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PREPARATION AND SOME PROPERTIES OF ANAPHYLATOXIN FROM HOG SERUM

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Abstract—Anaphylatoxin (AT) has been prepared by contact activation of hog serum with baker's yeast. A procedure of purification is described which is applicable to large scale preparation of highly active AT. The known biological actions of AT-containing sera are all recovered in the purified preparations. The active material is a peptide with an apparent molecular weight of 7000–8000 (as monomer). It contains essential disulphide groups.

THE CLASSICAL sources for anaphylatoxin (AT) formation are serum or plasma of guinea pig and rat. These species are not suitable for preparative work because of their small size. Stegemann, Vogt and Friedberg[1] prepared AT from hog plasma by incubation with the AT forming enzyme of cobra venom. They obtained small quantities of highly purified material. It was later found by Schwoerer[2] (see also [3]) that hog plasma contains not only the substrate but the enzyme also for anaphylatoxin formation. This made it possible to produce AT on a large scale by the original method of contact activation of serum, without interaction of any exogenous enzyme.

The final procedure of purification described below has been repeated several times and has proved to reproducibly yield highly active material which by all available criteria is identical, functionally, with AT from rat and guinea-pig sera.

METHODS

Hog serum and plasma. In the earlier experiments plasma was obtained from hog blood collected at the slaughter house with 100 mg/l. heparin. Later, serum was used. Hog blood was collected with 3 g di-sodium oxalate/l. and was centrifuged. The plasma from 1 l. blood was recalcified with 1·8 g $CaCl_2$ added as 10% solution with stirring. Ca-oxalate precipitated out and was trapped in the developing fibrin clot. To obtain the serum the mixture was poured through a polythene sieve which retained the clot.

Assay of AT. All assays were performed on isolated strips of guinea-pig ileum. For determination of active zones in chromatographic separations and for other qualitative assays the contractions produced were recorded isotonically with a frontal lever on smoked paper. Quantitative assays were done by isometric recording via strain gauge using four point assay arrangements as suggested by Rocha e Silva and Rothschild.[4] As a reference a standard preparation of purified AT was used which was kept as a 0·5% stock solution in 0·02 N acetic acid at −20°. For use samples were withdrawn and were diluted with saline.

Chromatography. CM-cellulose (Schleicher and Schüll, 1·06 mEquiv/g) was used for batch adsorption as supplied. CM-Sephadex C-50 was washed with 0·5 N NaOH, water, 0·5 N HCl, water in that order and was then equilibrated with 0·02 N Na-acetate buffer pH 5·6 before each use. The resin Amberlite XAD-2 (Roehm and Haas) was used as supplied. After chromatography it was regenerated by washing it with large volumes of water. All chromatographical separations were carried out at 0–4°.

Analytical tests

Carbohydrate. The anthrone, orcinol and Elson-Morgan reactions were carried out as described.[5–7] For detection of glycol groups 200 μg AT dissolved in 0·2 ml 0·05 M acetate buffer pH 5·2 were treated with 0·05 ml 0·33 M Na-periodate for 30 min at 0°. The solution was then made up to 2 ml with ice-cold saline and its activity was assayed biologically immediately afterwards.

Reduction of AT

AT was reduced with mercaptoethanol according to Anfinsen and Haber,[8] however without the use of urea. In some instances the reaction mixture was freeze-dried afterwards, in others *N*-ethylmaleimide was added to prevent re-oxidation.

Reduction with thioglycollate: To 1 mg AT dissolved in 1 ml 0·067 M phosphate buffer pH 7·2 was added 0·1 ml thioglycollate solution and the mixture was kept at 37° for 4 hr. As a control AT was kept in buffer alone under the same conditions. The thioglycollate solution was prepared by neutralizing 1 ml 80% thioglycolic acid with 11 ml 1 N NaOH.

