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Title: Determination of plasmin activity for clinical purposes (Bestimmung der Plasminakitivitat fur klinische Zwecke)

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Plasmin is a constituent of the proteolytic enzyme system of blood plasma. It is a proteinase which digests casein, gelatin, fibrin, and several other proteins into polypeptides. It is active over a pH range of 5 to 9. The optimum pH is dependent upon the substrate employed. For casein, it is about pH 7.

Plasmin was formerly known as serum tryptase. In order to avoid confusion with pancreatic trypsin and to denote its presence in serum, the name "plasmin" was gradually adopted about 20 years ago (1).

At neutral and basic pH, plasmin is almost completely inactive in fresh whole serum. In whole serum, plasmin is active only after treated as described in this paper. At acid pH values, on the other hand, plasmin is completely active. In the acid range, it exhibits a maximum at pH 5.8. This acid range maximum, however, is not identical with the actual pH optimum which is pH 7 in fresh whole serum (6).

Plasmin can be activated in the following ways:

(1) Shaking of serum with chloroform.

(2) Addition of very dilute acetic acid to the serum; this causes the coarsely dispersed globulins (euglobulin) to precipitate quantitatively at pH 5.6. The precipitated globulin contains almost all of the plasmin activity.

(3) Addition of a casein solution to the serum with consequent precipitation of the casein with an acetic acid-acetate buffer solution. In the casein precipitate, 80 % of the plasmin is present in active form.

(4) Incubation of the serum with streptokinase.

(5) Addition of certain dehydrating reagents such as alcohol, phenol, acetone, and others to the serum.

(6) Storage of the serum in toluene at low temperatures. This causes a gradual activation of the plasmin as a result of gradual physico-chemical changes (partly disaggregation of serum proteins). The alterations take place when whole serum

is incubated after the additon of a casein solution. There is no breakdown of the casein within the first 5 to 6 days. At longer incubations, however, a gradual, steady breakdown takes place resulting in almost complete breakdown of the casein in about 5 weeks (7).

As a result of the several possible means of activating plasmin, a variety of techniques for the determination of plasmin activity have been published by various authors. However, all of these techniques allow several different procedures for the preparation of the starting material used in the incubation procedure. This is true for the preliminary treatment of the serum with streptokinase, for the production of active plasmin by separation from the euglobin by acetic acid-acetate precipitation, or for the adsorption of plasmin onto casein and the consequent precipitation by the addition of acetic acid-acetate buffer (10).

Our own experiments employed a simple procedure whereby the plasmin was activated by the addition of acetone. For carrying this out, a buffer solution containing acetone is added and then a solution of casein is added. In this manner, the mixture can be prepared in just one step. The mixture is then divided into two equal aliquots. One of the aliquots is deproteinized immediately by the addition of trichloroacetic acid. The other aliquots is deproteinized in the same manner after 24 hours of incubation. The resulting breakdown products (tyrosine, tryptophane, and phenylalanine) are determined colorimetrically using the xanthoprotein reaction. This method is preferred over the Folin-phenol reagents because of the longer duration of the color that develops (8).

Based on the results that were obtained from over 1000 determinations, this method appears to be very well suited for routine clinical estimations. For the elimination of sources of error, we have prepared the necessary technical procedures to be followed. The following details are presented:

The following reagents are required for carrying out the determination:

(1) Acetone-buffer mixture. One part acetone (analytical grade) is mixed with 5 parts of phosphate buffer solution (pH = 7.0!). The buffer solution was prepared according to the procedure of "Sorenson" or by using Testol Standard buffer purchased from the firm "Feinchemie", Sebentiz (Sa.).

(2) 2 % casein solution in phosphate buffer (pH = 8.0 !).

(3) 10 % trichloroacetic acid pro anal.

(4) 25 % nitric acid pro anal.

(5) 33 % solution of sodium hydroxide pro anal. (prepared preferably from solid sodium hydroxide).

