A CHEMICAL STUDY OF THE MANOILOV TEST
FOR THE DIFFERENTIATION OF THE SEXES

BY K. GEORGE FALK AND I. LORBERBLATT.

(From the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

(Received 7th July 1926.)

The chemical differentiation between the sexes of substances derived from various animal and plant products, which is shown by the Manoilov reaction, has aroused considerable interest. An attempt to throw light on the chemical changes involved in this reaction, together with a simplification of the test, will be described in this paper. The test as described by Manoilov consists essentially of successive additions to an aqueous or 60 per cent. alcoholic extract of the substance in question of a definite number of drops of solutions of the following substances: papain, dahlia (or methyl green), potassium permanganate, hydrochloric acid, and thiosinamine. If the test is successfully carried out, the preparation from the female gives a pink to red or violet solution, while that from the male remains colourless. The directions for carrying out the tests include definite pauses after the addition of some of the reagents, and precautions against agitating the mixtures until after the addition of the first four.

In order to study the chemical reactions involved in the test, it was necessary to use considerable quantities of materials of constant properties. Such materials were available in ovarian and orchic substances dehydrated by a process developed at the Harriman Research Laboratory and the Chemical Engineering Laboratories of Columbia University. These dehydrated products were supplied by the Lehn and Fink Products Company.

EXPERIMENTS.

The following list of experiments will show the method used in studying the test and some of the results obtained. The solutions used were as follows:

- **Ovarian extract**: 2 gm. in 100 c.c. 60 per cent. alcohol were allowed to stand overnight (16 hours) and then filtered; 3 c.c. were used for each experiment.
- **Orchic extract**: ditto.
- **Papain**: 1 gm. in 100 c.c. water. Filtered.
- **Dahlia**: 1 gm. in 100 c.c. 95 per cent. alcohol.
- **Potassium permanganate**: 1 gm. in 100 c.c. water.
- **Hydrochloric acid**: 40 c.c. conc. hydrochloric acid made up to 100 c.c.
- **Thiosinamine**: 2 gm. in 100 c.c. water.
- **Gelatin**: 1 gm. in 100 c.c. water.
- **Albumin**: 1 gm. in 100 c.c. water. Filtered.
Peptone: 1 gm. in 100 c.c. water. Filtered.
Pararosaniline: 1 gm. in 100 c.c. 95 per cent. alcohol.
Paraleucaniline: 200 mg. in 20 c.c. 95 per cent. alcohol.
Ferric chloride: 1 gm. \( \text{FeCl}_3 \cdot 6\text{H}_2\text{O} \) in 100 c.c. water.
Sodium thiosulphate: 2 gm. in 100 c.c. water.

A. RESULTS OBTAINED WITH OVARIAN AND ORCHIC EXTRACTS.

I. Confirmation of Manoilov's technique.

It was found that the reaction as carried out by Manoilov distinguishes between ovarian and orchic extracts in that the former showed the presence of a distinct colour at the end of the reaction, whereas the mixture with orchic extracts was colourless. The test was carried out as follows: to 3 c.c. of each extract were added 10 drops of papain; after a pause of two minutes the following reagents were added: dahlia solution (3 drops), potassium permanganate (10 drops), hydrochloric acid (3 drops), and thiosinamine (5 drops). It was found that dahlia can be replaced by pararosaniline, which is the simplest representative of the triphenylmethane series of dyes.

II. Substitution of Proteins for Papain.

It was found that if the original papain solution was boiled before use, the test could still be carried out with positive results. It is therefore fairly certain that the enzymes contained in the unboiled solution play no essential role in the reaction.

When using ovarian and orchic extract it is entirely unnecessary to add papain, either boiled or unboiled. The original extracts together with \( \text{KMnO}_4 \) (10 drops), pararosaniline (3 drops), HCl (3 drops), and thiosinamine (5 drops) gave quite definite sex-differentiation. The ovarian extract gave a coloured solution, the orchic extract a colourless solution. It is, however, equally clear that the role played by papain usually consists in providing the presence of an adequate supply of protein substance in the mixtures. Three grams of ovarian extract were mixed with 100 c.c. 10 per cent. sodium chloride solution, allowed to stand overnight, and filtered. To 25 c.c. of this filtrate 10 drops of HCl were added. A dense white precipitate was formed, and after standing for a few minutes this was filtered off. From the final filtrate no more protein could be precipitated by either dilute acid or alkali. A similar solution of orchic extract was prepared, and 3 c.c. of each solution used for the following tests:

(a) Both extracts with pararosaniline (3 drops), \( \text{KMnO}_4 \) (8 drops), thiosinamine (5 drops) alone, gave colourless solutions.

