STUDIES BY ULTRACENTRIFUGE AND PAPER ELECTROPHORESIS ON THE EFFECTS OF HEPARIN AND HEPARINOID SUBSTANCE ON THE LIPOPROTEINS IN THE SERUM OF CHOLESTEROL-FED RABBITS

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The observation that heparin in vivo causes a rapid clearing of visible fat from the blood of lipemic animals has attracted widespread attention in recent years (1). This lipemic-clearing action requires extremely small intravenous doses of heparin, but it does not occur when heparin is added to lipemic blood or plasma in vitro (2).

A suggestion has been made on the basis of a number of observations that heparin may play a specific part in the physiology of lipoid metabolism. Graham et al. (1951) (3) found that in man during lipemia and in the cholesterol-fed rabbit, heparin injection produced a change in the distribution of low-density lipoproteins, with reduction in the Sf 10-50 component. This finding has led to a speculation whether individual and interspecific differences in the activity of the clearing-factor system may play a part in determining the difference of susceptibility to cholesterol atheroma (4).

The results obtained in an undertaking to observe this matter by means of paper electrophoresis and ultracentrifuge are here reported.

METHODS

The whole experimental period was 70 days. Young rabbits of mixed strain, weighing 1.9-3.2 kg. were divided into four groups. The animals of group I served as untreated controls. After the other three groups were fed 1 g. cholesterol daily for preliminary 50 days, groups II and III were then intravenously injected respectively with heparin (3 mg/kg.) and heparinoid substance (sulfated chitosan prepared by the writers, 15 mg/kg.) four times for 20 days. Group IV was untreated for the 20 days. Three rabbits were employed in each group, and the results shown in the tables represented their average value.

Electrophoresis of serum lipoproteins was carried out on paper by the method of Grassmann and Hanning (5) with subsequent staining of the protein with Bromphenolblue, and of the lipid with Sudan IV.

For the quantitative determination of the relative amounts of protein and lipids the paper strips after being stained were immersed for half an hour in paraffin oil. This produced sufficient clearing of the paper strips so that they

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could be read in a densitometer. The diagram thus obtained was analysed by torsion balance.

Ultracentrifuge of serum lipoprotein was carried out by the method of Gofman and co-workers (6). Plasma was diluted by an equal volume of 26.0% NaCl solution to yield a mixture with a density of about 1.413. This mixture was centrifuged at 56,100 r.p.m. (150,000 G) for three and one-half hours in the preparative rotor of a Spinco Model E ultracentrifuge. The top lipidrich layer was removed with a syringe and a needle which was hooked at the end. The lipoproteins of the top fraction were analyzed in the analytical rotor of the Spinco ultracentrifuge and classified according to their flotation rates, as recommended by Gofman and associates.

The analytical rotor was first brought to a speed of 39,460 r.p.m., and pictures were taken at two minute intervals. The speed of the rotor was increased to 50,740 r.p.m., and photographs were taken at intervals of four minutes, depending on the complexity of the observed pattern. The analysis of these patterns was made by the conventional planimeter method.

Paper electrophoresis of the isolated lipoproteins was carried out as described above. Animals were autopsied at the end of the 70 days.

RESULTS

1. Ultracentrifugation of lipoprotein concentrates of rabbit serum demonstrates two components in this experiment. Dependent on decreasing density, their flotation rates are $S_f 30-70$ and $S_f < 30$ (fig. 1, 2).

![Fig. 1. Ultracentrifugal patterns of lipoproteins of the normal (A) and cholesterol-fed rabbit serum (B).](image-url)
Fig. 2. Ultracentrifugal lipoprotein.

The \( S_f 30-70 \) component was significantly decreased and the \( S_f < 30 \) component was increased in cholesterol-fed and in untreated rabbit after cholesterol feeding. In the cholesterol-fed rabbit the injection of heparin and heparinoid substance, caused an increase of the \( S_f 30-70 \) component and a decrease of the \( S_f < 30 \) component; their values came near the normal value.

2. The electrophoretic serum protein patterns revealed 6 bands which corresponded in order of increasing mobility to the \( \gamma-, \beta_2-, \beta_1-, \alpha_2-, \alpha_1- \) globulins and albumin of free electrophoresis.

In cholesterol-fed rabbits albumin and \( \beta_2 \)-globulin decreased remarkably, on the contrary \( \alpha_2-, \beta_1- \) and \( \gamma- \) globulins increased, but injection of heparin and heparinoid substance in such a rabbit caused an increase of albumin and \( \beta_2 \)-globulin, and a decrease of \( \alpha_1-, \alpha_2-, \beta_1- \) and \( \gamma- \) globulins (table 1). The papers stained with the Sudan dye generally presented 2 distinct zones, the more prominent corresponding to the \( \beta- \) globulin region and the less intense area to the albumin.