PROCEDURE: To 1 ml of serum are added 3 ml of acetone-buffer mixture (1) and 2 ml of 2 % casein solution (2). This starting material contains 1/12 vol. % acetone which is the optimum concentration for the activation of plasmin and corresponds to a pH of 7.0-7.1 which is the optimum for activity.

From this 6 ml mixture that is obtained, one 3 ml aliquots is deproteinized at once by the addition of 3 ml of 10 % trichloroacetic acid (3). The remaining 3 ml aliquot is deproteinized in the same manner after 24 hours of incubation at 37°C (toluene addition).

The filtrates that are obtained after deproteinization must be water-clear. Exactly 2 ml of the filtrate are employed for performing the protein determination using the xanthoprotein reaction. To the 2 ml aliquot is added 0.5 ml of 25 % HNO_3. The mixture is heated in a boiling water bath for 3 minutes (the time is measured from the beginning of boiling of the contents of the tube). After cooling, 1.5 ml of 33 % NaOH is added. The slight turbidity which develops is the result of traces of earth alkalies. This does not interfer with the colorimetric measurements and can be removed by centrifugation. The color which

develops is measured in a "Stufen photometer" using the method of Pulfrich employing a S42 filter. The color readings are converted into tyrosine units by means of a standard curve. The difference in color intensity before and after incubation corresponds to the increase in degradation products, inasmuch as they contain tyrosine, tryptophane, and phenylalanine. This difference is a measure of the plasmin activity. Based on experience, the starting material before incubation gives values of 3-6 γ tyrosine per ml. This is the result of traces of substances present such as aromatic amino acids and potassium sulfate which give a positive xanthoprotein reaction. In addition, casein solutions which have been completely deproteinized contain very small amounts of substances which are present in the filtrates and give positive reactions.

After incubation, the increase due to plasmin activity in normal serum amounts to 12 to 20 γ tyrosine per ml of filtrate.

In order to determine the tyrosine values for all of the starting material, the results must be multiplied by a factor of 6, as the original 3 ml aliquots of starting material were diluted to 6 ml with trichloroacetic acid and the tyrosine values determined colorimetrically are for 1 ml of deproteinized starting material.

For the sake of simplicity, the results found in the following discussions will be given in γ tyrosine per 1 ml of starting material. In evaluating the results, only the ratios of the values are of significance and these are not changed by multiplying the values by 6.

Furthermore, the following technical details must be observed:

Preparation of the 2 % casein solution. "Casein according to Hammarsten". Casein obtained from the firms Merck or Nordmark is recommended because of improved solubility, more constant composition, and more consistent proteinase sensitivity.

The N-content of pure casein is 16 % (exactly 15.7 %).(2) Commercial preparations, however, contain less N because of the presence of some mineral constituents. As a result, it is necessary to prepare a 2 % solution which has the correct N content in order to achieve a reproducible baseline for the determinations. In order to accomplish this, a somewhat larger amount of the commercial product is weighed out and dissolved in 100 ml of buffer (pH 8.0). After the N content has been determined, this stock solution is diluted with an appropriate volume of the same buffer in order to obtain a 2 % casein solution with the correct N content. The volume of buffer solution required (x) is calculated using the following equation: $x = 50 \cdot C - 100$, where C is the concentration of casein in the stock solution. For example, if 3.5 gm of material had been used for preparation of the stock solution and a N content corresponding to 2.9 % casein was determined, then x would be 45 ml.

When the constant composition of the above-mentioned commercial products is taken into consideration, one can calculate the value of x which has to be weighed out in order to obtain a 2 % solution of casein. This calculation is carried out using the equation $x = 2a:b$ where a = a given weighed amount of the commercial product and b = the amount of pure casein which the commercial product contains. In the previously mentioned case, the calculations would be as follows: $x = 2 \cdot 3.5 : 2.9 = 2.4$. Thus, 2.4 gm of commercial product dissolved in 100 ml of buffer would result in a 2 % casein solution with the correct N-content. For the sake of reliability, a final N-determination is recommended.