(b) If to the original solution 10 drops of papain were added before the above reagents a typical colour was given by the ovarian extract, whilst the orchic extract was colourless. Papain can therefore re-establish the positive results which are lost by removing the proteins from the original extracts.

(c) Positive results were also obtained if, instead of adding papain to the acid treated extract, any of the following protein solutions were added, viz: albumin,
gelatin, or peptone. The addition of peptone, however, did not result in nearly as

It, therefore, seems clear that the essential reaction for sex-differentiation by
the Manoilov method can be simplified in that the papain can be replaced by
proteins such as albumin. It is also important to note that such compounds must,
however, invariably be present.

III. Replacement of Thiosinamine.

Since the original ovarian and orchic extracts contained an adequate amount of
protein, the role of the other reagents was investigated in mixtures containing no
papain or added protein. As far as thiosinamine is concerned, it was found that
complete replacement was possible by any of the following compounds. Sodium
thiosulphate (5 drops), 2 per cent. ferrous sulphate (5 drops), 2 per cent. oxalic
acid (5 drops), or 3 per cent. hydrogen peroxide (3 drops). It is difficult to avoid
the conclusion that the significance of thiosinamine in the original reaction is to
effect the reduction of excess potassium permanganate in the solution.

IV. Replacement of Pararosaniline by Paraleucaniline.

It was found that pararosaniline can be replaced by its reduction product
paraleucaniline. The addition of 10 drops of ferric chloride and 3 drops of para-
leucaniline to the original ovarian or orchic extracts gave well-defined sex-differ-
ettation, as the colour develops rapidly with the ovarian extract if the mixtures are
placed in hot water for a few minutes (see Table I).

V. Stability of the Essential Elements in the Original Extracts.

Positive results are still obtained if to 20 c.c. of the original extracts 5 c.c.
20 per cent. trichloracetic acid are added. Shaking 25 c.c. of the extracts with
20 gm. of alumina cream for one hour, the filtrates gave negative results, while
extracting the precipitates with 0·5 per cent. ammonia and testing these filtrates,
negative results were again obtained.

VI. Effect of Varying the Concentration of Original Extracts.

A variation in the concentration of the original extracts does not influence the
success, if the concentration of oxidizing agent is suitably adjusted. This is shown
in the following tables:

Table I.

<table>
<thead>
<tr>
<th>Concentration of original extracts used in 60 % alcohol</th>
<th>No. of drops of reagent required for positive results with 5 c.c. of extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paraleucaniline</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Table II.

<table>
<thead>
<tr>
<th>Concentration of original extracts used in 60 % alcohol</th>
<th>No. of drops of reagents required for positive results with 3 c.c. of extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pararosaniline</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

In carrying out the Manoilov reaction, other authors have also found that the amount of reagents, especially that of the permanganate, must be carefully adjusted to the particular substance undergoing the test. For example, with the ovarian and the orchic extracts it is possible to obtain a positive reaction for both if too little permanganate or other oxidizing agent is added. Similarly, if a large excess of reducing substance (thiosinamine or thiosulphate) is added finally, a negative reaction is obtained with both. Every pair of substances must be studied in order to obtain the best conditions for carrying out the reaction. This fact was emphasized by Satina and Blakeslee (3).

Conclusions based on the Study of Ovarian and Orchic Extracts.

Although ovarian and orchic preparations have thus proved to be of value in the chemical study of the Manoilov reaction, it must be remembered that they differ in many respects in their chemical and physical properties as well as in their physiological natures. The fact, however, that it is possible to differentiate between them by means of this reaction shows that in all probability the same factors of oxidation and reduction are involved as in the differentiation of the reaction of male and female blood. The experiments described above indicate clearly that the Manoilov reaction does not involve any action on the part of enzymes. As described by Manoilov, the initial chemical change appears to consist essentially of the oxidation of the dye (dahlia) by potassium permanganate to form a coloured acid. When permanganate acts on pararosaniline, an acid is formed which is red in colour. It is precipitated by other acids, and its salts or alkaline solutions are light yellow in colour. Ovarian substance, as a rule, does not interfere with the formation of the red acid and possibly actually aids in its formation by oxidation; orchic substance, on the other hand, appears to aid in the reduction of this acid or at least interferes with its formation. It must be remembered that the colour produced at the end of the reaction in the presence of ovarian extracts is not that of the original dye (dahlia or pararosaniline), since its behaviour to alkalies is quite different. The original dye with alkali forms an insoluble brown compound, whereas the end product of the Manoilov test yields with alkali a yellow solution.