Nitrogen content. Nitrogen was estimated by Nesslerization modified from Strauch.[9] Samples (up to 1 ml fluid) were pipetted into Fiolax glass tubes, 0·2 ml of Selenium-sulphuric acid were added and the tubes were slowly heated to 320° in a metal block and kept at that temperature for 30 min. After cooling the contents were transferred quantitatively with water to 25 ml volumetric flasks, 3 ml of Nessler's reagent were added and the mixture was made up to volume. The colour density was read in a Zeiss PMQ II spectrophotometer, at 420 mμ when less than 20 μg N was present, at 500 mμ when it was more. A calibration curve was prepared from a standard solution of ammonium sulphate. Selenium-sulphuric acid and Nessler's reagent were prepared as described by Strauch.[9]

RESULTS

Preliminary fractionation experiments

In the first experiments heparinized hog plasma (1–6 l) was activated with 10 g/l. Sephadex G-75 at 37° for 2–6 hr. After decantation and centrifugation the active principle was adsorbed by stirring the plasma with 3 g/l CM-cellulose for 30 min adjusting the pH to about 4 with formic acid. The cation exchanger was separated and stirred at 4° with 0·5 M ammonium formiate buffer pH 8·2 using a volume of 125 ml buffer/l. plasma started from. The pH was readjusted to 8·2 with concentrated ammonia. After 30 min the mixture was centrifuged, and the residue eluted once more with the same volume of ammonium formiate buffer. Immediately after separation the supernatants were slightly acidified, combined and dialyzed through Cellophan tubing (Kalle) against distilled water. The residue was freeze-dried, redissolved in 15–30 ml 0·02 M Na-acetate buffer pH 5·6 and fractionated on Sephadex G-100 (5·6 × 100 cm).

The fractions containing the biological activity were collected, freed from salts by dialysis and freeze-dried again.

By this procedure preparations of AT were obtained which contracted the guinea-pig ileum at doses of 2–5 μg/10 ml bath fluid. One of these preparations has been used for studying the actions of AT on respiration and circulation in guinea pigs.[10]

The yield of AT obtained by the method described was rather low. Rarely more than about 10 per cent of the original activity was recovered, often less. It became evident that most losses occurred during dialysis, and during the first adsorption-elution step.

Therefore other techniques were looked for to concentrate AT solutions and the first step of adsorption and elution was improved.

Final procedure for purification of AT

Hog serum (6–7·5 l) is incubated at 37° with 120–150 g baker's yeast, for 1 hr. Then the solution is centrifuged and the clear supernatant is mixed with an equal volume of water. The solution is cooled and formic acid is added until the pH is lowered to about 4. CM-cellulose (3 g/l.) is added and the solution is stirred for 30 min in the cold room. The cellulose is collected by centrifugation and 3 g/l. are added once more to the supernatant. After another 30 min stirring the second batch of CM-cellulose is collected, too, and the supernatant discarded.

The two batches of CM-cellulose are combined and washed with 0·1 M ammonium formiate buffer pH 4·0 (1/10 of the starting volume of serum), then with water. The material is then transferred to a chromatography tube and eluted first with 500 ml of 0·5 M acetic acid, followed by 0·5 M ammonium formiate buffer pH 7·0. During the second elution fractions of 10 ml each are collected. They are assayed for AT activity and the active eluate (usually the fractions from 250–450 ml) is collected. It is passed through a column of 40 g Amberlite XAD-2 suspended in water. The effluent is discarded, the column is eluted with water—acetic acid—methanol (1 + 2 + 1 vol.). Again the active fractions (eluted between 10–200 ml) are combined, concentrated by vacuum destillation at low temperature and freeze-dried.

Three batches of AT purified each from 6–7·5 l of serum to this stage are combined, dissolved in 15–20 ml of 0·02 M Na-acetate buffer pH 5·6 and are passed through a column of Sephadex G-100 (5·6 × 100 cm) with the same buffer. Fig. 1 shows a typical result of the fractionation. The AT containing fractions eluted between 1000 and 1800 ml are combined and passed through CM-Sephadex C-50 (1·6 × 20 cm) suspended in the same buffer. A linear gradient increasing in ionic strength and pH is applied for elution. The gradient starts with 0·02 M ammonium formiate buffer pH 5·5 and ends with 0·5 M ammonium formiate, pH 6·5. 200 ml each of the two buffer solutions are used and after the gradient has passed additional 200 ml of the second buffer are applied to the column. Fig. 2 shows the position of AT in the fractions obtained.