In order to obtain a complete and rapid solution of the casein, the weighed material is ground in a mortar with a small amount of buffer solution, rinsed into a beaker with more buffer solution, and then dissolved in a hot water bath. After cooling, the solution is filtered into a 100 ml volumetric flask and the

solution is diluted to the mark with buffer solution. Chloroform and toluene are added and the solution is stored in the refrigerator at 5°C. The solution is usable for not more than 6 days after preparation. Even in the absence of microorganisms, a spontaneous disintegration of the casein molecule occurs after a certain period of time. The result is that after complete digestion, increased amounts of degradation products can be detected. The proteinase-sensitivity is increased because the casein (in old solutions) is digested more rapidly than is casein in fresh solutions. As a result of this, the results of the experiments carried out using old solutions are no longer comparable and cannot be used.

Before the start of some experiments, the proteinase-sensitivity of the casein must be determined by carrying out several determinations using sera from healthy individuals. The values should lie between 12 and 20 tyrosine units. In addition to serum, one can also employ a freshly prepared solution of trypsin (1:50,000). In this case, the increase in degradation products corresponds to 26 - 36 γ tyrosine per ml according to our results. We have employed trypsin from Merck or Nordmark of which 1 gm corresponds to the effect of a minimum of 40,000 Gross-Fuld units. The solution is prepared in phosphate buffer (pH 8.0) and the final pH of such a solution lies exactly between 7.0-7.1, the same as in the case of serum.

In the clinical determinations, the determinations were usually carried out in duplicate. The instrument values were most always the same or differed from each other by not more than 0.01.

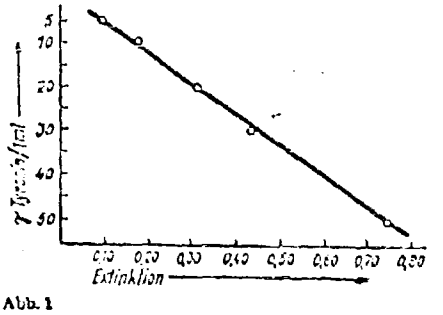

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Figure 1.

For every test series, the condition of the casein solution was examined in order to assure that it did not have any objectionable qualities. This was done by incubation of the starting material (in duplicate) with 1 ml of buffer solution in place of serum. Theoretically, no casein degradation should take place because of the absence of proteinases. In the actual tests, no increases in the photometric values were noted when the casein solutions were incubated for 24 hours with buffer.

Preparation of A Standard Curve. A standard curve was employed for the conversion of extinction values into tyrosine units. 0.1 gm of chemically pure tyrosine is dissolved in about 50 ml of N/10 HCl with light heating. The solution is rinsed into a 100 ml volumetric flask with N /10 HCl and then diluted to 100 ml. Using this 1:1,000 tyrosine solution, one prepares a series of dilutions with a volume of 10 ml and have a tyrosine content of 5, 10, 20, 30, 50, and 80 γ tyrosine per ml.

2 ml of each dilution are employed in the performance of the xanthoprotein reaction which has already been described. The extinction values that are obtained are plotted against the concentration of tyrosine and a standard curve, consisting of a straight line, is obtained (see Figure 1).

For carrying out the xanthoprotein reaction, only a 25 % HNO_3 solution can be used since at higher concentrations of acid, an additional, interferring yellow color is produced. As far as the purity of the other reagents is concerned, it should be pointed out that the trichloroacetic acid solution must be colorless and clear as water. A yellow color which seldom occurs, can be removed by shaking the trichloroacetic acid solution with activate charcoal and then filtering it.

Copious quantities of chloroform are added to the buffer solutions immediately after preparation. Thymol, sublimate, and other preservative should not be used.

The [illegible] should be [illegible]

Clinical Results. In various cases of [illegible] increased [illegible], diminished or increased plasmin activity can be observed. The clinical diagnosis is not always decisive unless it is carried in accordance with the general condition of the patient, the severity of the illness or the [illegible] of the illness.

