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# On some Properties of the Aromatic Constituent of Male Sperm Antagglutin and its "Active Group"

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Some properties of an aromatic constituent of the male sperm antagglutin have been studied spectrophotometrically. Its absorption properties like those of its *p*-quinone and its hydroquinone are similar to those of the known tocopherols. On account of many dissimilarities between the substance and the latter its identity with any of the known tocopherols is excluded.

The aromatic compound is part of a protein free complex also containing sulphuric acid, and sugar residues. In this complex it may also occur as quinone.

From male sperm antagglutin we obtained in 1954 [1] a substance which appeared to be aromatic by nature, and which by reason of its absorption curve and its behaviour at oxidation was suggested to be related to vitamin E. It was provisionally denominated as a "tocopherol derivative". Its non-identity with $\alpha$-tocopherol was, however, stressed, and will be further discussed below.

From samples of bull semen obtained during the spring of 1954 we rather frequently isolated antagglutin which on extraction with ethyl ether from water solution gave the above mentioned aromatic compound. More recently, however, this happened very sporadically only. Accordingly we now consider the occurrence of the aromatic constituent in extractable form as an artefact. So far we have not been able to establish the conditions provoking this state. As the possibilities of splitting off the aromatic residue from the rest of the molecule by means of chemical and physical agents is now being investigated in this laboratory it seems appropriate to publish some early observations on this spontaneously liberated material which has been available in such very minute quantities that it could be studied only by spectrophotometric methods.

## EXPERIMENTS AND RESULTS

The semen from which the antagglutin used in these experiments was obtained came chiefly from the A.I.-station of Eskilstuna. The antagglutin was isolated according to Lindahl and Kihlström [2], the procedure including twice repeated salting out with ammo-

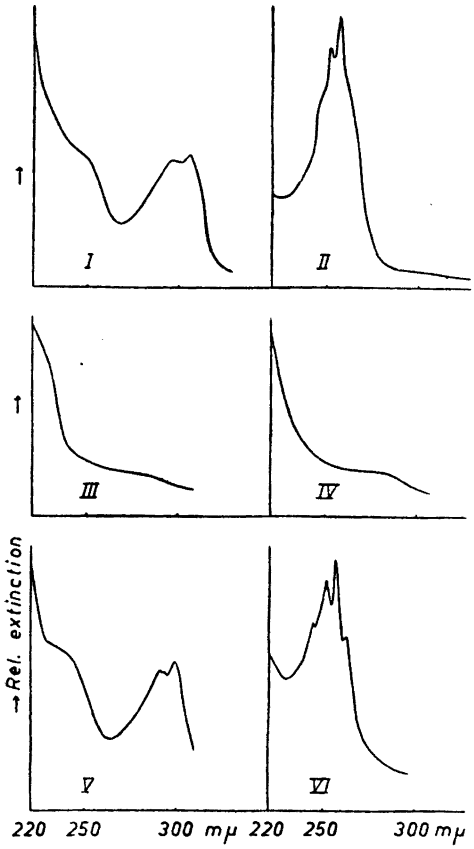


*Fig. 1. Absorption curves of the aromatic constituent and the "active group" of male spermantagglutin. I the aromatic constituent in its original form, II after mild oxidation as quinone, III after prolonged oxidation. IV the "active group" split from the protein residue and chromatographed (spectrum of aromatic constituent not visible), V after extraction with ethyl ether showing aromatic constituent in original form, and VI with the aromatic constituent as quinone. I—III in ethanolic, IV—VI in aqueous solution.*

nium-sulphate, and subsequent chromatography on calcium carbonate. The antagglutin was always in the reduced form when extracted with ethyl ether, the reduction being generally brought about with hydrogen, and platinum as a catalyst.

The absorption curve (I, Fig. 1) of the extracted compound was measured in absolute ethanol ("Spectroscopically pure", Vin och Spritcentralen, Ltd.) with maxima at 295 and 305 mμ, and a minimum at 268 mμ. Because of the similarity in the general trend of this curve with that of δ-tocopherol in ethanol (*cf.* [3]) a closer comparison is made. The single maximum [4] of δ-tocopherol is at 298 mμ, and its minimum at about 257 mμ. The α-, β-, and γ-tocopherols also have single absorption maxima in ethanol [3]. On the other hand the absorption curve of α-tocopherol in hexane has two maxima [5], and the same was found for δ-tocopherol (from Eastman Kodak Comp.). However, when transferred into hexane ("for spectroscopical use" B.D.H.) our substance yielded a smooth curve without maxima and minima [1] persisting also when the substance was brought back into ethanol again.

In order to provide further comparison our substance was subjected to mild oxidation by being aerated for 16 h at +5°C in ethanolic solution in the presence of $FeCl_3$ and $CuSO_4$ ($10^{-4}$ M). The absorption curve (II, Fig. 1) in ethanol of the product formed has a narrow double peak with maxima at 251 and 256 mμ, with about twice as high extinction coefficient as the substance before oxidation. These absorption data may be compared with those obtained in ethanolic solution for the *o*-quinone of α-tocopherol and the *p*-quinones of the α- and δ-tocopherols (Table 1).