**Table 1.** Paper Electrophoretic Profile of Serum Protein

<table>
<thead>
<tr>
<th>Table 1. Paper Electrophoretic Profile of Serum Protein</th>
<th>Protein values (% of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>53.6</td>
</tr>
<tr>
<td>Heparin injection after cholesterol feeding for 50 days</td>
<td>46.2</td>
</tr>
<tr>
<td>Heparinoid substance injection after cholesterol feeding for 50 days</td>
<td>41.3</td>
</tr>
<tr>
<td>No treatment after cholesterol feeding for 50 days</td>
<td>28.3</td>
</tr>
</tbody>
</table>
In cholesterol-fed rabbit, with respect to lipid content which corresponded to protein bands, $\beta_1$, $\beta_2$ and $\gamma$-globulin lipids increased, and albumin, $\alpha_1$ and $\alpha_2$-globulin lipids decreased, but heparin and heparinoid substance injection in cholesterol-fed rabbit caused an increase of $\alpha_1$, $\alpha_2$ and post-$\gamma$-globulin lipids and a decrease of $\beta_1$, $\beta_2$ and $\gamma$-globulin lipids (table 2).

<table>
<thead>
<tr>
<th>Lipid values (% of total lipid)</th>
<th>Albumin</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\gamma$</th>
<th>Post-$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.6</td>
<td>1.8</td>
<td>7.3</td>
<td>13.8</td>
<td>6.2</td>
<td>29.2</td>
<td>33.1</td>
</tr>
<tr>
<td>Heparin injection after cholesterol feeding for 50 days</td>
<td>3.9</td>
<td>2.9</td>
<td>6.8</td>
<td>14.8</td>
<td>5.9</td>
<td>18.8</td>
<td>46.9</td>
</tr>
<tr>
<td>Heparinoid substance injection after cholesterol feeding for 50 days</td>
<td>7.1</td>
<td>1.2</td>
<td>5.5</td>
<td>10.1</td>
<td>5.7</td>
<td>28.8</td>
<td>41.6</td>
</tr>
<tr>
<td>No treatment after cholesterol feeding for 50 days</td>
<td>3.2</td>
<td>1.6</td>
<td>2.3</td>
<td>15.9</td>
<td>9.9</td>
<td>35.4</td>
<td>31.7</td>
</tr>
</tbody>
</table>

3. The protein-stained paper strips obtained by paper electrophoresis of the lipoprotein isolated by flotation showed the $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\gamma$-globulins but no albumin band was present.

In cholesterol-fed rabbits $\alpha_1$, $\beta_2$ and $\gamma$-globulin decreased and $\alpha_2$ and $\beta_1$-globulins increased. Heparin and heparinoid substance injection in such a rabbit, caused an increase of $\alpha_1$ and $\gamma$-globulins, and a decrease of $\alpha_2$, $\beta_1$ and $\beta_2$-globulins (table 3, fig. 3).

<table>
<thead>
<tr>
<th>Protein values (% of total protein)</th>
<th>Albumin</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.0</td>
<td>1.9</td>
<td>6.7</td>
<td>27.4</td>
<td>10.3</td>
<td>53.7</td>
</tr>
<tr>
<td>Heparin injection after cholesterol feeding for 50 days</td>
<td>0.0</td>
<td>3.8</td>
<td>8.7</td>
<td>18.9</td>
<td>5.0</td>
<td>63.6</td>
</tr>
<tr>
<td>Heparinoid substance injection after cholesterol feeding for 50 days</td>
<td>0.3</td>
<td>2.7</td>
<td>9.2</td>
<td>26.7</td>
<td>7.5</td>
<td>53.6</td>
</tr>
<tr>
<td>No treatment after cholesterol feeding for 50 days</td>
<td>0.0</td>
<td>1.4</td>
<td>10.2</td>
<td>43.7</td>
<td>8.7</td>
<td>36.0</td>
</tr>
</tbody>
</table>

The lipid-stained paper strip showed the $\alpha_2$, $\beta_1$, $\beta_2$, $\gamma$- and post-$\gamma$-globulin lipids but no albumin and $\alpha_1$-globulin lipids were present. In cholesterol-fed rabbits $\beta_1$ and $\beta_2$-lipoprotein lipids decreased and the $\gamma$-globulin lipid increased.

Heparin and heparinoid substance injection in such a rabbit caused a decrease of $\beta_1$, $\beta_2$ and $\gamma$-globulin lipids, and an increase of post-$\gamma$-globulin lipid (table 4).
EFFECTS OF HEPARIN AND HEPARINOID SUBSTANCE ON LIPOPROTEINS IN SERUM

Normal

Heparin injection after cholesterol feeding for 50 days.

Heparinoid substance injection after cholesterol feeding for 50 days.

