THE FORMATION OF SPECIFIC PROTEOCLASTIC FERMENTS IN RESPONSE TO THE PARENTERAL INJECTION OF FOREIGN PROTEINS.*

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The so called Abderhalden reaction, dating from the appearance of Abderhalden's *Schutzfermente des tierischen Organismus*, has given rise to a vast amount of work on proteolytic ferments in the blood, and has produced an extensive literature. The fact that the reaction would be of diagnostic value, if practicable, has given additional impetus to the investigations. Few workers, except Abderhalden and his pupils, have obtained results definite enough to be of value. This lack of results has been attributed by Abderhalden to faulty technique.1 It was to avoid the use of the dialysis method2 and thus escape this criticism that the technique in the present instance was used.

HISTORICAL.

Ide,3 in 1902, came to the conclusion that "it was impossible to form antibodies in the body against the most important substance of bacteria," nucleoproteins; while Galeotti,4 working in the same year, reports the production of an antiserum to anthrax by injecting its nucleoproteins. MacCallum,5 Portis,6 and Yates7 stated that antithyroid serum produced by

* Dissertation for the degree of Ph.D., University of Pennsylvania, 1916.
them had no specific action, while Bierry\(^8\) stated that the action of such antiserum, while not specific, was more pronounced on the organ from which the nucleoprotein was derived. Levene\(^9\) concluded from his results on hemolytic sera produced by different constituents of erythrocytes that nucleoproteins may be effective in forming antibodies. Pearce\(^10\) in his early and in his most recent work has shown that specific cytotoxic sera cannot be produced by the injection of nucleoproteins. All these workers attacked the problems rather from the viewpoint of the immunologist than from that of the chemist.

Hedin\(^11\) first showed that the blood of the ox contained a weak proteolytic enzyme that acted in alkaline medium, but which was prevented from acting in the blood by the presence of antibodies. Delezenne and Pozerski\(^12\) found that the proteolytic powers were increased when the blood serum was incubated over chloroform. In 1904, Kawasoye\(^13\) applied his results to the diagnosis of pregnancy and reported that he obtained a placental antiserum by sensitization of the animal to placenta or albuminous urine from pregnant women. Wells and Osborne\(^14\) have shown that guinea pigs become immune to the chief vegetable proteins of their food, while on the other hand, Besredka\(^15\) could not succeed in sensitizing a guinea pig to milk either by oral or rectal administration. Abderhalden\(^16\) found on the injection of nucleoproteins and nucleins that a reinjection in no case caused death, inferring therefrom the production of protective ferments. Schawlow\(^17\) reported the existence of specific proteolytic ferments in the blood in specific pathologic conditions; he has used the Abderhalden test to diagnose carcinoma and sarcoma and reported failure in only 7 and 6 per cent.

Voelkel\(^18\) has been able to secure a ninhydrin reacting substance with antisera to diphtheria, typhoid and anthrax bacilli, trypanosomes, and spirochetes. Schwarz\(^19\) using the Abderhalden technique, reported the

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\(^12\) Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327.


positive diagnosis of cancer and pregnancy. Echols\textsuperscript{20} states that the dialysis method is of value when negative, showing the absence of pregnancy, but that a positive result may be due to errors in technique. Carlson,\textsuperscript{21} in answer to the last two statements, says that the Abderhalden test is not qualitative but quantitative, and that one could probably get a positive test in 100 per cent of the cases if the time for digestion were extended, as polyvalent ferments are present always in the blood. This latter position is supported by many investigators. Flatow\textsuperscript{22} finds that the splitting of placenta occurs not only in the pregnant serum, but also that the proteolytic ferments of the normal serum break up the proteins of the placenta unspecifically and quantitatively. He\textsuperscript{23} also objects to Abderhalden's results on the basis of his using "cooked" placenta, saying that "if specific ferments were present they would not show with cooked organs." In his later work\textsuperscript{24} with casein, he finds that casein is digested by the normal serum and more intensely with the pregnant serum. Michaelis and Lagermarck\textsuperscript{25} were not able to demonstrate a specific ferment for placenta, but obtained positive results even with men and old women in certain pathologic conditions. The results of many investigators indicate the presence of proteolytic ferments which evidently increase in quantity under certain conditions.

Herzfeld\textsuperscript{26} found the percentage of amino-acid greater in pregnant than in normal serum, indicating that the pregnant serum had increased fermentative powers. Schlimpert and Issel\textsuperscript{27} report increased ferments in animals during pregnancy, but they were not specific for the animals' own placenta. According to the recent investigations of Jobling, Eggstein, and Petersen,\textsuperscript{28} placental tissue is not digested but actually resists, in an increased degree, enzyme action. Kolmer and Williams\textsuperscript{29} are of the opinion that during pregnancy there is an increase of the general proteolytic ferment of the serum; and recently they\textsuperscript{30} state that there are two sets of ferments in pregnant serum; (1) normal non-specific ferment, and (2) specific; the former released through the adsorption of the antiferment by non-specific substances, the second released only through specific protein antigen.

