Study on the Influence of Temperature Upon the Peroxidase Reaction of Blood Cells.  
The 5th Report of Histochemical Study of Peroxidase  

By  

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All chemical reactions take place at a definite rate depending on the conditions, of which the most important are concentration of reactants, radiation, presence of a catalyst or inhibitor, and temperature. Increase of temperature almost invariably increases the velocity of a chemical reaction to a marked extent, and for homogenous process the velocity constant is approximately doubled or trebled for each 10°C rise of temperature: this is sometimes expressed in the form of the temperature coefficient, \( \frac{k_{t+10}}{k_t} = 2 \) to 3, where \( k_t \) is the velocity constant at \( t^\circ \) and \( k_{t+10} \) at 10°C higher.  

It is obvious that so-called peroxidase staining of blood cells is a histochemical reaction and temperature has much influence on it, i.e. the peroxidase staining fails very often at low temperature under 10°C. But, at a high temperature, the same result as in a case of a single chemical reaction, can never be obtained in the peroxidase staining. This is very clear from the fact that the peroxidase staining needs a definite temperature to get the best result.  

I attempted here to examine the influence of temperature upon the peroxidase staining with special reference to the following problems:  
a) The optimal temperature for the staining.  
b) High or low temperature as inhibitor of the staining.  
c) The meaning of room temperature for the staining.  

Methods of investigation  

1) The NAS-Benzidine solution (Mitsui & Ikeda) was adopted
here for the peroxidase stain because of its simplicity and sensibility. This solution was put into a tube instead of using a pipette. The blood smear was stained in it. The staining procedure was as follows:

a) The unfixed blood smear was stained with the NAS-Benzidine solution for one minute. The smear should be always stained in the tube.

b) Washed in tap water.

c) Counterstained with Pfeiffer's solution for one minute.

d) Washed, dried, and examined with oil lens.

e) Myelogenous leucocytes and erythrocytes were observed.

2) Low temperature in the experiments was obtained with an ice-safe, and high temperature with an incubator. These apparatus were specially made by the author for this study.

3) Blood smears were taken from the same healthy person, and the temperature of both the smears and the NAS-Benzidine solution was changed simultaneously in the apparatus, but in the experiments above 40°C only the smears were heated up to the various high temperatures, while the NAS-Benzidine solution as peroxidase stain was always kept at room temperature (25°C).

**Observations**

1) Change of the intensity of the peroxidase staining with decrease of temperature.

In order to evaluate the intensity of the staining, I used the peroxidase index of 0, 1, 2, and 3. Zero as negative, 1 as weak, 2 as middle-grade, and 3 as strong peroxidase reaction. The index of each neutrophilic leucocyte was first examined, and the average index of two hundred leucocytes was calculated. The average index should always lie between 3 and 0, as 3 is the strongest and 0 is an entirely negative reaction. The result obtained is shown in Table I. The temperature in the Table is that of the NAS-Benzidine solution used to stain blood smears.

It is clear that the lower the temperature, the lower the indices become, in other words, the reaction becomes weaker as the temperature decreases. Between 28°C and 37°C the strongest reaction is obtained, below 20°C there is a distinct decrease, and especially, at 1°C only one half of the index of 28°C is obtained. Therefore, we can say that extreme care must be taken when staining if the room temperature is under 20°C, for instance, the staining time must be
longer in winter than in summer. However, it should be noticed that the reaction never becomes negative even at 0°C. In Table I, the index of 0°C amounts to more than that of 1°C. I suppose this result may show the existence of various factors besides temperature in the peroxidase staining.

2) Change of the intensity of the peroxidase staining with increase of temperature.

As above described, the peroxidase reaction becomes stronger with increase in temperature below 37°C, while over 37°C the opposite results are shown. The reaction becomes weaker and weaker as the temperature rises, and yet, the reaction never occurs finally. The detailed result is shown in Table II. The peroxidase staining was carried out with the NAS-Benzidine solution at 25°C after cooling the heated smear is the air for one or two minutes. As Table II shows, high temperature from 40°C to 125°C inhibits the peroxidase reaction in various degrees, and of course the more the temperature rises the weaker the reaction becomes. Furthermore it never occurs at 140°C for five minutes although the peroxidase granules are still resistant to 130°C for five minutes.

It is generally believed in botany that the enzymes in dried systems are more resistant to high temperature (100°-120°C) than those in wet systems.

