FEULGEN NUCLEAL REACTION

I. QUANTITATIVE EXTRACTION OF FUCHSIN FROM FEULGEN-STAINED NUCLEOPROTEIN

BY TOSISUKE HIRAOKA, D.Sc.

(From the Botanical Institute, College of Science, Kyoto University, Kyoto, Japan)

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Recent advances in cytochemistry have revealed that two types of nucleic acids play important roles in the cell (for references, 1 and 2). Whereas these two types of nucleic acids, deoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA), have similar ultraviolet absorption properties, they are distinguished from each other by certain color reactions based on their constituent sugars. Among these color reactions, the Feulgen nucleal reaction has come to be known as the most specific one for deoxypentose nucleic acid (for references, 3). Owing to its high specificity, the Feulgen reaction has been used not only for the determination of DNA in tissue extracts (reaction in vitro, 4 and 5), but also for DNA determination in a single cell nucleus with the aid of microspectrophotometry (reaction in situ, for references, 6). While much effort has been made with noteworthy success on applications of this reaction to DNA determination in biological materials, less effort has been directed towards an experimental analysis of this reaction, thus leaving much to be learned about the mechanism of the reaction. In order to obtain some insight into this mechanism and at the same time to obtain further improvement in the reaction as a nuclear stain in the field of cytochemistry, it seems essential to determine the amount of fuchsin contained in a Fuelgen-stained sample as the result of the reaction, in terms of a fundamental unit of mass. With this in mind, the author has devised a new method for the quantitative extraction of fuchsin from Feulgen-stained samples (fuchsin extraction method, FEM). This paper deals with the description of this method. It also deals with some problems involving the Feulgen reaction, about which more may be learned by means of experimentation with the aid of the FEM.

Materials

Fuchsin and Schiff Reagent.—In the present investigation, para-D-fuchsin of G. Grüber was used. The dye gave a single spot of Rf 0.84, when its chromatogram was developed with 50

1 In microspectrophotometry, the amount has usually been expressed in an arbitrary unit (6).

525

J. BIOPHYSIC. AND BIOCHEM. CYTOL., 1957, Vol. 3, No. 4
526 FEULGEN NUCLEIC REACTION.

per cent ethanol on filter paper\(^3\). The Schiff reagent was prepared after the procedure given by Romeis (7) with certain modifications in fuchsin, HCl, and NaHSO\(_3\) content. It contained exactly either 10 \(\gamma\)/ml., 100 \(\gamma\)/ml., or 10 mg./ml. fuchsin, together with 0.1 \(\times\) HCl and 0.02 gm./ml. NaHSO\(_3\). The Schiff reagent with the highest fuchsin content was used unless otherwise stated.

Preparation of Raw Materials.—Crude nucleoprotein, containing about 1.2 per cent phosphorus, was prepared from testes of Scomberomorus niphonius after the method of Pollister and Mirsky (8), and thymus nucleic acid, containing 7.4 per cent phosphorus, was extracted from fresh calf thymus glands after the method of Feulgen (9). Egg albumen was the product of E. Merck.

Preparation of Nucleoprotein Samples.—Samples consisting of Scomberomorus nucleoprotein and egg albumen were used for analysis. Such samples were in the form of dry nucleoprotein films, each of which was prepared as follows: One ml. of an alkaline mixture containing 0.5 mg. of nucleoprotein and 0.5 mg. of albumen was neutralized in a centrifuge tube with 1 \(\times\) HCl (towards litmus) and acidified with 0.2 ml. of 30 per cent trichloracetic acid (TCA). The resulting precipitate was centrifuged down, washed twice with cold water, twice with ethanol, and then dried overnight at 30°C. In addition to samples of this kind, others consisting of thymus nucleic acid together with albumen were also used in some cases. The method of preparing the latter was similar to that described above.

Preparation of Albumen Samples.—Samples containing albumen but no nucleoprotein were subjected to the FEM for comparison. Dry albumen films, each prepared from one ml. of albumen solution (1 mg./ml.) by TCA precipitation, served as samples for this purpose.

Preparation of Feulgen-Stained Samples.—Samples for analysis were hydrolyzed with one ml. of 1 \(\times\) HCl at 70°C for exactly 15 minutes\(^4\). The hydrolyzed sample was washed twice with water, and blotted with filter paper. The blotted sample was then treated with one ml. of Schiff reagent of known fuchsin content in a centrifuge tube (which had been tightly closed with a rubber stopper) for 2.5 hours at 15°C. Thereafter, the sample was washed with a washing fluid consisting of 0.1 \(\times\) HCl and 0.02 gm./ml. NaHSO\(_3\), until the final washing fluid, which had been in contact with the sample for 5 minutes, was practically free from Schiff reagent. The absence of Schiff reagent in the final washing fluid was demonstrated in the following manner: A drop of formalin was added to the fluid and the absence of purple coloration was noted visually. Usually, 9 to 10 washings were enough to bring about a practically complete removal of excess Schiff reagent from the stained sample. The stained sample was then subjected to two further washings, this time with water, and served as material for the FEM.

RESULTS

1. Behavior of Fuchsin in Aqueous Media towards Extraction with Amyl Alcohol

Absorption Spectra of Fuchsin in Aqueous Media.—Parafuchsin dissolved in McIlvain buffer solution is purple at pH 2.2 and pink at pH 3.6–7.6. Spectrophotometric studies show that a buffered fuchsin solution has an absorption maximum at 546 mg at pH 2.2–3.6 and at 544 mg at pH 4.6–7.6. However the absorbency of the solution at the peak wave length varies with pH; that is, it reaches its maximum at pH 4.6 and decreases towards pH 2.2 and 7.6 (Table I).

