SERUM PROTEASES AND THE MECHANISM OF THE ABDERHALDEN REACTION.

STUDIES ON FERMENT ACTION. XX.*

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Since the Abderhalden method of dialysis has been available for clinical purposes, numerous reports of results have been published. Among these the more recent papers of Beumer (1), Fränkel (2), Csepai (3), Michaelis and von Langermarck (4), Bisgaard and Korsbjerg (5), Mosbacher and Port (6), Lange (7), von Domarus and Barsieck (8), and others, have tended to discredit the specificity of the reaction and so reflect upon its usefulness as a clinical method. The conflicting results have cast considerable doubt upon the mechanism of the reaction as first advanced by Abderhalden. It seems unfortunate that in the enthusiasm of the search for specific ferments, the proteases which might normally be present in serum and which had previously received some attention, have been neglected. We are inclined to believe that in the study of these non-specific proteases considerable information might become available which would aid in the elucidation of the points at issue in the Abderhalden reaction.

Hedin (9) first demonstrated that the globulin fraction of normal ox serum contained a weak proteolytic ferment. Later Delezenne and Pozerski (10) showed that when serum was incubated under chloroform the serum became actively proteolytic. Their experiments were not extended and received little attention. Opie (11) later demonstrated serum proteases by rendering the serum slightly acid (0.2 per cent. acetic). Delezenne and Pozerski offered no explanation for their results with chloroform, and we have only recently shown that protease action developed under these conditions is due to the fact that the serum antitrypsin is soluble in chloroform, and when so removed the serum proteases can become active (12). The method of Opie is based upon a similar phenomenon, for he found that the serum antitrypsin is inactivated in a slightly acid medium. In this manner the acid acting proteases only can become active. The inactivation of the antiferment is probably due to changes in the state of dispersion of the unsaturated lipoids induced by the acidity.

SERUM PROTEASE.

In our study of serum protease we have noted that the serum ferment of the common laboratory animals, guinea pigs and rab-

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bits, is not specific; for when freed from antitrypsin these sera are not only autolytic but digest casein, edestin, and similar substrata. Serum from the dog, cat, and ox show considerable variation. Thus in normal dog serum proteases could be demonstrated only occasionally, but appeared regularly under pathological conditions (distemper, pneumonia, and inanition). The serum protease of these animals acts both in a slightly acid as well as in an alkaline medium; its action, like that of trypsin and leucoprotease, is inhibited by diluted solution of the unsaturated soaps, and the thermal death point lies near 70° C. when heated for thirty minutes, although lower temperatures (56° C. for thirty minutes) cause marked impairment of the proteolytic power. The relative thermostability as compared to serum complement is of considerable importance both from a theoretical as well as a practical standpoint. The non-identity of serum complement and serum proteases should be emphasized, for throughout the literature these ferments are constantly confused. The simplest demonstration of the difference is made by the action of chloroform upon guinea pig serum. When this serum is thoroughly mixed with chloroform and incubated, the complement and esterase are rapidly destroyed, whereas the serum protease becomes active under these conditions, as first noted by Delezenne and Pozerski. Complement action is constantly referred to as proteolytic, and it is assumed that the temperature which inactivates complement also inactivates serum protease. Thus Lange (13) in his excellent study of the Abderhalden reaction is unable to account for the fact that frequently he noted that inactivated serum gave a positive reaction, although somewhat weaker than the active serum. Assuming that all proteolytic activity had been destroyed with the inactivation of the complement, he could not explain these reactions, although it becomes evident that such weak reactions will be observed frequently when tested for, inasmuch as at 60° C. the protease is impaired and not completely destroyed. Williams and Pearce (14) have recorded similar observations. It is shown with guinea pig serum incubated with placenta for twenty-four hours as follows:

	Dialysate. Ninhydrin reaction.
1. Normal guinea pig serum plus placenta	+++
2. Guinea pig serum (inactivated at 56° C. for 30 min.) plus placenta	+
3. Guinea pig serum (inactivated at 70° C. for 30 min.) plus placenta	0
4. Guinea pig serum (inactivated at 56° C.)	0

A similar result was obtained when the serum was incubated with starch or with chloroform.

We have previously indicated (15) that the complemental activity of the serum is probably related to the esterases and, therefore, bears no direct relation to the Abderhalden reaction. The effort has been made to reactivate heated specific sera by adding guinea pig complement, and in this several observers have claimed positive results. Inasmuch as they are in this way adding a large amount of a non-specific protease, these results can have no value.