Plasmin activity is reduced in certain forms of anemias, in nephroses, in cirrhosis of the liver, and cachectic conditions of various origins. It is increased in acute hepatitis, pancreatitis, prolonged cholecystitis, and several diseases of swelling or tumors. In these regards, the following observations are presented.

(1) The lowest values were found in a case of lipoid nephritis which developed after a case of acute glomerulonephritis. The clinical picture was clear. The diagnosis was later confirmed by autopsy. The treatment consisted mostly of a protein-rich diet, Precnisolon, and plasma infusions.

During the first weeks after admission of the patient, the plasmin activity was very much reduced and could hardly be detected. The level was around 1 γ tyrosine per ml. The patient was observed continually over a period of two years. After intense treatment, the plasmin activity remained around 4 γ and very seldom reached 7 - 8 γ. The values that were obtained were always considerably below the lower normal limit of 12 γ. The patient's condition improved but $\frac{1}{2}$ year later, he died with a nephrotic athropy of the kidney.

In other cases of nephropathies with pronounced proteinurias, the plasmin values were also markedly decreased, but not as much as in the above-mentioned case of lipoid nephrosis.

(2) In anemias which are the result of the lack of iron or the loss of blood, the values for plasmin usually ranged between 8 and 9 γ. In one case of agastric anemia where there was only 30 % hemoglobin, a value of 6 γ was found.

Anemias which are the result of tumor growths sometimes show decreased values. The results obtained with patients with tumors vary according to the extent of the tumor growth and with the general condition of the patient.

In one case of splenomegaly with moderate anemia (73 % hemoglobin), after removal of the spleen, values ranging between 7 and 9 γ were obtained. The histological examination revesled only non-specific, chronic inflammatory alterations.

Cirrhoses of the liver in advanced stages in most instances result in reduced values. The following levels were found: in one case, 2 γ; in 5 cases, 4-5 γ; in 4 cases, 6-8 γ; in 4 cases, 8-9 γ, and in 2 cases, 10-11 γ. The more advanced the illness is and the poorer the general health of the patient, the lower the plasmin values are apt to be.

In contradiction to the cases mentioned, we have found one case of painless, compensated cirrhosis, which was confirmed by biopsy, a normal value of 13 γ was found. The peptase reaction in the serum of 59 units was also normal. (the value here is usually around 80 units; increases usually indicate damage to the liver parenchyma, inasmuch as enzyme synthesis is not substantially restricted due to atrophy of the parenchyma (9)).

The etiology of the cirrhoses was varied. We had to deal partly with primary atrophic cirrhoses and partly with cholangiogene ones; some cases werewdue to not-completely cured hepatitis.

In cases of transition from chronic hepatitis to cirrhosis, one could observed decreases in plasmin activity while peptase values were still high. This indicates that there is no connection between plasmin activity and peptase activity.

(4) In cases of acute hepatitis, the plasmin activity is in most cases increased. We found values between 12 and 29 γ with a mean of 22-24 γ. In these cases, the peptase values are also increased and range from 80 to 120 units or higher. In contradiction to this fact, it was found that one case of subacute,

yellow liver atrophy with jaundice and ascites accompanied by widespread destruction of all elements had a plasmin value of 15 %. When the hepatitis was cured, the plasmin activity returned to normal.

In many cases of cholangitis resulting from severe cholecystitis but without detectable participation of the pancreas, the plasmin values ranged from 21- 30%. In most cases, the values were close to 23%. Also in cases of cholangitis which persisted after removal of the gall bladder, the plasmin values were elevated and were the peptase values also.

In contrast, a plasmin value of only 8% was found in long drawn out case of cholangitis which had led to a fine nodular cirrhosis of the liver. At the same time, there was tuberculosis of the intestines, the peritoneum, the pleura, and the spleen (autopsy results). In this case, the plasmin activity was markedly reduced as was true with all the other distinct cases of cirrhosis of the liver in spite of the continued cholangitis.

