SYNTHESIS OF FACTOR V (PYRIDINE NUCLEOTIDES) FROM NICOTINIC ACID IN VITRO BY HUMAN ERYTHROCYTES

BY HENRY I. KOHN AND J. RAYMOND KLEIN

(From the Department of Physiology and Pharmacology and the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

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We have shown that incubation of defibrinated human blood with nicotinic acid leads to a marked increase in the factor V content of the cells, and that ingestion of nicotinic acid by man leads to a similar increase in the factor V content of the blood cells (1). We believed that the synthesis occurred in the erythrocytes, because the factor V content of normal blood is practically confined to them. Axelrod, Gordon, and Elvehjem (2), who recently confirmed the synthesis in vivo, have also taken the same view. Vilter, Vilter, and Spies (3), however, although able to confirm the synthesis in vitro by blood cells, state that "...it seemed that the red cells stored and carried the enzymes instead of performing the synthesis." This statement was based on their inability to demonstrate synthesis with erythrocytes washed free of leucocytes. They concluded that, "The above findings are inconsistent with the statement of Kohn and Klein that the normal erythrocytes accomplish the synthesis...and suggest the tentative hypothesis that nucleated cells are essential for the synthesis..."

The object of this work is to reinvestigate the synthesis of factor V by erythrocytes.

EXPERIMENTAL

To prove that erythrocytes can synthesize factor V in vitro it is necessary to show that the amount of synthesis by a suspension

1 Factor V is the factor necessary for the cultivation of Hemophilus parainfluenzae. Of known compounds only di- and triphosphopyridine nucleotide can serve as factor V.
Synthesis of Factor V

of blood cells is independent of the number of leucocytes present. Obviously, it is essential to use a suspension of undamaged cells. Before gross injury such as hemolysis is evident, the ability of the erythrocytes to synthesize factor V can be decreased by washing with an unfavorable medium and undue handling; e.g., too frequent passage through capillary pipettes. The level of factor V can also be decreased by such treatment. These effects may be due in part to the release of a heat-labile system within the cells which destroys factor V. The existence of such a system may be demonstrated by hemolyzing the cells in water and following the progressive loss of factor V, as shown in Fig. 1. The technique of the factor V assay has been previously described (4, 5).

The following experiment illustrates how the synthesizing abil-

![Graph showing the disappearance of factor V from blood following hemolysis.](image)

**Fig. 1.** Disappearance of factor V from blood following hemolysis, at 25°. 1 ml. of venous blood was mixed with 9 ml. of water, and samples were assayed for factor V at various times thereafter. The rate of inactivation, which is first order with respect to time, is due chiefly to the heat-labile system liberated from the cells by hemolysis.
ity of the cells may be damaged by the use of unsuitable suspension media. Venous blood was defibrinated by shaking with glass beads. Two 1 ml. aliquots were placed in stoppered test-tubes and kept at room temperature during the preparation of the following cell suspensions. Four samples of the blood were centrifuged 10 minutes at 2400 R.P.M. The supernatant liquid was removed. One sample of cells was mixed by gentle pipetting with an equal volume of sodium chloride solution, 0.9 gm. per 100 ml., a second with Ringer-phosphate solution, 0.025 M phosphate, pH 7.4, a third with Ringer-phosphate solution containing 1 mg. of glucose per ml. In these cases care was taken not to remove the white cells. In the fourth case about two-thirds of the cells was removed from the bottom of the tube with a pipette whose tip was drawn out to a long capillary, transferred to a dry tube, and mixed with an equal volume of Ringer-phosphate solution containing glucose. The suspensions were centrifuged 8 minutes. The supernatant liquid was removed and the cells treated as before, with 4 volumes of medium. The suspensions were then centrifuged 5 minutes, the supernatant liquid removed, and the cells treated as before, with 1.5 volumes of medium. These operations were carried out under sterile conditions over a 5 hour period.

1 ml. of each suspension plus 0.05 mg. of neutralized nicotinic acid in 0.1 ml. of water and, as a control, 1 ml. of each suspension plus 0.1 ml. of its suspending medium were incubated 19 hours at 33-35°. After incubation, during which no detectable hemolysis occurred, the factor V in each mixture was assayed. The percentage increase in factor V in each mixture containing nicotinic acid over its control was calculated as a measure of synthesis. The results are given in Table I.