In contrast to the serum of the smaller laboratory animals, we have found that normal human sera contain little or no protease. Flatow (16), on the other hand, contends that all sera contain protease in considerable amounts, which he has demonstrated by adding casein to serum in dialyzing membranes. He found that the ninhydrin reaction was positive with all sera and was simply increased in pregnancy. These experiments have, however, the serious objection that casein is rather easily hydrolyzed to the higher splitting products without the presence of any protease, and the serum ereptase or peptase, which is undoubtedly present in all sera, would then digest the higher splitting products to dialyzable forms. The differentiation between serum protease and the peptase or ereptase is at times difficult to draw, although it is of primary importance.

It is at any rate interesting that animals which have a constant and strong protease in their serum (guinea pigs and rabbits) should be without such ferments in the leucocytes, whereas animals in which leucocytic proteases are well developed (dogs and man) are seemingly under normal conditions without much protease in the serum.

ABDERHALDEN REACTION.

From a theoretical point of view there are several phases in the explanation of the Abderhalden reaction which are at variance with

various facts in immunological research as developed in the past few years. It has been found that in those conditions in which the Abderhalden reaction has been most frequently tested for,—pregnancy, carcinoma, tuberculosis, and the various nervous lesions,—there is present a marked increase in the antiferment of the serum. The increase in pregnancy (and carcinoma) is so well recognized that numerous workers have suggested the use of the antitrypsin titer in place of the Abderhalden reaction.

Thus Franz (17) in a series of 223 cases and Heinemann (18) in a series of 50 cases report better results than with the Abderhalden method. This has been taken up fully by Freund and Brahm (19), who came to the same conclusion. Under such conditions we should expect less digestion of native placenta in the serum than normally. This, as a matter of fact, is exactly the condition found. Wilhelm and Szandicz (20) have recently shown that native placental cells (not boiled) autolyzed in normal serum, but that the serum of pregnant individuals retarded this autolysis, because of the increase in antiferment. These facts are, of course, contrary to the theory of the Abderhalden reaction. Again, the idea is advanced that as a result of tissue destruction or of infection, lytic bodies or proteases are found which are capable of splitting the infecting organism (as in tuberculosis) or the cell (as in carcinoma). Now it is a well established fact (reaction of Freund and Kaminer) that in carcinoma we have actually the reverse of this process; *i. e.*, the blood of the carcinoma patient has lost the power to dissolve carcinoma cells normally possessed by the serum. Yet the Abderhalden theory is based on the diametrically opposite supposition without any experimental basis in its support.

Even if we leave aside the question of the impairment of specificity of boiled tissues, one rather striking feature stands out in reviewing the results obtained in various laboratories, in that placental tissue is digested by practically all pathological sera irrespective of pregnancy, whereas the cross-digestion of caseous material and of carcinoma tissue is much less constant. It has been noted that normal lung tissue next to placental tissue is most frequently digested. The reaction is obtained only when a formed substrate is used, and seems to depend on the mechanical state of division of that substrate; or if a fluid substrate is used, it has been found that the reaction is positive only when a precipitin reaction occurs simultaneously. Thus there would seem to be some dependence of the reaction upon physical factors.

This is indicated in the work of Plaut (21) who found that with guinea pig serum the Abderhalden reaction was regularly obtained not only with placental tissue but also with inert substances such as kaolin, *Kieselguhr,* barium sulphate,

etc. This work, of course, showed that digested products might be derived from the serum and not from the added substrate. We have previously demonstrated such digestion if serum is extracted with lipoidal solvents (22). In a manner similar to that of Plaut, Peiper (23) and Friedemann and Schönfeld (24) regularly obtained a positive Abderhalden reaction when starch was added to the serum. Probably the most suggestive work is that of de Waele (25) who found that any agent which would cause an alteration of the physical state of the serum globulins would cause a most intensive Abderhalden reaction, and concluded that the reaction depended upon a globulinolysis, having an origin in processes possibly analogous to the precipitin reaction. In this it is interesting to note that Eggstein (26) found that the mere dilution of the serum in the dialyzing membrane with a large amount of distilled water would frequently give a positive reaction without the addition of any substrate, the result being probably due to a precipitation of the globulins. He also found that an acid reaction interfered with the reaction, whereas in an alkaline medium the digestion seemed increased. This has been noted by Goormaghtigh and Deheegher. In view of these results the view would seem warranted that dialyzable products responsible for the Abderhalden reaction might originate from the serum and that the phenomenon depended upon adsorption processes, the substrate added—placental tissue, caseous material, bacteria, etc.—acting as adsorbing media and not as substrata. On the basis of our work with the serum antiferment and on the serum lysis of bacteria, we have previously (15) suggested this explanation of the Abderhalden reaction.