*Table 1.*

| | λ in mμ | | max. of ox.prod. / max. of tocoph. | |
|---|---|---|---|---|
| α-Tocopheryl *p*-quinone | 261—263 | 267—268 | 6—7 | (6) |
| α-Tocopheryl *o*-quinone | 258 | (270) | 3 | (6) |
| δ-Tocopheryl *p*-quinone | 257 | | 2.5 | (4) |
| Ox. antagglutin constituent | 251 | 256 | 2 | |

As seen from Table 1 the oxidation product of the antagglutin constituent comes nearest to δ-tocopheryl *p*-quinone which, however, has only one maximum. Further it should be stressed that neither α- nor δ-tocopherol will be oxidized to a quinone under the conditions employed for the antagglutin constituent.

When subjected to catalytic reduction with hydrogen the absorption curve of the oxidized antagglutin constituent changes rapidly. The strong absorption band disappears, and a new weak one around 310 mμ appears, the whole absorption curve now being very similar to the curve of the originally extracted substance. There is, however, a single maximum, and the minimum as well as the maximum absorption occur at longer wavelengths. The existence of a transition stage in the reduction is suggested by the fact that during the first minutes of the reduction, when the high absorption-band of the oxidation product is beginning to decrease, much higher extinction values appear around 310 mμ than those remaining when the reduction is finished.

As the oxidation product appears to be a quinone its reduction should give the corresponding hydroquinone. On account of the different positions of the absorption bands of *ortho*- and *para*-diphenols in general, it may be concluded that we are dealing with a hydroquinone. This hydroquinone is very quickly oxidized to quinone on exposure to air, being in this respect similar to α-tocopheryl hydroquinone [7]. The latter has a single extinction maximum [7] at about 290 mμ, as compared with the maxima of α-tocopherol at 292 and 298 mμ. A corresponding comparison of the extinction maxima of our hydroquinone, about 310 mμ, with those of the originally extracted compound, 292 and 305 mμ, makes probable that besides the quinone-hydroquinone transformation also other changes must have occurred.

Treatment of the tocopherol-like constituent with 4 M hydrochloric acid at 40°C for 11 h in ethanolic solution under $H_2$-atmosphere did not cause any change in its extinction properties. Thus none of the aromatic hydroxyls may be engaged in an easily hydrolyzed ester bond. In a similar experiment the

$H_2$-atmosphere was replaced by air. Also here the extinction curve was unchanged, indicating a rather high stability against oxidation by air under these conditions. However, when exposed to air at the same temperature for 48 h with 0.5 M hydrochloric acid the absorption curve was very much changed (Fig. 1, III). It appears from these two experiments that high concentrations of hydrochloric acid protect the substance from being oxidized in air. This is of special interest as acids are known to promote ring closure in $\alpha$-tocopheryl hydroquinone which is by this process transformed into tocopherol.

Occasionally the *p*-quinone, produced by oxidation of the tocopherol-like substance, may instead of the latter be extracted from antagglutin solutions. In case both are present in a sample the quinone is extracted first, and seems thus to be the more hydrophobic substance of the two. — Also the absorption bands of this quinone are changed in hexane solution under the conditions in question.

The splitting-off of the active group from the protein constituent was performed as a dissociation, promoted by a high pH (9.6) and certain ions. In one type of experiment it was then separated from the protein by adsorption, in the other by ultrafiltration.

In an experiment of the first mentioned type a purified antagglutin sample, dissolved in water, was incubated at 5°C and pH 9.6 with $10^{-4}$ M $CuSO_4$ and $FeCl_3$ for 44 h. To minimize possibly occurring oxidation the sample was then reduced catalytically with hydrogen at pH 5.5. The solution was afterwards neutralized, brought to pH 8.5 with glycine-sodium hydroxide buffer, and filtered through a column of calcium carbonate, activated by heating for 3 h at 150°C. With $CO_2$containing water (pH 5.5) an eluate was obtained showing a spectrum without specific absorption (IV). This eluate was evaporated to dryness, and the residue extracted with ethyl ether. The latter was again evaporated, and the remaining substance extracted with water. Part of this residue did not dissolve, and remained as an unwettable coat on the inside of the vessel. This phenomenon was observed regularly when the "active group" had been treated with ether or any other slightly polar solvent. The aqueous extract showed an extinction curve (V) very similar to that (I) of the tocopherol-like substance described above, but with absorption maxima at 290 and 299 m$\mu$. This material contained some impurities, probably salts, which could be further removed by adsorption of the aromatic substance on quartz powder and subsequent washing out which ethyl ether. After this the absorption curve, measured in water, showed a third maximum at 240 m$\mu$ (*cf.* