CHEMICAL ANALYSIS OF A PARA-AMYLOID "TUMOUR"

by

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INTRODUCTION

Para-amyloid tumours are very rare; therefore each case encountered should be investigated as thoroughly as is possible with the amount of available material. The tumour we investigated was obtained from a patient hospitalized in the Department of Internal Medicine of the University Hospital at Utrecht.

Two kinds of amyloid are distinguished, a) the common or secundary amyloid, an abnormal deposition of material, diffusely distributed between the tissue-cells as a consequence of a primary chronic inflammation, the inflammation being considered as the "cause" of the deposit, b) the para- or primary amyloid, a sharply circumscribed deposition of material, giving roughly the same histological colour reactions, for which no "cause" was found and which for that reason was called "primary" amyloid.

According to APITZ[1], however, para-amyloid is always accompanied by plasmacytoma (multiple myelome or KAHLER's disease) or by smaller, less sharply circumscribed accumulations of plasma cells. It does not seem improbable that there is a causal relation between the occurrence of these cells and of the para-amyloid.

On the other hand there seems to be some connection between the occurrence of secundary amyloid and an abnormal ratio of the amounts of the plasma proteins. Amyloidosis was for instance observed in a horse which was used for antibody production during a very long time and whose plasma proteins consisted almost completely of globulins. Also in the case of plasmacytoma one of the plasma globulin fractions is considerably increased and the abnormal content of this protein, which possibly is produced by these cells themselves, might be the immediate cause of the pathological deposition of amyloid.

The common- or secundary amyloid is diffusely distributed in liver, spleen and kidney; the para-amyloid is formed as tumours, covered by a thin layer of connective tissue, in muscles and skin. Sometimes these tumours initiate from the tunica media of the small arteries. Swelling of the tongue as caused by accumulation of para-amyloid between the muscle fibrils of the tongue is called the syndrome of LUBARSCH and PICK.

The histological colour reactions by which these diseases are diagnosed show only very small differences between amyloid and para-amyloid. This is, as has already been mentioned above, the reason why the deposited substance is always called amyloid with a distinction between common- and para-amyloid. We do not know for certain if there is any chemical difference between these substances.

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EXPERIMENTAL PART

The tumour was removed from below the skin of the patient's back. The fresh weight was 34.5 g and it had an ellipsoidal shape. It was cut longitudinally into two more or less equal parts and freeze-dried. The dry weight was 4.5 g. The great bulk of the dry para-amyloid consisted of a powder which could be shaken from the shell and could easily be separated from a few fibres of connective tissue.

The powder could be moistened by water and retained this property during the months in which we were occupied with this investigation*. The particles of a suspension in water rapidly settled down in the same way as in the case of a microcrystalline protein.

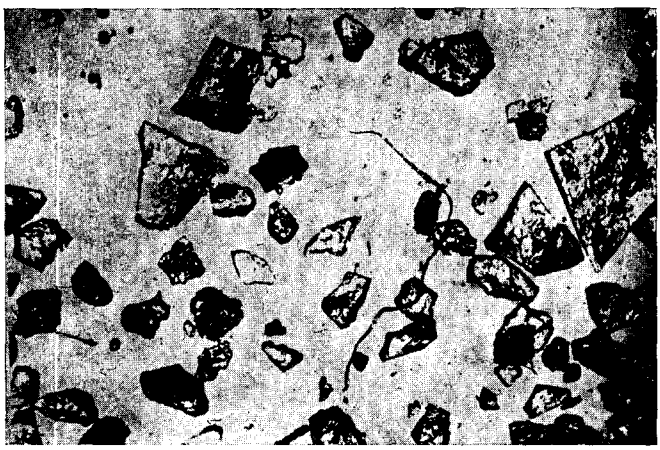

Fig. 1. Para-amyloid, largest particles of freeze-dried material, suspended in water, magnification 100 ×

Upon microscopical examination of the suspension particles with sharp edges, similar to crystals are observed. But these particles do not show double refraction. Hence it seems more probable that they are splinters of the dry material, which had been deposited in layers. It was possible to separate fractions of smaller and larger particles by decanting the suspension after only part of the solid matter had settled down (See Fig. 1).