The combined active fractions are freeze-dried, redissolved in a small volume of 0·02 N acetic acid and desalted on a column of Sephadex G-25 (1·6 × 100 cm) with the same solvent.

The final product is again freeze-dried and weighed. Table 1 shows the purification and yields after the various steps.

Some properties of the purified AT preparations

The dried preparations are white powders which readily dissolve in water, acetic

acid, phenol—acetic acid mixtures, and aqueous methanol. The Nitrogen content is about 15 per cent.

Purity. Sephadex chromatography gave no indication of heterogeneity of the purified material. In electrophoresis on cellogel strips (0·05 M barbital buffer, pH 8·6) two bands were stained with amido black and in disk electrophoresis on polyacrylamide three bands became apparent. They all moved to the cathode, lying closely together. Elution from the gel of active material has, as yet, not been possible.

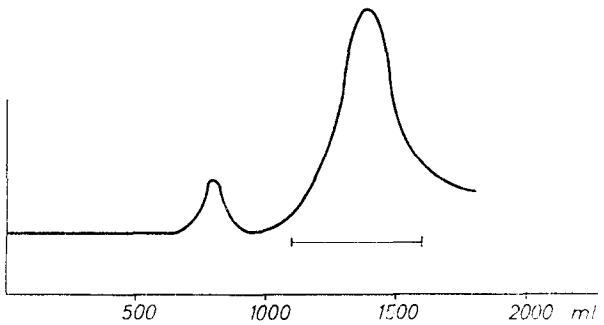

FIG. 1. Fractionation of AT on Sephadex G-100 in 0·02 M acetate buffer pH 5·6. Ordinate: extinction at 280 mμ (arbitrary units). Abscissa: elution volume (ml). —— zone containing AT activity.

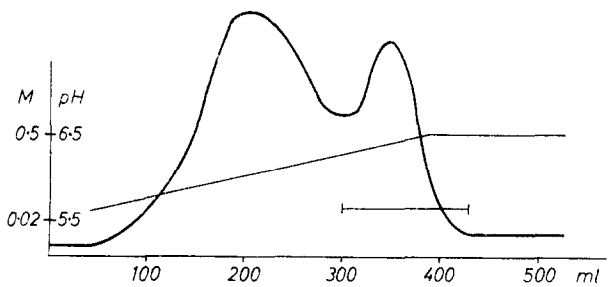

FIG. 2. Fractionation of AT on CM-Sephadex C-50 by gradient elution. Thick line: Extinction of the fractions at 280 mμ (arbitrary units). Thin line: Elution gradient (increase in molarity from 0·02 to 0·5, in pH from 5·5 to 6·5). —— distribution of AT-containing eluates.

TABLE 1. ACTIVITY AND YIELD OF ANAPHYLATOXIN OBTAINED AFTER THE VARIOUS STEPS OF PURIFICATION. THE FIGURES REPRESENT MEANS OF 6 EXPERIMENTS

Purification stage	Amount (mg)	Total activity*	Activity*/mg	% Yield
Activated serum	6650000	242000	0·036	100
CM-cellulose eluate		129000		53
Amberlite XAD-2 eluate	333	79000	237	33
3 Batches of XAD-2 eluate	(1000)	(237000)	(237)	(33)
Sephadex G-100 eluate		232000		32
CM-Sephadex G-50 eluate		140000†		19†
Sephadex G-25 eluate	85	174000	2042	24

* Activity estimated as μg equivalents of standard preparation A IV.

† The figures are apparently too low, probably because of interference of the buffer medium with the bioassay.

Stability. The purified preparations show the same high stability to acid environment at even elevated temperatures which has been described earlier for less pure AT of rat and hog plasma. Unexpectedly, several batches slowly lost biological activity in the lyophilized state, even at −20°. A partially purified preparation intended as biological standard deteriorated in the course of a few months. AT seems to be better preserved in 0·02 N acetic acid, at −20°. So far no serious loss of activity has become apparent after eight months storage.

Disulphide groups. After treatment with mercaptoethanol AT lost 50 per cent or slightly more of its smooth muscle contracting activity. The loss was not increased when the product was stabilized in the reduced form by adding *N*-ethyl-maleimide. Treatment of AT with *N*-ethyl-maleimide alone had no adverse effect on its activity. Thioglycollate reduced the activity of AT by about 90 per cent.