It has been shown that the essential features of the Manoilov reaction can reproduced with paraleucaniline and ferric chloride. With suitable quantities of the latter, reasonable time limits, and warming the mixtures, the extract of the
Chemical Study of the Manoilov Test

female material permits the oxidation to the coloured pararosaniline, that of the

The reaction as originally described has been recognized generally to involve
an oxidation reaction, and the simplified procedure takes advantage of this view
and attempts to use a simple oxidation reaction to bring out the essential features
of the test. At the same time, the exact chemical part played by the ovarian and
orchic substances, and by the various materials from other sources in analogous
tests, is uncertain. Manoilov, by using a dye, then an oxidizing agent, and finally
a reducing agent, made it more difficult to interpret the chemical reaction involved,
but in principle the changes appear to be based upon the concepts developed with
the simplified procedure.

An interesting parallelism, which however may have no real significance, may
be seen in the fact that the orchic substance was found to possess considerable
reducing action as shown by the methods of estimating glutathione developed by
Hopkins (3) and Tunnicliffe (4), while the ovarian substance possessed none. This
indicates that the orchic substance contains the reduced form of glutathione and
the ovarian substance does not. This does not prove that the Manoilov reaction is
due to glutathione in these materials, but, nevertheless, is suggestive.

B. Results obtained with other extracts.

As stated earlier, it is necessary to test each substance or each pair of substances
in order to obtain the most satisfactory conditions. The general reaction, as developed
with orchic and ovarian extracts, shows that the reaction in its simplest terms can
be reproduced with paraleucaniline and ferric chloride; those two reagents by
themselves cause the formation of pararosaniline with the ovarian extract, and non-
formation with the orchic extract. The same procedure applied to extracts of leaves
results in a reaction between the ferric chloride and some constituents of the leaves
which completely obscures the reaction. A satisfactory oxidizing agent for use in
place of ferric chloride has not been found in this investigation. It is therefore
necessary, in testing leaves, to add permanganate to the extract and then para-
leucaniline or the dye (pararosaniline or dahlia); this results in the formation of
the red acid (or corresponding substance of the series in certain cases), but a
reducing agent must finally be added (thiosulphate is perhaps best) to get rid of the
excess permanganate and allow the colour of the red acid to appear. It is not
necessary to add protein material in order to obtain the differentiation between
the sexes, although in some cases the colour is more intense in the presence of
papain, albumin, or peptone. It will be observed, however, that one set of leaves
consistently gave a positive result for the male, while another set gave it for the
female.

In testing human bloods, the same relations were found as with the leaves.
Owing to interference by the ferric chloride, it was necessary to use permanganate,
sulphuric (or hydrochloric) acid, and finally a reducing agent.
For the sake of completeness, a number of experiments with leaves and with blood are recorded.

The leaves were collected at the Brooklyn Botanical Gardens and dehydrated by the Lehn and Fink Products Company. 2 per cent. extracts in 60 per cent. alcohol were made by allowing to shake for one hour on a shaking machine and filtering. 3 c.c. of extract were used for each test.

1. Leaves of Salix cordata. In all experiments the subsequent reagents were as follows: Pararosaniline (2 drops), KMnO₄ (10 drops), HCl (3 drops), thiosinamine (5 drops).

<table>
<thead>
<tr>
<th>Protein used</th>
<th>Result of test</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain (10 drops)</td>
<td>∅ - +</td>
<td>Only slight differentiation</td>
</tr>
<tr>
<td>Albumin (10 drops)</td>
<td>∅ +</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Peptone (10 drops)</td>
<td>∅ +</td>
<td>Intermediate between papain and albumin</td>
</tr>
<tr>
<td>Gelatin (10 drops)</td>
<td>∅ +</td>
<td>Similar to papain.</td>
</tr>
</tbody>
</table>

With this material it was also found possible to replace thiosinamine by oxalic acid, sodium thiosulphate, ferrous sulphate, or hydrogen peroxide. Either pararosaniline or dahlia can be used as the original dye. Paraleucaniline with a slightly increased amount of permanganate also gave positive results.