The freeze-dried substance has a high affinity to crystal-violet and to congo-red. It is unsoluble in water and in a 50 % urea solution, even after adding alkali. It is dissolved to a yellowish-brown solution by boiling with 10 or 20 % caustic soda. Only part of the para-amyloid is dissolved in 20 % HCl. It dissolves completely, however, by allowing to stand for 24 hours in 38 % HCl, by which treatment a violet colour develops. We shall revert to this colour reaction below.

Chemical analysis of the freeze-dried powder:

1. Water content, 7.7 %.
2. Total nitrogen content (KJELDAHL) 13 %.
3. Non-protein nitrogen (KJELDAHL) 0.3 %.

* We have observed that freeze-dried heart-muscle lost the property of being moistened by water during storage for several months.

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4. Protein content, calculated from total N and non-protein N, 79.4 %. The protein shows positive reactions of MILLON (phenyl groups), ADAMKIEWICZ (tryptophane), SAKAGUCHI (arginine) and PAULY (histidine), while the xanthoprotein- and the biuret reactions were also positive.

5. Non-sterol lipoids (KUMAGAWA and SUTO), 1.4 %.

6. Cholesterol (LIEBERMANN-BURCHARDT), 0.85 %.

7. Glycogen (PFLÜGER), nil.

8. Total reducing substances liberated after boiling for 3 hours with 25 % HCl and determined by the HAGEDORN-JENSEN method, calculated as glucose, 8.5 %.

9. Total P, determined by destruction with H_2SO_4 and HNO_3 and P-determination according to FISKE and SUBBAROW, 0.015 %.

10. –SH sulphur, determined by boiling with 40 % NaOH, addition of Pb-acetate, dissolving the $Pb(OH)_2$ by adding acetic acid and filtering, drying and weighing the PbS, 0.6 %.

The presence of free or bound sulphate could not be demonstrated. Small amounts of sulphate ions in pure aqueous solution giving a distinct precipitate with $Ba(OH)_2$ did not give a trace of turbidity, if added to a protein hydrolysate and reacted upon with baryta. So small amounts of sulphate present or set free by treatment of para-amyloid with HCl can escape detection by the baryta reaction. The same holds for the benzidine reaction.

Lack of material prevented the determination of total sulphur.

The colour reaction with 38 % hydrochloric acid

As has already been mentioned, para-amyloid dissolves completely in 38 % hydrochloric acid on standing at room temperature for 24 hours. The dissolution is accompanied by the development of an intensive violet colour. This colour remains practically constant for months on standing at room-temperature.

It changes to deep brown by adding alkali until alkaline reaction. The latter colour also develops on boiling the acid solution. Melanine formation on boiling amyloid with hydrochloric acid has already been observed several years ago by HANSSEN[2].

The absorption spectrum of the violet solution is given in Fig. 2. The maximal extinction is situated at the wave length 560 mμ.

The colour disappears upon reduction by a trace of stannochloride; it returns after adding a small amount of hydrogen peroxide. Upon adding more hydrogen peroxide the colour fades to pale yellow.

Considering the ways in which the violet dye might be formed it appeared most probable that a similar dye is formed in the reaction of tryptophane with concentrated HCl and a hexose. The first observation concerning the latter reaction has been made by ROHDE[3], who observed a violet colour after mixing a protein solution with p-dimethyl-benzaldehyde or another aromatic aldehyde and a concentrated inorganic acid. This reaction appeared to be due to the presence of tryptophane in the protein. It was also given by tryptophane itself. The reaction of ROHDE as well as the well-known tryptophane reaction of ADAMKIEWICZ (with glyoxylic acid) are inhibited by adding acetaldehyde to the protein solution previous to mixing the latter with the reagents. Hence the reaction of ADAMKIEWICZ probably also depends upon the aldehyde group of the glyoxylic acid.