Carbohydrate. The anthrone and orcinol reactions were negative with 100 μg and 300 μg of AT, respectively. For comparison, the anthrone reaction was strongly positive with 100 μg of ovomucoid as well as 3 μg of glucose, and 5 μg of xylose gave a positive orcinol reaction.

The Elson–Morgan reaction for hexosamines was negative when applied to 600 μg AT, whereas a positive result was obtained with 10 μg glucosamine.

Treatment of purified AT with periodate reduced its activity by 80–90 per cent. In serum AT appears to be protected against periodate, for no serious loss of activity was observed when AT-containing serum was subjected to the same treatment.[3]

Molecular size. AT is generally regarded as being non-dialyzable. However, some activity passed through Visking tubing 27/100 especially when the tubing had been distended. In phenol—acetic acid—water solution AT penetrated slowly through regenerated cellulose.

An estimate of the molecular size was made by comparative gel filtration on Sephadex G-75. In order to eliminate absorption effects or aggregation, phenol—acetic acid—water (1/1/1 w/v/v) was used as solvent.[11] The column (1·4 × 100 cm) was run at 12°.

During preliminary runs the properties of the column changed. The flow speed declined and the elution volume for a given substance became larger in the course of some weeks. Some column material went into solution. Probably some cross-links were cleaved. The analytical runs shown below (Fig. 3) were therefore performed in the course of 3 weeks at a time when the column had been in use for 3 months already.

The compounds used for comparison and AT were applied to the column at suitable intervals, and fractions of 1·8 ml each were collected. They were analysed for nitrogen. AT fractions were also tested on the guinea-pig ileum, after lyophilisation. The elution volumes were calculated from the position of the peaks of nitrogen which in the case of AT differed from the position of maximal biological activity by one tube.

From the diagram in Fig. 3 it is apparent that the elution volume obtained with one sample of AT corresponds to a molecular weight of about 7600.

Actions. The purified preparations of AT show all biological actions characteristic of AT-containing rat or guinea-pig serum, as obtained by contact activation. The isolated guinea-pig ileum is contracted maximally by concentrations often as low as 0·05 μg/ml. Assuming a molecular weight of 7600 this corresponds to a concentration of 6×10^{-9} M. The activity of AT on guinea-pig ileum is thus definitely higher than that of histamine. Histamine concentrations of $2{\cdot}6 \times 10^{-8}$ M usually produced a

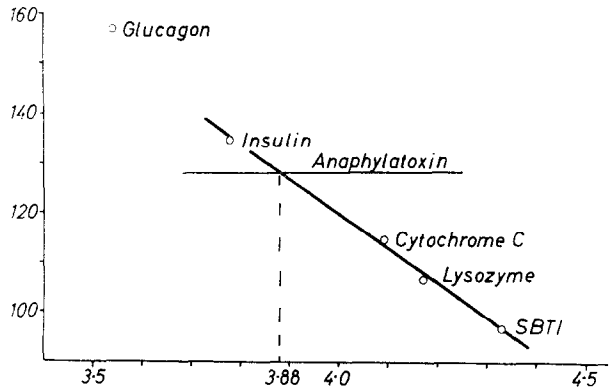

FIG. 3. Gel chromatography of hog AT and some compounds of known molecular weight on Sephadex G-75 in phenol—acetic acid—water (1:2:1 w/v/v). Ordinate: elution volume (ml). Abscissa: log molecular weight. SBTI: Soybean trypsin inhibitor.

medium response. The effect of purified AT on the guinea-pig ileum is blocked by antihistaminics and is subject to tachyphylaxis. With AT from rat and guinea-pig serum there is cross-desensitization.

Given i.v. to guinea pigs 10 μg/kg produce bronchospasm and 20 μg/kg are often lethal. The effects of purified AT on respiration and circulation in guinea pigs have been described in detail elsewhere.[10, 12] The preparations release histamine into the blood, in guinea pigs (personal communications by Dr. Giertz, Freiburg and Dr. Friedberg, Göttingen).