2. Leaves of Ginkgo biloba. On applying the test to this material, positive results were obtained but in this case the colour was developed with female material and not with male. This is illustrated below.

(i) 1 drop pararosaniline + 10 drops permanganate + 2 drops sulphuric acid + 5 drops thiosinamine.

∅ (+)  
♂ (−).

(ii) 1 drop pararosaniline + 10 drops permanganate + 2 drops sulphuric acid + 5 drops thiosulphate.

∅ (+)  
♂ (−).

2 per cent. and 3 per cent. extracts were also used.

(iii a) 2 per cent. Extract. 2 drops paraleucaniline + 15 drops permanganate + 2 drops sulphuric acid + 10 drops thiosulphate.

∅ (+)  
♂ (−).

(iii b) 3 per cent. Extract. 2 drops paraleucaniline + 20 drops permanganate + 2 drops sulphuric acid + 10 drops thiosulphate.

∅ (+)  
♂ (−).

Tests were also made on extracts from the male and female leaves of Diost... and of Ailanthus. Both sets of leaves gave positive results for the female leaves and negative results for the male leaves.
Chemical Study of the Manoilov Test

3. Experiments with Sheep Blood. 1 c.c. of 10 per cent. potassium oxalate solution was used with 100 c.c. blood, the mixture was filtered through gauze, and made up to a 5 per cent. solution with water. 3 c.c. portions used for the tests.

(i) 10 drops papain + 2 drops dahlia + 11 drops permanganate + 3 drops hydrochloric acid + 5 drops thiosinamine. Two minutes pause after addition of papain (Manoilov reaction).

\[ \varphi (-) \]
\[ \sigma (+) \]

(ii) 2 drops pararosaniline + 12 drops permanganate + 2 drops sulphuric acid + 5 drops thiosulphate.

\[ \varphi (-) \]
\[ \sigma (+) \]

(iii) 15 drops permanganate + 2 drops paraleucaniline + 2 drops sulphuric acid + 10 drops thiosulphate.

\[ \varphi (-) \]
\[ \sigma (+) \]

(iv) 15 drops permanganate + 2 drops sulphuric acid + 10 drops thiosulphate.

\[ \varphi (-) \]
\[ \sigma (+) \] brown, faded on standing.

The results with leaves and sheep blood need very little additional explanation. One set of leaves consistently gave so-called reversed actions for the chemical test, as well as for the test as described by Manoilov. Similarly, the sheep blood gave positive tests for the samples which were stated to be male, and negative tests for those stated to be female. It may be stated that a number of human bloods which were tested gave positive results for the female and negative results for the male. Here also, however, some bloods gave so-called reversed actions, negative for the female and positive for the male.

This study was undertaken at the suggestion of Dr C. B. Davenport of the Carnegie Institution of Washington, Department of Genetics. Thanks are also due to Dr A. F. Blakeslee, Miss S. Satina, and Dr O. Riddle, of that Institution, as well as to Dr Davenport, for their advice in connection with this problem.

SUMMARY.

In the Manoilov reaction it is unnecessary to add an enzyme extract such as papain. A protein material must, however, be added if the solution does not already contain it. The addition of papain in the original Manoilov reaction appears to provide the necessary protein.

Pararosaniline, the simplest of the triphenylmethane series of dyes to which dahlia and methyl green belong, is as satisfactory as the more complex members of the series. With substances which do not form precipitates on the addition of
ferric chloride it was found that paraleucaniline, the reduction product of para-
rosaniline, and ferric chloride alone give the same results as the Manoilov reaction. With solutions of blood and extracts of leaves it is necessary to use permanganate and then, in order to reduce the excess permanganate, a reducing agent such as thiosinamine must be added. Other substances which will reduce permanganate can be used in place of thiosinamine; sodium thiosulphate was found to be a satisfactory substitute.

The probable chemical mechanism of the Manoilov test is discussed, and the procedure simplified. Cases of reverse reaction are recorded.

REFERENCES.