WEEHUIZEN[4] has shown that a similar reaction is given by scatol or indol, instead

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of tryptophane, and glucose, lactose, saccharose and polysaccharides, instead of the aromatic aldehyde. According to WEEHUIZEN the reaction mixture must be heated. He does not mention differences between the various carbohydrates examined by him.

We have carried out a few experiments on this reaction. If the reaction is carried out with scatol and fructose an intensive violet colour develops at room temperature; glucose, lactose, galactose, mannose and glucuronic acid give a pale yellow colour, only becoming violet in the upper layer which is in contact with the air.

We have performed this reaction by adding 18.5 ml 38 % HCl and 0.5 ml of a 1 %

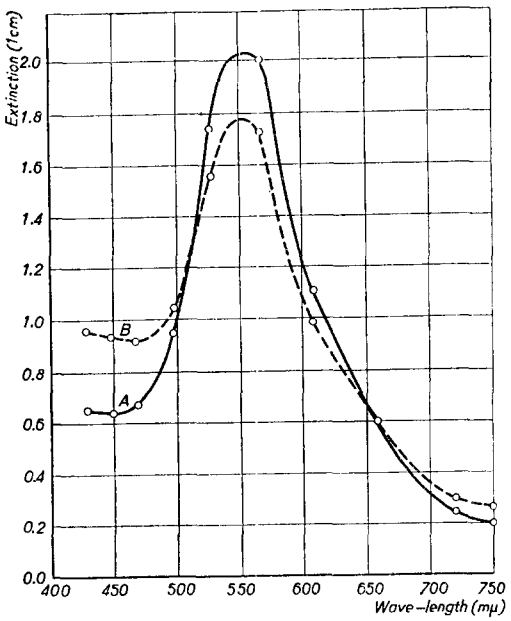

Fig. 2. A. Absorption curve of a solution of 10 mg para-amyloid in 10 ml 38 % HCl after 3 days standing at room temperature (measurements with ZEISS, "Stufenphotometer"). B. Absorption curve of the same solution 12 days later.

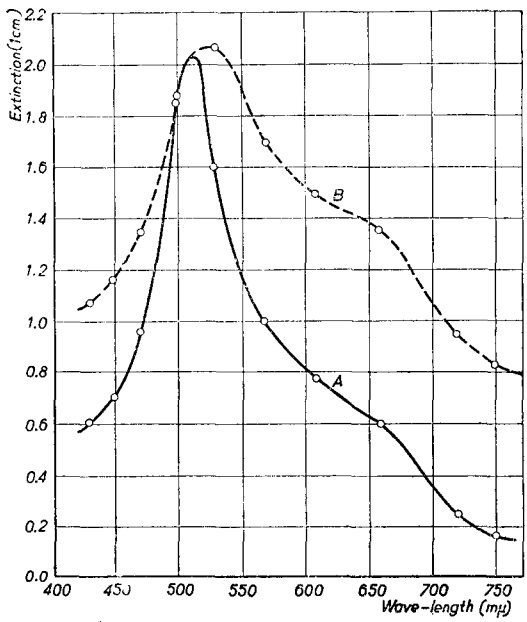

Fig. 3. A. Absorption curve of reaction product of 1 ml fructose solution (8 mg fructose) + 18.5 ml 38 % HCl + 0.5 ml 1 % alcoholic scatol solution. B. Absorption curve of reaction product of 1 ml fructose solution (8 mg fructose) + 18.5 ml 38 % HCl + 0.5 ml 1 % tryptophane solution (measurements with ZEISS "Stufenphotometer").

alcoholic solution of scatol to 1 ml of fructose solution, or one of the other sugar solutions, containing 8 mg of the sugar. The violet colour formed in the case of fructose was so strong that the solution could be diluted many times with the concentrated hydrochloric acid before the colour became too weak to be observed. The absorption spectrum is given in Fig. 3 (curve A). It has two bands, a strong one at 520 mμ and a very weak one at 660 mμ.