DISCUSSION

As starting material for the preparation or AT hog serum activated with yeast was used. In some experiments the serum had been activated with agar. In these it became apparent that the AT activity of the serum declined in the course of some hours, unless adsorption of AT on to CM cellulose followed straight after the activation. Possibly, the acidic polysaccharides contained in agar (agaro-pectin) activated serum proteases which destroyed AT. Neutral compounds like sephadex or zymosan proved more suitable as activators. Fresh baker's yeast was successfully substituted for zymosan. Care was taken to go through the steps of activation, adsorption to and elution from CM-cellulose without delay.

The use of the adsorbent Amberlite XAD-2 allowed to circumvent dialysis or ultrafiltration of AT containing solutions, which procedures caused heavy losses of activity. As buffer salts did not interfere with the adsorption eluates of ion exchange columns etc. could be applied directly to XAD-2. For elution of AT the salt-free, easily evaporable solvent mixture methanol-acetic acid was found suitable.

By using contact-activated hog serum it has now become practicable to prepare highly purified AT in sufficient quantities to study its biological actions *in vivo* and its chemical properties. Electrophoresis has revealed that the final preparation is not yet uniform in its composition. As the biological actions of the AT-containing sera and of the purified preparations are the same there is so far no indication that the purified hog AT is not functionally pure. It produces cross-desensitization with rat and guinea

pig AT as obtained by contact activation of the respective sera. So it can be regarded as a true AT.

Our finding that AT contains disulphide bridges which are essential for the biological activity is at variance with the absence of cystine postulated earlier.[13] Possibly the preparation used in the earlier analytical investigations was contaminated with other (cystine-free) material. Thus cystine may not have been detected in the hydrolysates which were prepared from rather small samples of AT, anyway. The contamination is also suggested by the low *N*-content and large content of sugars in the former preparation.[13] The AT as obtained now contains 15% N and although periodate destroys the activity there is no analytical evidence for the presence of carbohydrates. Thus AT may well have a pure peptide structure. The inactivation of purified AT by periodate is probably an unspecific effect.

Stegemann, Vogt and Friedberg[1] assumed that AT is a peptide rather than a protein, but as it did not dialyze a larger molecule seemed conceivable, too. From preliminary results of ultracentrifugation Stegemann[13] deduced a molecular weight of about 30,000. The present gel filtration experiments indicate a molecular weight of 7000–8000 for hog AT. In the solvent mixture used for these investigations (phenol—acetic acid–water) AT should be present as a monomer and adsorption should be prevented[11] suggesting that AT moved on the column according to its true molecular size. In aqueous solution AT possibly forms association products. This would explain the non-dialyzability.

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REFERENCES

1. H. Stegemann, W. Vogt and K. D. Friedberg, *Hoppe-Seylers Z. physiol. Chem.* **337**, 269 (1964).
2. D. Schwoerer, Thesis Göttingen (1966).
3. W. Vogt, *Ergebn. Physiol.* **59**, 160 (1967).
4. M. Rocha e Silva and A. M. Rothschild, *Br. J. Pharmac.* **11**, 252 (1956).
5. R. L. Whistler and M. L. Wolfrom, *Methods in Carbohydrate Chemistry*, vol. 1, p. 479. New York (1962).
6. R. L. Whistler and M. L. Wolfrom, *Methods in Carbohydrate Chemistry*, vol. 1, p. 485. New York (1962).
7. D. Glick, *Methods of biochemical analysis*, vol. 2, p. 292. New York (1955).
8. C. B. Anfinsen and E. Haber, *J. biol. Chem.* **236**, 1361 (1961).
9. L. Strauch, *Z. Klin. Chem.* **3**, 165 (1965).
10. G. Bodammer and W. Vogt, *Int. Archs. Allergy appl. Immun.* **32**, 417 (1967).
11. M. Bagdasarian, N. A. Matheson, R. L. M. Synge and M. A. Youngson, *Biochem. J.* **91**, 91 (1964).
12. G. Bodammer, *Naunyn-Schiedebergs Arch. exp. Path. Pharmak.* in press
13. H. Stegemann, R. Hillebrecht and W. Rien, *Hoppe-Seylers Z. physiol. Chem.* **340**, 11 (1965).