3) Difference between myeloid leucocytes and erythrocytes according to change of temperature.

a) The blue peroxidase positive granules never occur within erythrocytes below 70°C when they are stained with the NAS-Benzidine solution for one minute. But these granules begin to appear within erythrocytes when the smear is heated at 80°C for 35 minutes or at 100°C for 15 minutes, and yet, at 130°C for ten minutes, the neutrophils never show granules, while eosinophils distinctly show peroxidase positive brown granules, and also erythrocytes show distinct peroxidase positive blue granules. However eosinophils and erythrocytes never show peroxidase positive granules at 130°C for 25 minutes. In low temperatures, erythrocytes never show such blue granules in cytoplasm even at 0°C. Therefore it is suggested to us that the peroxidase reaction of erythrocytes becomes positive only at high temperature by this staining method (one minute method).

b) There are many differences between eosinophils and neutrophils as seen in the results of NAS-Benzidine reaction at various temperatures. Eosinophilic granules are more resistant to high temperature
than neutrophilic, namely, at 130°C for 10 minutes the neutrophilic become negative, while the eosinophilic still remain positive. Furthermore, at 2°C for 2 hours or at 125°C for 5 minutes peroxidase granules of neutrophils are blue, while those of eosinophils entirely brown. Therefore the eosinophilic granules generally seem to be more difficult to stain blue than the neutrophilic.

c) The stained substance of eosinophilic granules with the NAS-Benzidine solution probably differs from the stained substance with May-Giemsa stain. This presumption is due to the fact that the eosinophilic granules in leucocytes, at 130°C for 25 minutes do not stain with the NAS-Benzidine solution, but stain red with May-Giemsa stain. Furthermore the shape, size, and localisation of the granules are not quite the same with the above two staining methods at various temperatures.

d) Mitsui and Ikeda have already made it clear that the method of showing blue granules such as Sato's or Osgood's does not differ essentially from that of showing brown granules such as Graham's, because the reactions of the two methods are linked with each other. In other words, the method of showing blue granules in the myelogenous blood cells indicates brown granules at first, which are gradually changed into blue according to the staining time at room temperature from 20°C to 30°C. The very same conclusion was drawn here also from the following experiments. At room temperature from 20°C to 30°C for one minute, the eosinophilic granules stain blue or in the state of double phase of blue and brown with the NAS-Benzidine solution, while the latter granules stain only brown at 2°C for 2 hours or at 125°C for five minutes. Again, at 90°C for 10 minutes the neutrophilic granules stain only brown when the NAS-Benzidine solution is added to the heated smear just immediately after taking out of the incubator lest the smear should be cooled in the air, while the latter granules stain diffusely blue when the NAS-Benzidine solution is added after the heated smear is cooled in the air for several minutes. The temperature of the NAS-Benzidine in the above experiments must be nearly 25°C as already described. As these results show, both the blue and brown granules do not differ essentially from each other, but indicate each a phase of the peroxidase reaction (cf.: Mitsui & Ikeda: On the Four Phases of Peroxidase Staining of Blood Cells Using Benzidine and Salts, 1951).
Table I. Change of peroxidase index of neutrophils with low temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Peroxidase index</th>
<th>Temperature</th>
<th>Peroxidase index</th>
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<tbody>
<tr>
<td>28°C</td>
<td>2.10</td>
<td>6°C</td>
<td>1.35</td>
</tr>
<tr>
<td>20°C</td>
<td>2.05</td>
<td>5°C</td>
<td>1.46</td>
</tr>
<tr>
<td>15°C</td>
<td>1.80</td>
<td>4°C</td>
<td>1.30</td>
</tr>
<tr>
<td>10°C</td>
<td>1.75</td>
<td>3°C</td>
<td>1.42</td>
</tr>
<tr>
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<td>1.75</td>
<td>2°C</td>
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</tr>
<tr>
<td>8°C</td>
<td>1.70</td>
<td>1°C</td>
<td>1.05</td>
</tr>
<tr>
<td>7°C</td>
<td>1.45</td>
<td>0°C</td>
<td>1.40</td>
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Table II. Change of peroxidase reaction of neutrophils with high temperature and its heating time

<table>
<thead>
<tr>
<th>heating time (minutes)</th>
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<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
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<td></td>
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<tr>
<td>37°C</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>±</td>
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<td>-</td>
</tr>
<tr>
<td>90°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100°C</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>110°C</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>120°C</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>125°C</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>130°C</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Summary

Extreme care must be taken of room temperature when staining peroxidase granules of leucocytes, since change of temperature exerts a certain influence upon the peroxidase staining. The optimal temperature of the staining lies between 28°C and 37°C, and both lower and higher temperatures than this inhibit the staining in various degrees. The peroxidase reaction becomes entirely negative at 140°C for 5 minutes, however it is still positive at 130°C for 5 minutes. The
reaction never becomes negative even at 0°C for 10 hours, although
the peroxidase index used to express the intensity of the reaction
numerically, decreases clearly. The brown and blue granules shown
in the peroxidase reaction do not differ essentially with each other
judging from the results of the experiments on the effect of change
in temperature.

(cf.: The 7th Report of Histochemical Study of Peroxidase)

References