In the case in which fuchsin is dissolved in an acidic medium (0.3 \(\times\) perchloric acid), PCA, the solution is colorless or faint yellow when the dye concentration is

\[ R_f = \frac{\text{Distance moved by the dye from its starting point}}{\text{Distance moved by the solvent front}} \]

\(^3\) Defined as

\(^4\) Under these conditions, optimal staining of the sample was obtained.
high, and shows a strong absorption in the ultraviolet near 220 μm, together with two absorption maxima, a major one at 260 μm, and a minor one at 430 μm. In the case in which the dye is dissolved in an alkaline medium (0.1 N NaOH), the resulting solution is also colorless and again shows a strong absorption in the same region near 220 μm, and two absorption maxima, a major one at 242 μm and a minor one at about 550 μm. The fuchsin, which has been decolorized by acid or alkali, is reversibly restored to its original pink colored state when the pH of the acidic or the alkaline solution is adjusted to 4.6. The restored fuchsin shows an absorption peak at 544 μm.

Behavior of Fuchsin in Aqueous Media towards Extraction with Amyl Alcohol.—When solutions of fuchsin (pH 2.2-7.6), either untreated or restored as described above, are thoroughly mixed with amyl alcohol, the dye is transferred to the amyl alcohol phase. The extracted fuchsin is pink and shows an absorption maximum at 554 μm, irrespective of the pH values of the aqueous solution from which the fuchsin has been extracted. But the amount of fuchsin extracted by amyl alcohol from an aqueous solution of fuchsin varies with the pH of the solution. Table I shows that the highest absorbency (i.e., highest fuchsin concentration) of the amyl alcoholic extract is attained when fuchsin is extracted from aqueous solutions ranging in pH 3.6-5.4.

<table>
<thead>
<tr>
<th>pH of aqueous fuchsin solutions from which fuchsin is to be extracted</th>
<th>Absorbency of aqueous fuchsin solutions (2 7/ml.) before extraction</th>
<th>Absorbency of amyl alcoholic extracts* at 554 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>0.156 (546 μm)</td>
<td>0.469</td>
</tr>
<tr>
<td>3.6</td>
<td>0.394 (&quot; )</td>
<td>0.525</td>
</tr>
<tr>
<td>4.6</td>
<td>0.407 (544 μm)</td>
<td>0.526</td>
</tr>
<tr>
<td>5.4</td>
<td>0.403 (&quot; )</td>
<td>0.525</td>
</tr>
<tr>
<td>6.6</td>
<td>0.356 (&quot; )</td>
<td>0.512</td>
</tr>
<tr>
<td>7.6</td>
<td>0.216 (&quot; )</td>
<td>0.500</td>
</tr>
</tbody>
</table>

* Each extract was clarified with ethanol and made up to 5 ml.

When amyl alcohol is mixed with acidic (0.75 N PCA) or alkaline (0.1 N NaOH) fuchsin solution, amyl alcohol and aqueous phases result. The amyl alcohol phase from the PCA-amyl alcohol mixture is only faintly pink and no marked change in color intensity occurs when it is alkalized with a drop of 0.75 N NaOH. However, the colorless amyl alcohol phase from the NaOH-amyl alcohol mixture turns pink when it is acidified with a drop of 0.1 N HCl, and the resulting pink fuchsin shows an absorption peak at 554 μm after the phase is clarified with ethanol. On the other hand, when the pH is adjusted to 4.6, the colorless aqueous phase from the former mixture turns pink, while that from the latter remains almost colorless. These facts indicate

* When colorless fuchsin was extracted from 5 ml. of its alkaline solution (0.1 N NaOH) with 4.5 ml. of amyl alcohol, an admixture of a drop (0.05 ml.) of 0.05 or 0.1 N HCl with the alcoholic extract, followed by an addition of 0.45 ml. of ethanol, resulted in a quantitative recovery of pink colored fuchsin.
that fuchsin is almost incapable of being extracted by amyl alcohol from its strongly acidic solution, probably due to a decrease in number of undissociated dye molecules in the solution (cf. 10), whereas it is capable of being transferred into amyl alcohol from its alkaline aqueous solution.

**Distribution of Fuchsin between Amyl Alcohol and Aqueous Media.**—Varying amounts of fuchsin, ranging from 10 to 125 γ, were dissolved in 10 ml. of McIlvain buffer of pH 4.6 or 0.1 N NaOH. The aqueous fuchsin solution, either buffered or alkaline, was mixed with an equal volume of amyl alcohol and the mixture was centrifuged to bring about a complete separation of amyl alcohol and aqueous phases. The aqueous phase from the acid mixture was kept buffered at pH 4.6, while that from the alkaline was adjusted to pH 4.6 to bring about a color restoration of the fuchsin. The amyl alcohol phase from the former mixture was clarified by an addition of ethanol, whereas that from the latter was first acidified with HCl and then clarified with ethanol. In all cases, the fuchsin concentration in the aqueous phase was determined from its absorbency at 544 mμ, and that in the amyl alcohol phase from its absorbency at 554 mμ. Since the partition coefficient of fuchsin between aqueous and amyl alcohol phases and that of colorless fuchsin between alkaline aqueous and amyl alcohol phases are constant as shown in Table II, it follows that fuchsin, either pink colored

### TABLE II

**Distribution of Fuchsin between Buffer Solution and Amyl Alcohol (a) and between 0.1 N NaOH and Amyl Alcohol (b) at 21–22°C.**

(Average of triplicate operations)

<table>
<thead>
<tr>
<th>Amount of fuchsin to be distributed between two phases</th>
<th>Concentration in aqueous phase (9.6 ml.)</th>
<th>Concentration in amyl alcoholic phase (10.4 ml.)</th>
<th>Partition coefficient $K = C_1/C_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(γ)</td>
<td>$γ$/ml.</td>
<td>$γ$/ml.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.006</td>
<td>0.950</td>
<td>0.0063</td>
</tr>
<tr>
<td>25</td>
<td>0.014</td>
<td>2.387</td>
<td>0.0059</td>
</tr>
<tr>
<td>50</td>
<td>0.028</td>
<td>4.751</td>
<td>0.0059</td>
</tr>
<tr>
<td>75</td>
<td>0.039</td>
<td>7.093</td>
<td>0.0055</td>
</tr>
<tr>
<td>100</td>
<td>0.056</td>
<td>9.502</td>
<td>0.0059</td>
</tr>
<tr>
<td>125</td>
<td>0.067</td>
<td>11.877</td>
<td>0.0056</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td>0.0059</td>
</tr>
</tbody>
</table>