We have recently demonstrated that the serum antiferment is a readily adsorbable substance, being adsorbed by bacteria, kaolin, starch, agar, etc., and that following such adsorption the serum proteases normally present may split the serum proteins to toxic products, as in anaphylatoxin formation(27). It would then seem logical to investigate whether similar processes underlie the Abderhalden reaction. Under such circumstances we should expect that (a) the placental tissue would not be decreased in amount during digestion, (b) the placental tissue used in the reaction would become more resistant to tryptic digestion because of adsorption of antiferment, (c) the serum in the dialyzing membrane would show some lowering of its antiferment titer, (d) the developed protease action would be non-specific, and (e) that other means of adsorption of the antiferment would reveal the presence of proteases.

EXPERIMENTAL.

LACK OF PLACENTAL DIGESTION BY SERUM.

In order to demonstrate that the placental tissue is not digested we have carried out the following experiment.

Placental tissue was dried according to the method of Lindig (28). 70 mg. were made up into an emulsion, boiled, and carefully divided into ten centrifuge tubes. 2 c.c. of pregnant serum were added to five tubes, and an equivalent amount of salt solution to the five control tubes. After digesting for twenty-four hours the placental tissue was centrifuged from the supernatant fluid, and after two washings the total nitrogen of the placental rest was determined. The non-coagulable nitrogen of the supernatant fluids and washings was also determined.

Total non-coagulable nitrogen per c.c. of serum..... 0.25 mg.
Total non-coagulable nitrogen of the salt solution from placenta..... 0.05 mg.
Total non-coagulable nitrogen per c.c. of serum from placenta..... 0.37 mg.
Total nitrogen of placental tissue from salt solution controls..... 0.58 mg.
Total nitrogen of placental tissue from serum tubes..... 0.74 mg.

Instead of a lessened amount of substrate, as would be demanded if the placental tissue were actually digested, there is an actual increase in the amount of nitrogen-containing material which has been derived from the serum, while there is at the same time an increase in the total non-coagulable nitrogen of the supernatant serum, the split products being formed from the serum proteins.

RESISTANCE OF PLACENTAL TISSUE.

That the placental tissue becomes more resistant to enzyme action following the dialysis is shown in the following experiment.

Dried placental substrate was suspended in salt solution so that 1 c.c. contained 2 mg. of nitrogen. A similar preparation was made from placental tissue after it had been subjected to the action of serum and thoroughly washed free from the serum. The non-coagulable nitrogen in each suspension amounted to 0.03 mg. per c.c. Both suspensions were made alkaline with sodium carbonate, and 0.1 c.c. of trypsin solution was added, an amount sufficient to digest 2 c.c. of 1 per cent. casein solution in one hour. The amount of digestion noted was as follows.

	Digestion in per cent. after	
	1 hr.	4 hrs.
Untreated placenta.....	2.5%	18%
Serum placenta.....	0.6%	10%

The serum-treated placenta had become almost twice as resistant as the normal placenta.

ADSORPTION OF ANTIFERMENT.

Protease action in the serum must take place under conditions

of antiferment deficiency, for if the ferment action were not overbalanced by an antiferment the organism would die immediately from intoxication from the protein split products. We believe that the antiferment deficiency need not be expressed by a lowering of the titer of the whole serum unless the adsorption has been very extensive. Indeed, it seems probable that the protease action can take place in what might be termed local areas of antiferment deficiency, such as must occur at the point of contact of the serum and adsorbing substance. A complete absence of antiferment is, therefore, not essential for protease action provided some adsorbing surface is present on which the relative balance of ferment-antiferment may be altered. The titer of the serum is, however, lowered as a whole during the dialysis, as is shown in the following experiment with pregnant serum which was incubated for thirty-six hours and which gave a ++++ Abderhalden reaction.

Serum dilution.	Inhibition of serum.	
	Before dialysis.	After dialysis.
0.10 c.c.	90%	91%
0.075 c.c.	91%	90%
0.05 c.c.	91%	91%
0.025 c.c.	91%	33%
0.01 c.c.	24%	10%

It will be observed that an excess of serum is able to cause almost complete inhibition of the trypsin unit, the lowering of the titer being evident in the greater dilutions.

It is probable that the dialyzing membrane itself acts to a certain extent as an adsorbing membrane for the antiferment, so that frequently a serum, which in itself does not contain sufficient dialyzable substances to give a positive reaction, as evidenced by the negative control of inactive serum, and also of inactive serum plus placenta, will give a positive reaction. When placed in glass such a serum will not undergo autolysis. Paul Lindig (28), among others, has noted this phenomenon, and concludes that the dialyzable products are due to enzyme action and not to preformed dialyzable products. Such an experiment is shown in the following protocol.