A similar reaction is given by tryptophane and fructose instead of scatol and fructose (see Fig. 3, curve B). The intensity of the violet colour increases in the course of several days. This reaction is also given by glucose, lactose and mannose, but the colour obtained with fructose was much more intensive than with the other sugars.

Hence it seems possible that the violet colour developed by para-amyloid with concentrated hydrochloric acid upon standing for 24 hours depends upon the presence of tryptophane and fructose or some other sugar in relatively high concentration.

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We have tried the reaction of a number of proteins of different origin with 38 % HCl, *viz.* human casein, common crude casein from cow's milk, serum albumin, an aqueous extract of rabbit's muscle, thymus nucleoproteid, thrombin, gelatin, BENCE-JONES protein. The samples of these proteins were more or less pure and part of them had been stored for many years in the collection of this laboratory. With the exception of gelatin, which contains no tryptophane, they all gave a faint violet colour upon standing with 38 % hydrochloric acid for 24 hours and longer*. The colour with BENCE-JONES protein was extremely weak ; a beautiful colour reaction was given by the crude cow's casein, and to a somewhat lesser degree by the human casein. Fig. 4 gives the absorption spectrum of the violet reaction product of the latter protein. It shows bands at 530 mμ and 660 mμ and is similar to the spectrum of the violet solution resulting from the reaction of fructose, tryptophane and hydrochloric acid.

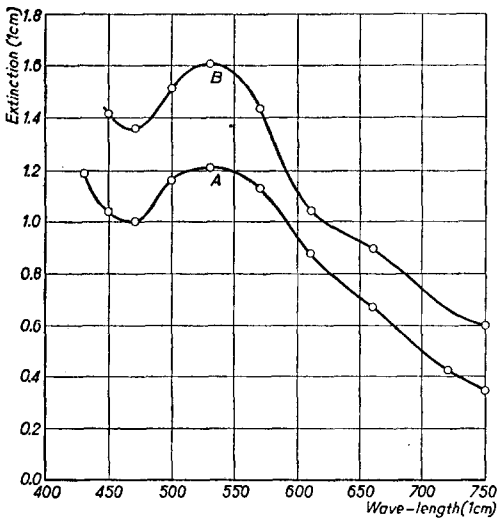

Fig. 4. A. Absorption curve of a solution of 500 mg human casein in 20 ml 38 % HCl after 8 hours standing at room temperature. B. Absorption curve of the same solution after 24 hours (measurements with ZEISS "Stufenphotometer").

We think it possible that in all these cases the reaction is analogous to the reaction of para-amyloid with concentrated HCl. Comparing curves A and B of Fig. 2 one observes a crossing of the curves at 660 mμ, which suggests that a very weak absorption band, similar to the weak bands at this wave-length in Figs. 3 and 4, develops in the hydrochloric acid solution of para-amyloid on standing at room-temperature for several days.

But there are differences, *viz.*:

1. in the case of para-amyloid the chief absorption maximum is not situated at 530 mμ, but at 560 mμ;

2. in the case of para-amyloid the colour is much more stable than with the other proteins.

The much greater intensity of the colour in the case of para-amyloid (compare extinctions and concentrations in Figs. 2 and 4) may simply be explained by higher concentrations of the reaction components.

If we accept the view that the reactions of the proteins and the para-amyloid all depend upon the presence of an indol-derivative and an aldose — and indeed, in addition to all arguments mentioned already we can still record the fact that the reaction of para-amyloid with HCl, as well as the reaction of tryptophane and fructose with HCl are inhibited by formaldehyde —, we must be aware that this conclusion only concerns the general type of reaction, for we have no certainty about the identity of both the indol derivate and the aldose in all these cases. In view of the intensity of the violet colour of the para-amyloid reaction we think that in this case most evidence is in favour of fructose. The intensities of the violet colours are equal if we compare corresponding

* In the case of rabbit's muscle extract the reaction was carried out by saturating the extract with gaseous HCl.