| (b)                             |                                 |                                 | 0.0984                          |
| 10                              | 0.081                          | 0.833                          | 0.0972                          |
| 25                              | 0.200                          | 2.142                          | 0.0934                          |
| 50                              | 0.416                          | 4.351                          | 0.0956                          |
| 75                              | 0.677                          | 6.560                          | 0.1032                          |
| 100                             | 0.866                          | 8.702                          | 0.0995                          |
| 125                             | 1.110                          | 10.911                         | 0.1017                          |
| **Mean**                       |                                 |                                 | 0.0984                          |
or decolorized by alkali, has a constant molecular weight in both aqueous and amyl alcohol phases. This table also shows that a practically complete transfer of fuchsin into amyl alcohol is easily attained from its buffered solution (pH 4.6) but with some difficulty from its alkaline solution, when the extraction procedure is repeated several times.

Conformity of Amyl Alcoholic Extracts of Fuchsin to the Bouguer-Beer Law.—A quantity of fuchsin, ranging from 0.1 to 20.0 %, was dissolved either in buffer solution (pH 4.6) or in 0.1 N NaOH, and was then extracted with amyl alcohol. The amyl alcoholic extracts of the buffered solution were clarified with ethanol, and those of the alkaline solution were acidified with 0.1 N HCl and clarified with ethanol. All the alcoholic extracts were brought to a final volume of 5 ml. and the absorbency measured at 554 μm. A plot of the absorbencies of the amyl alcoholic extracts of fuchsin against the fuchsin concentrations in the buffered or alkaline solutions before the extraction gives a straight line and shows a good conformity of the extracts to the Bouguer-Beer law.

Stability Tests of Fuchsin.—10 % of fuchsin were dissolved in McIlvain buffer (pH 4.6), in 0.75 N PCA, and in 0.1 N NaOH. Each fuchsin solution was divided into two equal aliquots; one was kept at room temperature, while the other was heated at 98°C. for 15 minutes in a water bath. In the case of the buffered and alkaline fuchsin solutions, the fuchsin was extracted with amyl alcohol directly from these aliquots, whereas in the case of the acidic solution, it was transferred into amyl alcohol after the pH of the aliquots was adjusted to 4.6. The amyl alcoholic extracts of pink colored fuchsin from the buffered aliquots or from the pH adjusted acidic aliquots were clarified with ethanol immediately, while those of colorless fuchsin from the alkaline aliquots were acidified with a drop of 0.1 N HCl (to restore color in the colorless fuchsin) and then clarified with ethanol. Each alcoholic extract was made up to a constant volume (5 ml.) and the absorbency measured at 554 μm. All the extracts gave a constant absorbency irrespective of differences in the preparative procedures; deviations among their absorbencies were statistically insignificant. This fact clearly indicates that neither the decolorization by 0.75 N PCA nor by 0.1 N NaOH nor the treatment at 98°C. for 15 minutes resulted in a detectable decomposition of fuchsin molecules.

2. Liberation of Fuchsins from Fuchsin—Sulfurous Acid Compound

Wieland and Scheuing (11) are of the opinion that when Schiff reagent is prepared, fuchsin reacts with SO₂ producing a colorless fuchsin-SO₂ compound. This compound is labile and readily loses SO₂ to reform the fuchsin (12). A dilute Schiff reagent of low SO₂ content, prepared by diluting Schiff reagent of 10 mg./ml. fuchsin content 1,000-fold with water, is faint pink, but turns pink when it is heated at 98°C. The resulting pink solution shows an absorption maximum at 544 μm at pH 4.6. The diluted Schiff reagent becomes colorless when it is acidified with PCA and heated at 98°C., but turns pink again when its pH is adjusted to 4.6. When the diluted Schiff reagent (pH 4.6) is mixed with amyl alcohol, the pink component is dissolved in the alcoholic extract, which shows an absorption peak at 554 μm. When the diluted Schiff reagent is alkalized with NaOH and heated at 98°C., a colorless solution results. An admixture of this solution with amyl alcohol gives rise to colorless aqueous and alcoholic phases. The alcoholic phase turns pink on
acidiﬁcation with a drop of 0.1 N HCl, and shows an absorption peak at 554 m\(\mu\) after it is clariﬁed with ethanol. These results indicate that the pink colored product derived from the diluted Schiff reagent is nothing other than the fuchsin, which is re-formed from the fuchsin-SO\(_2\) compound on removal of SO\(_2\).

The quantitative recovery of fuchsin from fuchsin-SO\(_2\) upon removal of SO\(_2\) was examined. An aliquot of the diluted Schiff reagent of low SO\(_2\) content, containing an equivalent of 10 \(\gamma\) of fuchsin, was heated at 98°C. for 15 minutes and cooled. Another aliquot was acidiﬁed with PCA (ﬁnal concentration, 0.75 \(\%\)), heated at 98°C. for 15 minutes, and cooled. The pH of these two aliquots was adjusted to 4.6, and the aliquots were mixed with amyl alcohol to extract the fuchsin recovered from the fuchsin-SO\(_2\) compound. The amyl alcohol extract was separated from each mixture and clariﬁed with ethanol. Another aliquot was alkalized with NaOH (ﬁnal concentration, 0.1 \(\%\)), heated at 98°C. for 15 minutes, cooled, and mixed with amyl alcohol to extract the colorless fuchsin which had been recovered from the fuchsin-SO\(_2\) compound. The amyl alcohol extract separated from this mixture was acidiﬁed with a drop of 0.1 N HCl and clariﬁed with ethanol. After all the amyl alcohol extracts were made up to a deﬁnite volume, their absorbencies were measured at 554 m\(\mu\) with an amyl alcoholic fuchsin solution as standard. In each extract, 10 \(\gamma\) of fuchsin were found, showing that the recovery of fuchsin from the fuchsin-SO\(_2\) compound is quantitative under the conditions used.

