reaction of absorbed beef antiserum with meat extracts.

1 = Beef extract; 2 = Pork extract; 3 = Lamb extract; 4 = Horse extract; 5 = Heated beef extract; 6 = Heated horse extract.

Fig. 3. Quantity of protein extracted at various fat levels.