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amounts of para-amyloid and fructose, *viz.* 50 mg para-amyloid and 4 mg fructose + 2.5 mg tryptophane, assuming that the reducing power of para-amyloid is entirely due to fructose (comp. Figs. 2 and 3). But we feel that this evidence is not conclusive. For instance we do not know the tryptophane content of our para-amyloid and lack of material prevented a tryptophane determination. EPPINGER[5], however, found 4.2 % tryptophane in the dry para-amyloid examined by him.

The carbazol reaction

We have tried to identify the sugar by means of the carbazol reaction of DISCHE[6]. This reaction was carried out in the following way: 6 ml of H_2SO_4 (8 vol. of concentrated H_2SO_4 + 1 vol. of H_2O) are added to 1 ml of the sugar solution. The mixture is placed in a boiling waterbath for 20 minutes; after cooling to room-temperature 0.2 ml of an 0.1 % solution of carbazol in ethyl alcohol is added.

If the reaction is carried out with glucuronic acid a violet colour develops immediately after adding the carbazol. According to DISCHE this colour reaches its maximum after about 2 hours. In our experiments the colour appeared immediately, but the maximum was only reached after 24 hours. Glucose gives a pink-brown colour with H_2SO_4 in the boiling waterbath; this colour is not changed immediately by adding carbazol, but a violet colour develops slowly in about 24 hours. This colour is first seen in the upper layer which is in contact with the air, from which the sulphuric acid absorbs some water. Indeed the reaction could be inhibited by closing the tube with a glass stopper.

Para-amyloid dissolved in 38 % HCl and discoloured by adding a trace of $SnCl_2$ and fructose gave about the same reaction as glucose. In all cases, glucuronic acid included, the absorption curve had a maximum at 530 mμ. So neither by this means have we been able to demonstrate which sugar is present in para-amyloid. The only point which seems to be quite certain is that it does not contain glucuronic acid in appreciable amounts. In general our observations are far from complete, but we think it right to plublish them in view of the scarceness of the cases of para-amyloid encountered and the possibility that they may be of some use to other workers in the field of clinical chemistry.

DISCUSSION

We have given considerable attention to sulphate and glucuronic acid in para-amyloid as there is some controversy in literature on the occurrence of chondroitin sulphuric acid here in. We did not find any indication of its presence in para-amyloid. Many years ago, HANSSEN[2], who investigated mechanically isolated grains of secundary amyloid, and later on EPPINGER[5], investigating a sharply circumscribed amyloid tumour, arrived at the same conclusion. ODDI[7], however, and later on KRAWKOW[8] and recently HASS[9] found an increased content of chondroitin sulphuric acid in liver, spleen and other tissues with diffusely distributed amyloid. But the chondroitin sulphuric acid has never been found when the amyloid was isolated mechanically from the surrounding tissue in which it had been deposited. Hence we are inclined to agree with HANSSEN that the deposition of amyloid is accompanied by the accumulation of chondroitin sulphuric acid apart from the amyloid grains in the diseased organ.

We believe the chief result of our work has been the discovery of the violet reaction with 38 % hydrochloric acid. If another para-amyloid tumour becomes available we

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think the material should be primarily used for the determination of the tryptophane content and the identification of the sugar.

It will further be interesting to determine the indican excretion of the patients as in the course of two months our patient's urine twice gave a stronger indican reaction than the urine of 13 control subjects examined at the same time. There might be some connection with a presumed high tryptophane content of the para-amyloid.

SUMMARY

1. A para-amyloid tumour was preserved by freeze-drying; some properties of the dry material are given. It was analyzed for total N, non-protein N, protein, non-sterol lipoids, cholesterol, glycogen, reducing substances after boiling with HCl, total P, –SH sulphur, and sulphate.

2. The dry para-amyloid contains about 80% protein, which gives possitive reactions of MILLON, ADAMKIEWICZ, SAKAGUCHI, PAULY and positive xanthoprotein and biuret reactions.