No treatment after cholesterol feeding for 50 days.

FIG. 3. Paper electrophoretic patterns of serum lipoprotein isolated by ultracentrifugal flotation.

TABLE 4. Paper Electrophoretic Profile of Serum Lipoprotein Lipid Isolated by Ultracentrifugal Flotation

<table>
<thead>
<tr>
<th>Lipid values (% of total lipid)</th>
<th>Globulin</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$</td>
<td>$\alpha_2$</td>
</tr>
<tr>
<td>Normal</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Heparin injection after cholesterol feeding for 50 days</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Heparinoid substance injection after cholesterol feeding for 50 days</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>No treatment after cholesterol feeding for 50 days</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

4. Atheroma were found in the aorta and aortic valves by autopsy in untreated cholesterol-fed rabbits, but only slight changes occurred in heparin and heparinoid substance injected rabbits.

DISCUSSION

Several authors (7, 8, 9) have expressed the opinion that heparin causes a transformation of $\beta$-lipoprotein into $\alpha$-lipoprotein and an increase in the $S_f$ classes representing the $\alpha$-lipoprotein. Nikkilä (9) found by analysis with moving boundary and paper electrophoresis an increase in the amount of the $\alpha$-lipoprotein lipids in alimentary hyperlipemia following the injection of heparin. Herbst et al. (10) reported that heparin has direct effect only on $\beta$-lipoproteins of high $S_f$ values causing their transformation into those of lower $S_f$ values, but apparently has no effect on $\beta$-lipoproteins of low $S_f$ values and on the $\alpha$-lipoproteins. The present use of paper electrophoresis and subsequent staining in analysis of whole serum for lipid showed an increase of the $\alpha$-lipoprotein lipid and a decrease of the $\beta$-lipoprotein lipid, after the injection of heparin or heparinoid substance, but analysis for isolated lipoprotein showed no $\alpha$-lipoprotein lipid band, though $\alpha$-lipoprotein increased.
Accordingly, it is doubtful that there is any in vivo transformation by heparin of β-lipoproteins into α-lipoproteins.

Evidence presented by several investigators (11-13) would appear to establish the fact that in the presence of clearing factor, triglycerides are hydrolyzed in vitro systems with the liberation of glycerol and free fatty acids. Shore et al. (14) observed that in vivo the addition of heparinized plasma caused a release of fatty acids from β-lipoproteins. There is no evidence that clearing factor is consumed in the reaction; in other respects it appears to function in an enzymatic role. The presence of other activity factor for the reaction is suspected, but such substances have as yet not been isolated, and their functions are unknown. Lever, Smith and Hurley (15) noted that the administration of heparin to a lipemic individual resulted in a decrease of the quantity β-globulin and a compensatory increase in the amount of α-globulin or albumin, or the formation of a new pre-albumin component.

Gofman and associates (3, 16) have shown that during cholesterol feeding of rabbits the Sf 5-8 class component, and the Sf 10-30 class component which appeared to be related to atherosclerosis increased. They also showed that heparin administration causes a shift in the lipoprotein of high Sf values to successively lower ones in the serum of hypercholesterolemic rabbit.

In the present experiments of isolated lipoproteins, heparin and heparinoid substance administration caused an increase of high Sf values 30-70, a decrease of low Sf value <30, an increase of α-lipoprotein, a decrease of β-lipoprotein a decrease of β-lipoprotein lipid, and a remarkable increase of post-γ-globulin lipid which presented a more diffuse appearance, extended in anodal direction and adsorbed nearly at the origin.

It is concluded that heparin and heparinoid substance administration transforms the high density of lipoprotein into low density of lipoprotein, subsequently causes an increase of post-γ-globulin area lipid and therefore suppresses the rate of development of atheroma in cholesterol-fed rabbits.

**SUMMARY**

The action of heparin and heparinoid substance (sulfated chitosan) on the cholesterol-fed rabbit serum was as follows:

1. An increase of the Sf 30-70 component and a decrease of the Sf <30 component.
2. An increase of albumin and β2-globulin and a decrease of α1+, α2+, β1- and γ-globulins in serum.
3. An increase of α1, α2- and post-γ-globulin lipid and a decrease of β1-, β2- and γ-globulin lipids in serum.
4. An increase of α1- and γ-globulin, and a decrease of α2-, β1- and β2-globulins in isolated lipoprotein.
5. A decrease of β1-, β2- and γ-globulin lipids and an increase of post-γ-globulin lipids in isolated lipoprotein.
6. Slight atheroma was found on the aorta and aorta valves.

Heparin and heparinoid substance administration protect against atheroma and suppress the rate of development of atheroma.
The authors wish to thank Prof. T. Minoshima cordially for his helpful suggestions and criticism during the course of this investigation.

REFERENCES