\textsuperscript{22} Flatow, L., \textit{Münch. med. Woch.}, 1914, lx, 468.
\textsuperscript{23} Flatow, \textit{Münch. med. Woch.}, 1914, lxi, 1168.
\textsuperscript{24} Flatow, \textit{Münch. med. Woch.}, 1914, lxi, 1500.
\textsuperscript{25} Michaelis, L., and Lagermarck, L. v., \textit{Deutsch. med. Woch.}, 1914, xl, 316.
\textsuperscript{26} Herzfeld, E., \textit{Biochem. Z.}, 1914, lxi, 249.
\textsuperscript{27} Schlimpert, H., and Issel, E., \textit{Münch. med. Woch.}, 1913, ix, 1758.
\textsuperscript{29} Kolmer, J. A., and Williams, P. F., \textit{Am. J. Obst.}, 1915, lxxi, 899.
\textsuperscript{30} Kolmer and Williams, \textit{Am. J. Obst.}, 1915, lxxii, 1.
The immunologists have also offered explanations for this increased activity during pregnancy and after injection of protein, as an immunity reaction. Vaughan\textsuperscript{31} states that he procures in guinea pigs, by immunization, a serum which will digest egg protein. He explains immunity as development of a serum which will digest foreign bodies and render them harmless. Jobling and Petersen\textsuperscript{32} explain this digestion in blood as due to non-specific proteolytic ferments or proteases normally present in blood and held in check by antiferments, which they believe to be unsaturated fatty acids. Bronfenbrenner\textsuperscript{33} explains the reaction by saying that the results obtained by Abderhalden are due to the presence in this serum of specific substances that are not fermentative in nature, and that the substratum is not digested, but that autolysis takes place and serum proteins are liberated, thus giving rise to protein digestion products in the blood. On the other hand, we have the evidence of Frank\textsuperscript{34} that no experimental specific immune reaction to placenta can be demonstrated. Lake\textsuperscript{35} also regards the possibility of producing an immune serum of therapeutic value in chorion epithelioma, by use of human placenta, as extremely slight. Abderhalden\textsuperscript{36} regards the diagnosis of pregnancy and carcinoma as dependent on the presence of ferments produced by the injection of the protein or tissue in question. De Waele,\textsuperscript{37} however, did not succeed in preparing a serum that would give digestion with specific tissues, but he did find that the addition of even an inorganic substance will cause the liberation of dialyzable, ninhydrin-reacting substances. DeWaele,\textsuperscript{38} and Heilner and Petri\textsuperscript{39} show that ferments appear very quickly after parenteral injection of the protein, in intervals hardly sufficient for the elaboration of new and specific ferments; they support the theory that the ferments are preformed and that the substratum serves to activate rather than to bring about the production of new ferments.

The most recent work done on proteoclastic ferments tends to favor the opinion that the production of ferments during pregnancy and after the injection of protein is a quantitative and not a qualitative reaction. Sloan\textsuperscript{40} observed that there may be an increase of proteolytic activity un-
der the above conditions. Van Slyke, Vinograd-Villchur, and Losee\textsuperscript{41} found nearly or quite as much power to digest placenta in normal as in pregnant sera. Malone\textsuperscript{42} reported that he has found ferments, even in the urine, which would digest placenta. The clinical literature on the subject, supporting the efficiency of the test and also denying its usefulness, would fill volumes and has been omitted here, because we are concerned with the reaction from the chemical rather than from the clinical viewpoint. A full list of references may be found in Abderhalden's \textit{Abwehrfermente}.

**EXPERIMENTAL.**

As stated in the introduction, the object of this investigation was to determine whether proteoclastic ferments or activity develop in the blood in response to the injection of a foreign protein and cause digestion when allowed to act on the protein injected, using a method which would not be open to the criticism of the dialysis method or of the ninhydrin reaction. The method followed in each case was practically the same. White rabbits were injected with an amount of protein varying from 100 to 800 mg. in solution or suspension, depending on its solubility in Ringer's solution. The Ringer's solution was boiled and cooled to about 40°C. The protein was placed in this and after the hair had been cut from the rabbit and the skin disinfected with iodine, this solution was injected parenterally. Each rabbit received three successive injections, 1 week apart. The day following the last injection the rabbit was etherized and bled to death from the carotid. The blood was caught in a beaker and was stirred continuously to defibrinate it. The control animals were bled in the same way. The defibrinated blood was centrifuged, the serum decanted off, and portions were used to obtain the figures for the non-protein nitrogen according to the method of Folin,\textsuperscript{43} except that we used, for some of the later tests, absolute ethyl alcohol instead of methyl alcohol.