3. It contains –SH groups, but no sulphate, free or bound, could be detected.

4. The amount of reducing substance after hydrolysis, calculated as hexose, amounted to 8.5% of the dry material. The sugar(s) present could not be identified. Glucuronic acid is absent.

5. The solubility in various concentrations of strong acid and alkali was studied. The para-amyloid dissolves at room temperature in 38% hydrochloric acid, giving a solution of an intense violet colour. The absorption spectrum of this solution was determined. The reaction is suggested to be analogous to the reaction of tryptophane and a hexose with hydrochloric acid. The violet colour was also observed by treating other proteins with hydrochloric acid, but its intensity was of a much lower order of magnitude than in the case of para-amyloid.

RÉSUMÉ

1. Une tumeur para-amyloïde, conservée par dessication après congélation, a été analysée en ce qui concerne: N total, N non protéique, protéines totales, lipoïdes non stéroïdes, cholestérol, glycogène, substances réductrices après hydrolyse chlorhydrique, P total, soufre des SH et sulfates.

2. La substance contient environ 80% de protéines réagissant positivement avec les réactions de MILLON, ADAMKIEWICZ, SAKAGUCHI, PAULY et du biuret.

3. Elle contient des groupes SH, mais aucun sulfate, libre ou combiné.

4. La proportion de substances réductrices après hydrolyse, calculées en hexose, correspond à 8.5% de matière sèche. Le ou les sucres présents n'ont pu être identifiés. Il n'y a pas d'acide glucuronique.

5. La solubilité dans les acides et les alcalis forts à différentes concentrations, a été étudiée. La substance para-amyloïde se dissout à la température ordinaire dans l'acide chlorhydrique à 38% en donnant une solution violet intense. Le spectre d'absorption de cette solution a été mesuré. Il s'agit ici d'une réaction analogue à celle que donne le tryptophane et un hexose avec l'acide chlorhydrique. La couleur violette a été également obtenue en traitant d'autres protéines par l'acide chlorhydrique, mais son intensité était beaucoup plus faible que dans le cas de la substance para-amyloïde.

ZUSAMMENFASSUNG

1. Para-amyloid wurde durch Gefriertrocknung konserviert; einige Eigenschaften des getrockneten Materials werden angegeben. Es wurde untersucht auf Gesamtstickstoff, Nicht-Eiweissstickstof, Eiweiss, Nicht-sterol-lipoide, Cholesterol, Glykogen, reduzierende Stoffe nach Kochen mit HCl, Gesamtphosphor, –SH Schwefel und Sulfat.

2. Das getrocknete Para-amyloid enthält ungefähr 80% Eiweiss, das bei den Reaktionen von MILLON, ADAMKIEWICZ, SAKAGUCHI, PAULY und bei der Xanthoproteinreaktion und der Biuretreaktion positiv reagiert.

3. Es enthält –SH Gruppen; Sulfat konnte jedoch, weder frei noch gebunden, entdeckt werden.

4. Die Menge der reduzierenden Stoffe nach Hydrolyse beträgt, als Hexose berechnet, 8.5% des getrockneten Materials. Der (Die) anwesende(n) Zucker konnte(n) nicht identifiziert werden. Glukuronsäure ist nicht vorhanden.

5. Die Löslichkeit in verschiedenen Konzentrationen starker Säuren und Basen wurde untersucht. Para-amyloid löst sich bei Zimmertemperatur in 38 prozentige Salzsäure, und ergibt dabei eine Lösung von stark violetter Farbe. Das Absorptionsspektrum dieser Lösung wurde gemessen.

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Die auftretende Reaktion scheint analog an die Reaktion von Tryptophan und einer Hexose mit Salzsäure zu sein. Die violette Farbe trat auch bei Behandlung anderer Eiweisskörper mit Salzsäure auf; ihre Intensität war dann aber von einer viel geringeren Grössenordnung als im Falle des Para-amyloids.

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Received February 24th, 1948