3. Liberation of Fuchsin from Formaldehyde-Schiff Reagent Compound

Wieland and Scheuing (11) have ascribed the color reaction of aldehyde with Schiff reagent to the formation of a colored addition compound in which one mole of the reagent links with two moles of aldehyde. In view of the fact that an additional compound of aldehyde with bisulﬁte is readily decomposed by the action of acid or alkali which results in the recovery of the original aldehyde (13 and 14), treatment of the formaldehyde-Schiff reagent with acid or alkali will bring about liberation of fuchsin-SO\(_2\). The fuchsin-SO\(_2\) once liberated, can be transformed into fuchsin on removal of SO\(_2\), and thus the recovered fuchsin can be extracted quantitatively with amyl alcohol under appropriate conditions.

When a mixture consisting of one ml. of Schiff reagent (10.0 mg./ml. fuchsin content) and an excessive amount of formaldehyde (80 mg.) is diluted with water to make one liter, the resulting solution contains a formaldehyde-Schiff compound equivalent to 10 \(\gamma\) of fuchsin per ml. It is reddish violet and shows an absorption peak at 562 m\(\mu\) at pH 4.6. The formaldehyde-Schiff reagent compound is not transferred into the amyl alcohol at all, when its solution is mixed with amyl alcohol.

When the formaldehyde-Schiff reagent solution is acidiﬁed with PCA and heated at 98°C., it becomes bluish violet on acidiﬁcation and colorless on heating. The resulting solution turns pink when its pH is adjusted to 4.6, and shows an absorption peak at 544 m\(\mu\), just as in the case of fuchsin. This pink colored product can be transferred into the amyl alcohol, when its solution is mixed with amyl alcohol, and gives an absorption peak at 554 m\(\mu\). An addition of NaOH to the formaldehyde-Schiff

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\(^4\) The recovery of fuchsin from a fuchsin-SO\(_2\) compound was not complete when Schiff reagent rich in SO\(_2\) was used as material.
reagent solution, followed by heating at 98°C., makes the solution colorless. When
the resulting solution is mixed with amyl alcohol, a colorless amyl alcohol extract
results, but turns pink when it is acidified with HCl and clarified with ethanol. It
shows an absorption peak at 554 mµ. All these facts suggest that these hot acid and
alkali treatments liberate fuchsin-SO₂ from the formaldehyde-Schiff reagent com-
pound, and that they also effect a recovery of fuchsin from the liberated fuchsin-SO₂.

TABLE III
Liberation of Fuchsin from Formaldehyde-Schiff Reagent Compound, Equivalent to 10 γ Fuchsin,
by Hot Acid (a) and Alkali (b) Treatments
(Average of duplicate operations)

(a)

<table>
<thead>
<tr>
<th>No. of hot acid or alkali treatment</th>
<th>Fuchsin amount liberated by each treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ</td>
</tr>
<tr>
<td>I</td>
<td>9.91</td>
</tr>
<tr>
<td>II</td>
<td>0.14</td>
</tr>
<tr>
<td>Residue</td>
<td>0.00‡</td>
</tr>
<tr>
<td>Total</td>
<td>10.05</td>
</tr>
</tbody>
</table>

(b)

| I  | 4.50 |
| II | 1.13 |
| III| 0.57 |
| IV | 0.52 |
| V  | 0.46 |
| VI | 0.42 |
| VII| 0.41 |
| VIII| 0.35 |
| IX | 0.32 |
| Residue | 1.32‡ |
| Total | 10.00 |

* Determined from absorbencies at 554 mµ.
† Not directly determined.

One ml. aliquot of the formaldehyde-Schiff reagent solution was acidified with
PCA (final concentration, 0.75 n), heated at 98°C. for 15 minutes, cooled, and its
pH adjusted to 4.6. The resulting pink colored solution was mixed with amyl alcohol,
and after the mixture was separated into two phases, the amyl alcohol extract was
transferred into another container. The extract was clarified with ethanol, whereas the
remaining aqueous phase was again subjected to the procedure described above.
Thus, the amount of fuchsin transferred to amyl alcohol as the result of each hot
acid treatment was determined (Table III(a)). Another one ml. aliquot of the form-
aldehyde-Schiff reagent solution was alkalized with NaOH (final concentration,
0.1 N), heated at 98°C. for 15 minutes, and cooled. The resulting solution was mixed with amyl alcohol, the mixture being separated into amyl alcohol and aqueous phases. The amyl alcohol phase was acidified with a drop of 0.1 N HCl, and then clarified with ethanol, while the aqueous phase was subjected 8 times to the procedure described above. Thus the resulting amyl alcohol extracts were used as material for determination of the fuchsin amount liberated by each hot alkali treatment (Table III(b)).

The results given in Table III show that both the recovery of fuchsin-SO₂ from the formaldehyde-Schiff reagent compound and that of fuchsin from the fuchsin-SO₂ are practically quantitative under the present experimental conditions, though the hot acid treatment is more effective than the hot alkali one for this purpose.

4. Liberation of Fuchsin from Feulgen-Stained Nucleoprotein

Overend and Stacey (15) are of the opinion that fuchsin-SO₂ links with hydrolyzed nucleic acid through sugar aldehyde groups in the manner stated by Wieland and Scheuing (11), not only in the Feulgen reaction in vitro but also in the reaction in situ. If this be the case, a Feulgen-stained nucleoprotein sample is none other than an addition compound of fuchsin-SO₂ bound to the hydrolyzed nucleic acid moiety of nucleoprotein through aldehyde groups. Thus, liberation of fuchsin from the sample should be possible by hot acid and alkali treatments, just as occurs with the formaldehyde-Schiff reagent compound.