The rest of the serum was divided into 5 or 10 cc. portions ac-


\textsuperscript{43} Folin, O., and Denis, W., \textit{J. Biol. Chem.}, 1912, xi, 527.
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cording ho the amount obtained, and these were diluted up to 50 cc. with Ringer's solution, made alkaline with 2 cc. 0.1 N NaOH and placed in a flask with 1 gm. of the particular protein, and incubated at 37.5°C. The first three were incubated for 24 hours, the next two for 48 hours, and the last two for 42 hours. After incubation the contents of the flask were mixed with ten volumes of alcohol, and allowed to stand for 24 hours. The filtrate was treated with a saturated alcoholic solution of ZnCl₂, allowed to stand 24 hours, and a Kjeldahl run on the filtrate. Bearing in mind the fact that Greenwald could not recover all the amino-acid from an alcoholic filtrate, we did some check analyses with 2.5 per cent trichloroacetic acid, as recommended by him, but since the results checked so closely with the alcohol figures, we continued with the alcohol method.

The results are summarized in the tables.

**TABLE I.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Animal</th>
<th>Injection</th>
<th>Non-protein N in 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.</td>
<td>2.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>Protamine</td>
<td>Experimental</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Casein</td>
<td>Experimental</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bence-Jones</td>
<td>Experimental</td>
<td>250</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaseolin</td>
<td>Experimental</td>
<td>800</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edestin</td>
<td>Experimental</td>
<td>500</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gliadin</td>
<td>Experimental</td>
<td>350</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy bean globulin</td>
<td>Experimental</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
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</tr>
</tbody>
</table>

DISCUSSION.

The results show plainly in all cases that under the conditions of the experiment there is practically no digestion with the blood of the injected animal in excess of that which takes place with the serum of the normal control animal. The greatest amount of digestion takes place in the case of the Bence-Jones protein and milk albumin, which would naturally be expected, as these two proteins are more nearly related to mammalian proteins. With casein, the blood of the injected animal on the addition of casein gave a precipitin reaction, so that this might account for the greater increase of nitrogen in the normal animal; the precipitin reaction might preclude the possibility of a proteolytic action taking place. The results would also lead one to suppose that the blood of the rabbit possesses to a slight degree proteolytic activity, but that this activity toward a particular protein is not increased by injecting the animal with that protein. The criticism might be offered that these proteins are too foreign; but if the reaction must be discriminated on the basis of the protein being just foreign enough, it would seem that the possibility of usefulness becomes remote.

The very premise that ferments (free or restrained) exist preformed in the blood is not strongly borne out in these data. The ferments are best regarded as endocellular entities; and under certain conditions, not well understood, the cellular activity may be so stimulated that an excess of ferment is produced and overflows...
into the blood stream (just as frequently trypsin occurs in the urine), but exists there not primarily for the purpose of digestion directly in the circulating blood. The fact that such varying results on the presence or activity of ferments in the blood have been obtained might be due to the fact that some of the investigators have accidentally reproduced the conditions under which the cells are stimulated to greater activity and the excess ferments then appeared in the blood. Our results would lead us to believe that pure proteins at least need not call forth such increased activity. Even though there be in pregnancy a specific serum reaction, it is probably not one due to placental digestion in the blood.

It is not demonstrated that the normal hydrolysis of the protein of the body occurs in the circulating blood; indeed, it is much more likely that this occurs largely within the cells of the tissues. It is easily possible to imagine introduced foreign protein being taken into the body cells and hydrolyzed there, without any reaction appearing in the circulating plasma. It may be shown that foreign protein introduced into the venous blood may be taken up by the tissues. In like manner, it might be imagined that the placental cells entering the maternal circulation would be held and hydrolyzed within the tissues, and leave no trace of any enzymic or other activity in the circulating plasma. In theory, therefore, one must separate the question of hydrolysis of foreign proteins from the question of the reaction of the maternal body to placental cells. It is entirely possible that there is a specific reaction of the host to placental cells or to neoplasms, without this being a hydrolysis of their proteins in the circulating blood dependent on the presence there of enzymic activity.

SUMMARY.

Protamine, phaseolin, and gliadin are not digested to any degree by either normal serum or that of an animal injected with these substances.

Casein and soy bean globulin are digested to a greater extent by the normal serum than by that of the injected animal.

Unpublished data.
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Edestin and milk albumin are digested to the same degree by the normal and experimental serum.

Bence-Jones protein is digested to a marked degree by both sera and equally well in each case.

I take this opportunity to thank Professor Alonzo E. Taylor, at whose suggestion this work was undertaken and under whose direction it was carried out, for his help, both in the experimental work and in reading and revising the manuscript. I also thank Professor Mendel and Dr. Osborne for the proteins\textsuperscript{46} supplied by them, Dr. Isaac F. Harris,\textsuperscript{47} for those supplied by him, and Drs. Taylor and Miller for the Bence-Jones protein.

\textsuperscript{46} Casein, edestin, gliadin, phaseolin.

\textsuperscript{47} Soy bean globulin, milk albumin.
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