The data in Table I show that the synthesizing ability of the cells is greater in Ringer-phosphate solution containing glucose than in Ringer-phosphate or sodium chloride solution.

The increase in factor V in the case of the preparation made from the lower layer of cells was 27 per cent. The decrease in leucocytes was 99.5 per cent; the decrease in synthesis of factor V with respect to its similarly washed control was 44 per cent. That the decrease in factor V synthesis was not a function of the decrease in white cells, but of the manipulations employed in preparation, is shown by the experiment given below.
Venous blood was defibrinated by shaking with glass beads. Two 1 ml. aliquots of the blood were placed in stoppered tubes and kept at room temperature during the preparation of the following suspensions. In order to remove the leucocytes 8 ml. of blood were mixed with 5 ml. of Ringer-phosphate solution containing glucose, and then centrifuged 5 minutes at 2400 R.P.M. The upper layer of cells and the supernatant liquid were removed, leaving 4 ml. of cells which were mixed with 8 ml. of medium and centrifuged for 5 minutes. The upper layer of cells and supernatant liquid were removed, leaving 2.5 ml. of cells which were finally suspended in 2.5 ml. of medium. There were ten leucocytes per million erythrocytes in this preparation. Another sample of

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Effect of Suspension Medium upon Synthesis of Factor V by Human Blood Cells</td>
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</table>

<table>
<thead>
<tr>
<th>Medium</th>
<th>Per cent increase in factor V</th>
<th>Leucocytes per million erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>68</td>
<td>1000</td>
</tr>
<tr>
<td>Sodium chloride solution</td>
<td>18</td>
<td>1000</td>
</tr>
<tr>
<td>Ringer-phosphate solution</td>
<td>37</td>
<td>1000</td>
</tr>
<tr>
<td>&quot; containing glucose</td>
<td>48</td>
<td>1000</td>
</tr>
<tr>
<td>&quot;</td>
<td>27</td>
<td>5</td>
</tr>
</tbody>
</table>

the blood was treated similarly except that only the supernatant liquid was removed.

1 ml. of each suspension plus 0.05 mg. of neutralized nicotinic acid in 0.1 ml. of water and, as a control, 1 ml. of each suspension plus 0.1 ml. of its suspending medium were incubated 19 hours at 33-35°. The time required from venepuncture to the beginning of the incubation was 3 hours. After incubation the factor V assay of each mixture was made, and the percentage increase in each mixture containing nicotinic acid over its control was calculated.

The increase was 63, 64, and 65 per cent for the original blood, the cell suspension from which most of the leucocytes were removed, and the washed cells respectively. There were 700, 10, and 535 leucocytes per million erythrocytes in the preparations in the order given.
This experiment indicates that the synthesis of factor V was not a function of the number of leucocytes. It must be concluded, therefore, that human erythrocytes can synthesize factor V from nicotinic acid in vitro.

Comparison of the experiments shows that the difference in the extent of synthesis in serum and in Ringer-phosphate solution containing glucose obtained in the first experiment was due to the excessive handling of the cells.

However, even when the separation of the two types of cells was made under conditions unfavorable for synthesis, i.e. suspension in saline and excessive handling, synthesis of factor V by the erythrocytes to the extent of a 15 to 25 per cent increase could always be obtained. Such obvious damage as hemolysis or the presence of an inhibitor, e.g. 1 to 2 mg. of potassium oxalate per ml. of suspension medium, inhibits the synthesis. The oxalate inhibition occurs even with fresh untreated blood.

On the basis of the above experiments it may be suggested that the inability of Vilter, Vilter, and Spies (3) to demonstrate an increase in factor V when "carefully washed" erythrocytes were incubated with nicotinic acid was due to two factors. First, the cells were damaged. Secondly, the relatively small synthesis performed by these cells could not be detected by the published method of Vilter, Vilter, and Spies (6) which is based upon the visual comparison of the bacterial growth elicited by dilutions of blood of 1, 1.5, 3, 6, and 12 ml. per 12,000 ml.

SUMMARY

Human erythrocytes can synthesize factor V from nicotinic acid in vitro. The presence of oxalate, the absence of glucose, suspension of the cells in sodium chloride solution, and excessive handling of the cells tend to diminish the synthesis.

BIBLIOGRAPHY

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