A Feulgen-stained nucleoprotein sample, homogenized and suspended in 0.75 N PCA, became blue. When the suspension was heated at 98°C. and cooled, it turned colorless, but when the pH of the resulting suspension was adjusted to 4.6, the dispersion medium of the suspension turned pink, while the suspended phase remained colorless. This pink colored product was capable of being transferred into the amyl alcohol added to the suspension; the amyl alcohol extract showed an absorption peak at 554 mμ. Another stained nucleoprotein sample, dissolved in 0.1 N NaOH and heated at 98°C., gave a colorless solution. When the solution was mixed with amyl alcohol, a colorless amyl alcohol extract resulted. The extract turned pink on acidification with HCl, and showed an absorption peak at 554 mμ, after being clarified with ethanol. From these facts, it is concluded that the linkage between fuchsin-SO₂ and the hydrolyzed nucleic acid moiety of nucleoprotein through aldehyde groups can be split by the action of hot acid or alkali, and that the liberated fuchsin-SO₂ can be transformed into fuchsin, which in turn is capable of being transferred to amyl alcohol and thus extracted.

A Feulgen-stained Scomberomorus nucleoprotein-albumen sample was homogenized, and suspended in 0.75 N PCA or dissolved in 0.1 N NaOH. The resulting acidic suspension was subjected 4 times to the hot acid treatment followed by extraction with amyl alcohol, while the resulting alkaline solution was subjected 8 times to the hot alkali treatment followed by extraction with amyl alcohol. The recovery of fuchsin was not attained from a formaldehyde-Schiff reagent solution rich in SO₂.

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*The recovery of fuchsin was not attained from a formaldehyde-Schiff reagent solution rich in SO₂.*
alcohol (as described in the case of the formaldehyde-Schiff reagent compound). After completion of the last extraction with amyl alcohol, the aqueous phase was clarified by addition of dilute alkali in the former case or by mild heating in the latter case, and its pH adjusted to 4.6. The amount of fuchsin transferred to amyl alcohol from the stained sample, as the result of each hot acid

TABLE IV

Liberation of Fuchsin from Feulgen-Stained Samples by Hot Acid (a) and Alkali (b) Treatments
(Average of triplicate operations)

(a)  

<table>
<thead>
<tr>
<th>No. of hot acid or alkali treatment</th>
<th>Nucleoprotein sample</th>
<th>Albumen sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuchsin amount</td>
<td></td>
</tr>
<tr>
<td></td>
<td>liberated by each</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treatment*</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12.35</td>
<td>0.06</td>
</tr>
<tr>
<td>II</td>
<td>0.21</td>
<td>0.00</td>
</tr>
<tr>
<td>III</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>IV</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Residue</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>12.62</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fuchsin amount liberated, as percentage of total amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>97.9</td>
</tr>
</tbody>
</table>

(b)  

<table>
<thead>
<tr>
<th>No. of hot acid or alkali treatment</th>
<th>Nucleoprotein sample</th>
<th>Albumen sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuchsin amount</td>
<td></td>
</tr>
<tr>
<td></td>
<td>liberated by each</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treatment*</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8.25</td>
<td>0.07</td>
</tr>
<tr>
<td>II</td>
<td>3.56</td>
<td>0.00</td>
</tr>
<tr>
<td>III</td>
<td>0.56</td>
<td>0.00</td>
</tr>
<tr>
<td>IV</td>
<td>0.17</td>
<td>0.00</td>
</tr>
<tr>
<td>V</td>
<td>0.10</td>
<td>0.00</td>
</tr>
<tr>
<td>VI</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>VII</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>VIII</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Residue</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>12.89</td>
<td>0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fuchsin amount liberated, as percentage of total amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.0</td>
</tr>
</tbody>
</table>

* Calculated from absorbencies at 554 mμ in amyl alcoholic extracts and from those at 544 mμ in aqueous extracts.

or alkali treatment, together with the amount not liberated from the sample by the repeated treatments, is given in Table IV. As shown in this table, the fuchsin contained in the sample as the result of the Feulgen reaction is recovered quantitatively from the sample by either the hot acid or alkali treatment under the present experimental conditions.

In order to determine the amount of fuchsin which may be found in a Feul-
FEULGEN NUCLEAR REACTION. I

gen-negative sample after treatment with Schiff reagent, hydrolyzed and Schiff-treated albumen samples were subjected to the analytical procedures for fuchsin mentioned above. Table IV indicates that a minute amount of fuchsin, which does not originate in the Feulgen reaction, is found attached to the sample. The existence of this kind of fuchsin, non-specific to the reaction, cannot be ignored in the case of a sample of poor DNA content treated with Schiff reagent of high fuchsin content.

5. Removal of Excessive Schiff Reagent from Stained Sample

In order to estimate the amount of fuchsin contained in a Feulgen-stained sample by means of the FEM, excess Schiff reagent must be completely re-

### TABLE V

Removal of Excess Schiff Reagent from Hydrolyzed and Schiff-Treated Nucleoprotein and Albumen Samples

(Average of duplicate operations)