(6) In cases of pancreatitis with more or less severe courses, frequently increased plasmin levels were detected. After recovery, the levels usually returned to normal and in some cases even became subnormal. Nine cases of actue pancreatitis were observed which had plasmin values ranging from 22 to 28%. In one case, a value of 54% was found. The extremely strong breakdown of casein may be due in part to the infiltration of pancreatic trypsin into the blood stream. Since the optima of plasmin and trypsin both lie between pH 7.0 and 8.0, with regards to the degradation of casein, it would be very difficult to determine to what degree active pancreatic trypsin participates in the process since trypsin is still active at the pH of the assay.

After healing has taken place in cases of pancreatitis, the plasmin activity usually returns to normal. In one case, it returned to 15%. In other case, it went as low as 7%.

In a case of extremely severe pancreatic necrosis with septic temperatures,

no increases in plasmin activity was observed and it remained at 1h % . The patient died two weeks after admission. In this case, the necrosis was so strong that only small fragments of the pancrease were left by the time of autopsy. It is quite likely that the relatively low plasmin activity that was observed was the result of advances atrophy of the tissue.

(7) Many other diseases exhibited various plasmin activities. From the results, it was concluded that the clinical diagnosis in itself could not be considered decisive and that one must also consider special individual factors and the peculiarities of the course of the disease. With regards to malignant tumors and leukemias, the following results were obtained:

(a) Nine cases of malignant tumors (mostly carcinomas) gave increased values ranging between 21 and 27% . Among these cases, there were four cases of stomach carcinoma and one case each of the following: carcinoma of the rectum, of the colon, and of the pancreas, ovarian carcinoma, and mesothelium of the peritoneum. In most cases, there was advanced metastasis of the liver and in some cases also of the peritoneum.

(b) In the following three cases, there were normal plasmin values which were between 18 and 20 % : bronchial carcinoma with purulant bronchitis and extrusive metastasis in various organs; pancreatic carcinoma with liver metastasis and jaundice; ovarian carcinoma with metastasis of the lung and skeleton.

(c) Subnormal plasmin values were observed in eight cases: stomach cirrhosis with metastases in the peritoneum and cachexia; colon carcinoma; pancreatic carcinoma; stomach carcinoma with ulcers and extended metastases in various organs; hypernephroma; carcinoma of the bile duct; mammary carcinoma with skeletal metastasis; bronchial carcinoma with carcinomas of the lymphatic ganglions.

Strikingly frequent are increased plasmin values in the case of malignant tumors of the abdominal organs with metastases of the liver and the peritoneum; subnormal values are frequently observed in cases of advanced cachexia.

(8) In four cases of lymphadenitis with more than 350,000 leucocytes per ml of peripheral blood, increased plasmin activity values ranging from 21 to 25 % were found. Another four cases gave normal values ranging between 12 and 18 %. Two of these cases had more than 120,000 leucocytes per ml and two non-leukemic cases had 3,000 and 3,600 leucocytes per ml.

Values between 4 and 11 % were found in four cases with quite different leucocyte counts in the peripheral blood. Correlations between plasmin values, cell counts, and hemoglobin content could not be made.

Two cases of myelomic leukemia with 176,000 and 296,000 leucocytes per ml gave normal values of 17 and 18 % respectively.

(9) Sporadic determinations of plasmin levels were also performed in cases involving numerous other diseases. Partly increased, partly normal, and partly subnormal levels were found here also. Generally, there were no characteristic patterns of increased or decreased plasmin activity. In this regards it should be mentioned that phlegmonas or other purulent processes with surgical treatment gave no increased plasmin activities.

With regards to cases of pneumonia, it was found that there were normal levels in the febrile stage and increased levels after the fever.

We are confident that after thorough group determinations, characteristic patterns of increased or decreased plasmin activity will be found.