	Abderhalden reaction.	Antiferment titer after dialysis, for 0.025 c.c.	Total nitrogen in dialysate.
Pregnant serum	+	45%	0.118 mg.
Pregnant serum inactive	0		0.13 mg.
Pregnant serum plus placenta	+++	33%	0.25 mg.
Pregnant serum inactive plus placenta	0		0.17 mg.
Placenta	0		0.07 mg.
Undialyzed serum		90%	Total non-coagulable nitrogen in serum 0.12 mg.

There has been a considerable reduction of the titer of the serum in the dialyzing membrane even without the addition of substrate. It will be recalled that this adsorption of the antiferment by dialyzing membranes led to an error on the part of Rosenthal, who sought to show that the antiferment of the blood consisted of the dialyzable protein split products, and concluded that the reduction of antiferment following dialysis was due to diffusion of the split products.

NON-SPECIFICITY.

The fact that pregnant and various pathological sera will, when placed in the dialyzing membrane with inorganic or organic agents, give a positive Abderhalden reaction has already been demonstrated, the most recent series being that of Freund and Brahm (19), who noted that in fifty-eight cases they obtained positive results in forty cases. Similar results have recently been described by Bronfenbrenner (29) with the use of chloroform. He concludes that the serum itself is the source of the dialyzable products, that the placental tissue is not digested, that substances which removed the antiferment,—chloroform, kaolin, starch,—cause a positive reaction, while the addition of antiferment causes the inhibition of the reaction. Bronfenbrenner has noted that the intensity of the Abderhalden reaction is inversely related to the complement activity, thus offering additional evidence of the fact which we have endeavored to emphasize; namely, the non-identity of the serum complement and serum protease. Bronfenbrenner does not, however, draw the same conclusion from this observation.

In our own experiments we have obtained positive results with pregnant, tuberculous, and pneumonic sera, whether chloroform, agar, starch, or placenta were used as adsorbing media. With agar, starch, and chloroform the effects were less uniform when these substances were placed directly into the dialyzing membrane, probably because they interfere more or less with the rapidity of diffusion. When, however, these substances were permitted to act

upon the serum at an incubator temperature for a period of time, and the serum was then placed in the thimbles, a positive result was invariably obtained. The interference of chloroform and of the colloids with the dialysis is shown in the following experiment in which 1.5 c.c. of Seiden peptone were permitted to dialyze in one membrane, and chloroform and agar were added to an equal amount of peptone in other membranes.

	Abderhalden reaction.	Per cent. of nitrogen dialyzed.
Peptone	+++	82%
Chloroform and peptone	++	58%
Agar and peptone	+	52%

This effect of the colloids must be considered when experiments are made by adding such substances directly to the thimbles. We have found it advisable to permit the antiferment adsorption to take place before the serum is dialyzed, the adsorbing substances being centrifuged from the serum as much as possible before the serum is placed in the thimbles. Even a short period (three hours at 37° C.) of the adsorbing action may in some cases be sufficient to give a positive reaction, as is shown in the following experiment with pneumonia serum.

	Abderhalden reaction.	Total nitrogen in dialysate.
1. Serum	0	0.34 mg.
2. Inactive serum	0	0.3 mg.
3. Serum and placenta	++++	0.56 mg.
4. Inactive serum and placenta	++	0.41 mg.
5. Serum and chloroform	+	0.33 mg.
6. Serum and agar	0	0.28 mg.
7. Serum and starch	+	0.37 mg.
8. Serum and kaolin	0	0.2 mg.

1.5 c.c. of serum were used. The placental tests were permitted the usual time of twenty-four hours. Tests 5, 6, 7, and 8 were mixed with chloroform (equal volume), agar (equal volume of 0.1 per cent. solution), starch (equal volume of 10 per cent. solution), and kaolin (0.05 gm.) and incubated for three hours. They were then centrifuged and the clear sera placed in the thimbles over night.

It is probable that the negative reactions with the agar and kaolin were due to insufficient amounts of adsorbing substances. We have discussed this point in a previous paper (27). In all the sera

tested (pregnancy, pneumonia, and tuberculosis) the placenta has given a positive reaction, while caseous material has given a cross-reaction with both pregnant and pneumonia serum. An extended series of sera from various pathological cases has been reported by Falls (30), who in eighty-four pathological sera obtained positive reactions varying from weak to strong in sixty-eight, using placental tissue as a substrate. Under such circumstances the specificity of the reaction is at least highly doubtful.