<table>
<thead>
<tr>
<th>No. of washing</th>
<th>Amount of Schiff reagent found in each washing fluid, as percentage of the total amount removed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleoprotein sample treated with Schiff reagent of 10 mg./ml. fuchsin content</td>
</tr>
<tr>
<td></td>
<td>100 µg/ml. fuchsin content</td>
</tr>
<tr>
<td>I</td>
<td>99.5902</td>
</tr>
<tr>
<td>II</td>
<td>0.3833</td>
</tr>
<tr>
<td>III</td>
<td>0.0100</td>
</tr>
<tr>
<td>IV</td>
<td>0.0020</td>
</tr>
<tr>
<td>V</td>
<td>0.0015</td>
</tr>
<tr>
<td>VI</td>
<td>0.0013</td>
</tr>
<tr>
<td>VII</td>
<td>0.0010</td>
</tr>
<tr>
<td>VIII</td>
<td>0.0008</td>
</tr>
<tr>
<td>IX</td>
<td>0.0007</td>
</tr>
<tr>
<td>X</td>
<td>0.0007</td>
</tr>
<tr>
<td>XI</td>
<td>0.0007</td>
</tr>
<tr>
<td>XII</td>
<td>0.0007</td>
</tr>
<tr>
<td>XIII</td>
<td>0.0008</td>
</tr>
<tr>
<td>XIV</td>
<td>0.0007</td>
</tr>
<tr>
<td>XV</td>
<td>0.0007</td>
</tr>
<tr>
<td>XVI</td>
<td>0.0007</td>
</tr>
<tr>
<td>XVII</td>
<td>0.0007</td>
</tr>
<tr>
<td>XVIII</td>
<td>0.0007</td>
</tr>
<tr>
<td>XIX</td>
<td>0.0007</td>
</tr>
<tr>
<td>XX</td>
<td>0.0007</td>
</tr>
<tr>
<td>XXI</td>
<td>0.0006</td>
</tr>
<tr>
<td>XXII</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Total: 99.9990 100.0000 100.0000 100.0000
moved from the stained sample prior to the extraction. Two kinds of sample, one nucleoprotein and the other albumen, were hydrolyzed and treated with one ml. of Schiff reagent of either 100 μg/ml. or 10 mg./ml. fuchsin content at 15°C. for 2½ hours. The Schiff reagent was then decanted and the samples were washed for 5 minutes with a washing fluid consisting of 0.02 gm./ml. NaHSO₃ and 0.1 N HCl. The fluid which had been used for washing the samples was combined with the decanted Schiff reagent and brought to a known volume to make the first washing fluid of a definite volume. The samples were washed as often as 21 times with known volumes of fresh washing fluid and the fluid was decanted after each 5 minutes' washing. Since Schiff reagent turns purple when formaldehyde is added to it, the resulting colored product having an absorption peak at 576 mμ, each washing fluid, after addition of formalin, was analyzed photometrically at 576 mμ.

As shown in Table V, excess Schiff reagent is easily removed from the lightly stained nucleoprotein sample by the first washing fluid. The removal of excess Schiff reagent from the heavily stained sample is rather difficult; indeed the amount of Schiff reagent washed out from the sample by the washing fluid decreases rapidly as the washing procedure is repeated, but it remains constant after the ninth washing. Judging from the fact that the removal of excess Schiff reagent from an albumen sample which has been hydrolyzed with HCl and treated with Schiff reagent of 10 mg./ml. fuchsin content, is complete at the ninth washing, it seems reasonable to consider 9 washings sufficient to bring about a practically complete removal of excess Schiff reagent from the heavily stained sample; extraction of Schiff reagent from the stained sample is practically negligible in the ninth and subsequent washings (cf. 16).

The geometry (size and shape) of a stained sample affects the ease with which removal of excess Schiff reagent from the sample is accomplished. In general, a sample which consists of one mg. of nucleoprotein and is in the form of a thin film should facilitate the complete removal of excess Schiff reagent from it.

6. Proposed Method of Fuchsin Extraction from Stained Sample

There are two kinds of procedure for fuchsin extraction from Feulgen-stained samples; one based on hot acid treatment and the other based on hot alkali treatment. As the former procedure is more effective than the latter, it will be illustrated first (Fig. 1).

In most cases, the fuchsin contained in a Feulgen-stained sample is completely liberated by the procedure mentioned above, but in the cases where a minute amount of the fuchsin is retained, not liberated from the sample after the completion of the procedure, the following method based on hot alkali treatment is recommended (Fig. 2).

The absorbencies of the amyl alcoholic extracts obtained by hot acid or alkali treatments are determined at 554 mμ with reference to an appropriate blank and standards. The blank is prepared from a nucleoprotein sample,
hydrolyzed and treated with a blank Schiff reagent (i.e., reagent without fuchsin) by the above mentioned procedures (Figs. 1 or 2). The standards (0.01 to 5.0 μg/ml. fuchsin content) are prepared by diluting fixed volumes of amyl alcoholic fuchsin solutions having dye concentrations ranging from 0.1 to 50.0 μg/ml. with the blank extract.

7. Behavior of DNA and Protein in the Course of the FEM

Dry nucleoprotein films, each of which contained 2.0 mg. of Scomberomorus nucleoprotein and 0.5 mg. of albumen, were prepared from aqueous solutions
FIG. 2. Procedure based on hot alkali treatment

Aqueous suspension
Centrifuged

Precipitate
Dissolved in 1 ml. of 0.1 N NaOH
Colorless solution
Heated at 98°C. for 2 to 8 minutes
Cooled
Colorless solution
Mixed with 1 ml. of amyl alcohol
Emulsified by rapid shakings
Centrifuged

Colorless amyl alcohol phase
Protein gel
alkaline aqueous phase
Combined
Heated at 98°C.
Cooled
Extracted with amyl alcohol
Centrifuged

Amyl alcohol phase
Protein gel
Alkaline aqueous phase
Combined
Treated as above, if necessary, or discarded

Combined
(Total volume, 4.5 ml.)
Acidified with a drop of 0.1 N HCl
Pink extract
Clarified with ethanol
(Final volume, 5 ml.)
Analyzed spectrophotometrically for fuchsin

by TCA precipitation, and DNA and protein amounts were determined with these films at each step of the Feulgen and the fuchsin extraction procedure. For these experiments, a blank Schiff reagent, consisting of 0.02 gm./ml. NaHSO₃ and 0.1 N HCl, was substituted for Schiff reagent, since the presence of fuchsin interfered with certain color reactions for DNA and protein. The
The amount of DNA was determined colorimetrically with the Dische reaction (17), or from phosphorus determinations (18) after the samples were incinerated by PCA (19). The amount of protein was determined from the biuret reaction. The absorbencies of the analytical samples were measured with reference to