GENERAL CONCLUSIONS

Plasmin values determined in the case of a variety of diseases show a wide range of differences. They can be as low as 1 to 2 % (Lipoid nephrosis, liver cirrhosis) or as high as 30 % (hepatitis, cholangitis, pancreatitis). The single case of pancreatitis which gave a value of 54 % should not be considered here since the possibility exists that active pancreatic trypsin had entered the blood stream.

The interpretation of all the results using a common basis is not possible at this time. Proteinases, which are active at neutral or alkaline pH's, have been found in leucocytes, spleen, and in other organs in trace amounts (5). It is possible that in addition to other mesenchymic elements, the reticuloendothelial systems also participates in the biosynthesis of plasmin. This is confirmed by the action of polypeptidase and plasmin in cases of hepatitis and cirrhosis of the liver.

Generally, one can assume that in cases of infectious hepatitis, the reticuloendothelial systems is affect first before the parenchyma. In acute stages of the disease, plasmin activity as well as the polypeptidase activity is markedly increased as a result of the inflammatory process. As the hepatitis progresses into the chronic stage and ultimately into cirrhosis, the values decreas for both plasmin activity and polypeptidase activity. By this time, the polypeptidase activity has returned to normal and ranges between 30 and 65 units. The plasmin values, however, become subnormal and can be extremely low in cases of progressive cirrhosis.

It can be seen, therefore, that the activities of plasmin and polypeptidase do not decrease at the same rate. In this respect, there is no correlation between these enzymes. This fact is also confirmed by the observation that during the transition from hepatitis to the chronic stage and then to cirrhosis, the plasmin activity can become markedly decreased while the polypeptidase activity is still above normal. A reasonable conclusion to draw from these observations is that plasmin and polypeptidase are synthesized by different tissue elements. In this respect, one can assume that the polypeptidase and other enzymes also are synthesized in the liver parenchyma. In the case of plasmin, it is likely that the mesenchymic elements are involved particularly the reticuloendothelial system, which is also destroyed in cases of progressive cirrhosis.

In this connection, it should be noted that the reticuloendothelial is considered to be a relatively primitive tissue from a phylogenetic viewpoint. It may be possible, therefore, that plasmin can be considered to be a proteinase which has not yet been differentiated and whose pH optimum lies near neutrality like those of the bacteria. This is in contrast to the digestive proteinases and the cathepsins which have pH optima at significantly high or low pH's. As a result, a close relationship between biochemistry and morphology is assumed.

The fact that in cases of leukemia and purulent inflammations, the plasmin activity is only rarely increased or not at all does not comply with one's expectations, as one is dealing here with irritations of the leucocyte system. One must remember, however, that treatment with bacteriostatic, antibiotic, and cytostatic agents, the biochemical and immunobiological processes are most certainly altered.

During recent years, Tolkmitt has been extensively engaged in plasmin determinations. The values that he obtained in cases of hepatitis and other diseases correspond closely with the one that we have obtained. A critical summary of the domestic and foreign literature can be found in his papers.

Determinations of plasmin levels should not be carried out sporadically. Considering the sensitivity of the method, reliable results can only be obtained when the determinations are performed continously in series and by using controls. In this way, one can determine often plasmin levels in sera from patients with cirrhosis of the liver and extended cholangitis. Thus, one would generally find subnormal or elevated values when using unobjectionable techniques.

Moreover, it must be remembered that the plasmin level changes very little in the case of chronic diseases. The case of lipoid nephrosis which was previously described served as a confirmation of the dependability of our method for determining plasmin activity.

SUMMARY

In this article is described a method for plasmin determination which is suitable for clinical serial investigations. In this method, plasmin is activated by the addition of acetone to the native serum. The experimental setup is carried out in one step. This results in a simple technique and a saving of time. The technical pecularities and the sources of error have been described in detail.

In most cases of infectious hepatitis, protracted cholangitis, and pancreatitis, the plasmin activity is increased. It is usually decreased in cases of nephroses and cirrhosis. In other diseases, deviations from normal can often be noted. However, it has not yet been possible characterize regular patterns in these diseases. In part, individual factors may determine the level of plasmin activity.

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