In view of the experimental data presented above, together with those given in our previous papers, we are inclined to believe that the Abderhalden dialysis method and the theory underlying it, in so far as it is applicable to protease action, is without warrant of specificity, and probably depends upon purely fortuitous mechanical factors. It seems probable that in various pathological conditions proteases normally confined to the leucocytes in the human being appear in the blood where their presence can be demonstrated by a method which removes the antiferment without injuring the ferment. The proteases are not specific, the placental tissue being found most efficacious, possibly because of purely mechanical factors (surface exposure), as is indicated by the wide range of clinical conditions in which the placental substrate gives positive results.

CONCLUSIONS.

1. Normal serum protease is not specific; it is active in both dilute acid as well as alkaline media. It is destroyed by heating to 70° C. for thirty minutes. It is markedly impaired when heated at 56° C. for thirty minutes. It is inhibited by the unsaturated soaps and lipoids.

2. Guinea pig and rabbit serum contain relatively much protease; the leucocytes are without proteolytic ferments.

3. Normal human and dog serum contain little or no protease; the leucocytes are strongly proteolytic.

4. Serum complement and protease are not identical.

5. During various pathological conditions the non-specific protease is increased in both human and dog serum.

6. An increase in antiferment is in many instances coincident.

7. During the Abderhalden reaction the placental tissue becomes more resistant to enzyme action, because of the adsorption of the antiferment from the serum.

8. The dialyzed serum loses antiferment because of adsorption by the placental tissue or other adsorbing substances, including probably the dialyzing membrane.

9. The digestive substrate is the serum protein made available for protease action by the adsorption of the antiferment.

10. The proteases in pathological conditions investigated by us (pregnancy, tuberculosis, and pneumonia) are non-specific.

BIBLIOGRAPHY.

1. Beumer, H., *München. med. Wchnschr.*, 1914, lxi, 1999.
2. Fränkel, E., *Deutsch. med. Wchnschr.*, 1914, xl, 589.
3. Csepai, K., *Wien. klin. Wchnschr.*, 1914, xxvii, 804.
4. Michaelis, L., and von Langermarck, L., *Deutsch. med. Wchnschr.*, 1914, xl, 316.
5. Bisgaard, A., and Korsbjerg, A., *Deutsch. med. Wchnschr.*, 1914, xl, 1367.
6. Mosbacher, E., and Port, Fr., *Deutsch. med. Wchnschr.*, 1914, xl, 1410.
7. Lange, C., *Berl. klin. Wchnschr.*, 1914, li, 785.
8. von Domarus, A., and Barsieck, W., *München. med. Wchnschr.*, 1914, lxi, 1553.
9. Hedin, S. G., *Jour. Physiol.*, 1904, xxx, 195.
10. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. de biol.*, 1903, lv, 327.
11. Opie, E. L., *Jour. Exper. Med.*, 1905, vii, 316.
12. Jobling, J. W., and Petersen, W., *Jour. Exper. Med.*, 1914, xix, 459.
13. Lange, C., *Biochem. Ztschr.*, 1914, lxi, 193.
14. Williams, P. F., and Pearce, R. M., *Surg., Gynec. and Obstet.*, 1913, xvi, 411.
15. Jobling and Petersen, *Jour. Exper. Med.*, 1914, xx, 321.
16. Flatow, L., *München. med. Wchnschr.*, 1914, lxi, 1500.
17. Franz, R., *Arch. f. Gynäk.*, 1914, cii, 579.
18. Heinemann, F., *Monatschr. f. Geburtsh. u. Gynäk.*, 1914, xxxix, 768.
19. Freund, R., and Brahm, C., *München. med. Wchnschr.*, 1914, lxi, 1664.
20. Wilhelm, R., and Szandicz, S., *Biochem. Ztschr.*, 1914, lxv, 219.
21. Plaut, F., *München. med. Wchnschr.*, 1914, lxi, 238.
22. Jobling and Petersen, *Jour. Exper. Med.*, 1914, xix, 480.
23. Peiper, A., *Deutsch. med. Wchnschr.*, 1914, xl, 1467.
24. Friedemann, U., and Schönfeld, A., *Berl. klin. Wchnschr.*, 1914, li, 348.
25. de Waele, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1914, xxii, 170.
26. Eggstein, A. A., *Jour. Tennessee State Med. Assn.*, 1914, vii, 115.
27. Jobling and Petersen, *Jour. Exper. Med.*, 1914, xx, 37.
28. Lindig, P., *München. med. Wchnschr.*, 1914, lxi, 1668.
29. Bronfenbrenner, J., *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 90.
30. Falls, F., *Jour. Am. Med. Assn.*, 1914, lxiii, 1172.