**TABLE VI**

<table>
<thead>
<tr>
<th>Behaviors of DNA and Protein Moieties of Nucleoprotein Sample in the Course of the Feulgen and the Fuchsine Extraction Procedure</th>
<th>(Average of triplicate operations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme in Feulgen procedure and FEM</td>
<td>Fraction analyzed</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact sample (1)</td>
<td>100.0</td>
</tr>
<tr>
<td>Hydrolysis of (1) with 3/4 N HCl at 70°C, for 15 min.</td>
<td>Hydrolysate</td>
</tr>
<tr>
<td></td>
<td>Hydrolyzed sample (2)</td>
</tr>
<tr>
<td>Treatment of (2) with blank Schiff reagent at 15°C. for 2 1/2 hrs.</td>
<td>Acid extract, adjusted to pH 4.6 (3)</td>
</tr>
<tr>
<td>Hot acid treatment with 0.75 N PCA, and pH adjustment of the acid extract</td>
<td>Residue on extraction</td>
</tr>
<tr>
<td>Treatment of (3) with amyl alcohol</td>
<td>Amyl alcoholic extract</td>
</tr>
<tr>
<td></td>
<td>Aqueous phase</td>
</tr>
<tr>
<td>Treatment of (2) with blank Schiff reagent at 15°C. for 2 1/2 hrs.</td>
<td>Alkaline solution (4)</td>
</tr>
<tr>
<td>Hot alkali treatment with 0.1 N NaOH</td>
<td>Acidified alcoholic phase</td>
</tr>
<tr>
<td>Treatment of (4) with amyl alcohol</td>
<td>Protein gel</td>
</tr>
<tr>
<td>Acidification of the amyl alcoholic phase</td>
<td>Alkaline aqueous phase</td>
</tr>
</tbody>
</table>

* Not directly determined.

The results obtained are given in Table VI, in which the DNA and protein amounts are represented by percentage of the initial amount of each found in the intact sample.
In the course of the Feulgen procedure, appreciable amounts of DNA phosphorus, deoxypentose, bases (21-23), and protein were removed from the sample by hydrolysis, but no further detectable loss of DNA and protein moieties occurred on treatment of the hydrolyzed sample with blank Schiff reagent. When the blank Schiff-treated sample was heated in an acidic medium, the heat treatment caused a splitting of the sample into DNA and protein moieties (24), and as a result, the DNA moiety and a minor portion of the protein moiety were found in the acid extract, while the majority of the protein moiety remained unextracted in the residue. When the pH of the acid extract was adjusted to 4.6 and amyl alcohol added, the DNA and protein moieties in the extract were not transferred to the amyl alcohol. Since fuchsin can be extracted from its aqueous solution by amyl alcohol, it should be possible to make concurrent determinations of the amounts of DNA and of fuchsin contained in a Feulgen-stained sample.

When the blank Schiff-treated sample was heated in an alkaline medium, an alkaline solution containing both DNA and protein moieties resulted (25). An admixture of this solution with amyl alcohol, followed by centrifugation, gave rise to three phases, viz., amyl alcohol phase, protein gel phase, and alkaline aqueous phase (26); thus the protein moiety and most of the DNA moiety were found in the latter two phases, only a minor portion of the DNA moiety being transferred into the amyl alcohol phase. From the behavior of the DNA and the protein moieties of the sample during the hot acid or alkali treatment and successive extraction with amyl alcohol, insight can be gained into the modus operandi of the FEM.

8. Precision and Sensitivity of the FEM

*Scomberomorus* nucleoprotein-albumen samples were subjected to the Feulgen reaction under controlled conditions, viz., hydrolysis with \( \frac{1}{6} \) \( \text{HCl} \) at 70°C for 15 minutes and treatment with Schiff reagent containing 10 mg./ml. fuchsin at 15°C for 2\( \frac{1}{2} \) hours. After complete removal of the excess Schiff reagent, the fuchsin contained in each sample was extracted by means of the FEM. Fivefold extractions and measurements gave an absorbency of 0.652 ± 0.003 at 554 nm with an allowance of 5 per cent error (27).

Thymus nucleic acid-albumen samples, in which the nucleic acid amount was varied over a wide range (5 to 250 \( \gamma \)) while the albumen amount remained constant, were prepared by TCA precipitation from nucleic acid-albumen mixtures. In addition, samples containing albumen, but no nucleic acid were also prepared. A group of the samples was stained under the controlled conditions mentioned above. From samples, which had been freed from excess Schiff reagent, fuchsin was extracted by hot acid or alkali treatment. The amounts of fuchsin liberated from the stained samples were then determined. The other group of samples was hydrolyzed and treated with a blank Schiff reagent under similar controlled conditions, incinerated by PCA (19),
Fig. 3. Relation between fuchsin content of Feulgen-stained samples and initial DNA contents (Line 1) and that between fuchsin and DNA P contents of stained samples (Line 2).

TABLE VII
Fuchsin and DNA Phosphorus Contents of Feulgen-Stained Samples of Varying DNA Content
(Average of duplicate operations)

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample composition</td>
<td>DNA amount</td>
<td>Albumen amount</td>
<td>DNA P amount in untreated samples (calculated)</td>
<td>DNA P amount in stained samples</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>γ</td>
<td>γ</td>
<td>γ</td>
</tr>
<tr>
<td>0.0</td>
<td>1,000.0</td>
<td>0.0</td>
<td>—</td>
<td>0.00</td>
</tr>
<tr>
<td>5.0</td>
<td>1,000.0</td>
<td>0.4</td>
<td>—</td>
<td>0.48</td>
</tr>
<tr>
<td>7.5</td>
<td>1,000.0</td>
<td>0.6</td>
<td>—</td>
<td>0.60</td>
</tr>
<tr>
<td>10.0</td>
<td>1,000.0</td>
<td>0.7</td>
<td>0.5</td>
<td>0.92</td>
</tr>
<tr>
<td>25.0</td>
<td>1,000.0</td>
<td>1.9</td>
<td>1.4</td>
<td>3.04</td>
</tr>
<tr>
<td>50.0</td>
<td>1,000.0</td>
<td>3.7</td>
<td>2.7</td>
<td>6.20</td>
</tr>
<tr>
<td>75.0</td>
<td>1,000.0</td>
<td>5.6</td>
<td>4.1</td>
<td>9.80</td>
</tr>
<tr>
<td>100.0</td>
<td>1,000.0</td>
<td>7.4</td>
<td>5.4</td>
<td>13.30</td>
</tr>
<tr>
<td>250.0</td>
<td>1,000.0</td>
<td>18.5</td>
<td>13.6</td>
<td>33.30</td>
</tr>
</tbody>
</table>

* Not determined due to the poor sensitivity of the P-determination.
and analyzed for phosphorus (18). The results obtained are given in Table VII and illustrated in Fig. 3.

In the hydrolyzed and Schiff-treated albumen sample, a minute amount (0.08 γ) of fuchsin was retained. This amount of non-specific fuchsin was subtracted from the fuchsin amount found in each stained DNA albumen sample to obtain the amount of fuchsin ascribable to the Feulgen reaction. The hydrolyzed and blank Schiff-treated albumen sample contained 0.4 γ phosphorus, which did not originate in DNA. The DNA phosphorus content of each hydrolyzed and blank Schiff-treated DNA albumen sample was obtained by subtracting 0.4 γ from the total phosphorus amount found. This corrected value was taken as the amount of DNA phosphorus contained in each Feulgen-stained sample. As the DNA here contained 7.4 per cent phosphorus, the DNA phosphorus content of each original, untreated sample was estimated by calculation. From comparison of the DNA phosphorus contents of samples before hydrolysis (Table VII, column 3) and after hydrolysis (Table VII, column 4), it can be seen that hydrolysis consistently brings about a loss of 26 to 27 per cent DNA phosphorus, irrespective of a variation in nucleic acid content of the samples. As shown in Fig. 3, line 1, a plot of the fuchsin amounts contained in the stained samples as the result of the Feulgen reaction against the nucleic acid contents of the intact samples (5 to 250 γ) results in a straight line. A linear relationship is also obtained by plotting the fuchsin amounts against the DNA phosphorus contents of the stained samples (0.5 to 13.6 γ DNA phosphorus, line 2). These experiments demonstrate two points: (1) the amyl alcoholic extracts of fuchsin obtained by the FEM from the stained samples conform to the Bouguer-Beer law; (2) the 50-fold range in concentration of DNA over which the FEM may be utilized.

DISCUSSION

Although Feulgen and Rossenbeck (28), the discoverers of the nucleic reaction, observed in pus cells that the coloration due to the reaction is fairly stable against acid and alkali, Milovidov (29) has reported that Feulgen-stained cell nuclei gradually lose their reaction color when they are treated with acid and alkali, and turn colorless when their caryotin has been dissolved by acid and alkali. Owing to such instability of a Feulgen-stained sample, determination of the fuchsin content by simple extraction of the sample appears justified. Recently, Sibatani (30) has succeeded in determining the ratio of DNA to fuchsin in a strongly acidified extract from Feulgen-stained cell nuclei, but unfortunately has failed to make a quantitative extraction of the fuchsin from the nuclei. Our extraction method presented in this paper permits a quantitative determination of the fuchsin content of a Feulgen-stained nucleoprotein sample in terms of an absolute unit of mass, not of an arbitrary one.

1 The treatment with the blank Schiff reagent did not cause any loss of DNA phosphorus from the hydrolyzed samples.
There exist a number of problems concerning the Feulgen reaction in situ, which may be approached through the use of the FEM. Some of them are considered below. In the first place, the influence of various steps in the Feulgen procedure, such as hydrolysis, treatment with Schiff reagent, etc., upon the fuchsin content of a Feulgen-stained sample in situ may be easily examined by means of the FEM. Thus, it is now possible to determine the precise conditions under which the Feulgen reaction can be made to yield the maximum color intensity when used as a cytochemical nuclear stain. In the second place, stoichiometrical analysis of the Feulgen reaction in situ may now be carried out by means of the FEM. We have demonstrated that a quantitative relationship exists between the amount of fuchsin bound and the DNA phosphorus content of a nucleoprotein sample treated with Schiff reagent at a certain stage of hydrolysis. In the third place, the FEM permits physicochemical examinations of the modus operandi of the Feulgen reaction in situ. Evidence has been presented that in this reaction, fuchsin-SO$_2$ is bound with the hydrolyzed nucleic acid moiety of nucleoprotein according to the mode of Langmuir's adsorption isotherm. Though valuable contributions to an understanding of the mechanism of the Feulgen reaction have been made by Feulgen and Voit (31), Overend and Stacey (15), Overend (16), and others, there remains much to be learned about the problem; quantitative chemical and physicochemical studies utilizing the FEM should provide valuable information hitherto inaccessible. In the fourth place, a microdetermination of DNA with the Feulgen reaction followed by the FEM may be carried out in milligram quantities of biological materials, since the fuchsin amount liberated from a Feulgen-stained sample of minute DNA content (of the order of 10 $\gamma$) can be determined by means of the FEM (Fig. 3). We have already been able to make DNA determinations on small quantities of plant and animal tissues in this way. In the fifth place, the FEM now makes possible investigations aimed at checking and improving microphotometric DNA determinations based on the Feulgen reaction (cf. 30). The results of our investigations of such problems will be reported later in separate papers.

**SUMMARY**

A new extraction method for the quantitative determination of the fuchsin contained in a Feulgen-stained nucleoprotein sample has been introduced. The method is based on the following facts: (1) Treatment of a Feulgen-stained nucleoprotein sample with hot acid or alkali brings about a splitting of the linkage between fuchsin-SO$_2$ and the hydrolyzed nucleic acid moiety of nucleoprotein through aldehyde groups. (2) It also effectuates the formation of fuchsin from the liberated fuchsin-SO$_2$. (3) The fuchsin is made colorless by the treatment, but is restored to its original pink colored state when the pH of
the acidic or alkaline medium is adjusted to 4.6. (4) The fuchsin, either pink colored or decolorized by alkali, can be extracted from an aqueous phase by amyl alcohol. A linear relationship was found to exist between the amount of fuchsin extracted by the FEM from a Feulgen-stained nucleoprotein sample and its DNA content. This relationship holds over a wide range of DNA concentration. From experiments utilizing this method, knowledge may be gained about the mechanism of the Feulgen reaction in situ which can lead to an improvement of the reaction in the field of cytochemistry.

REFERENCES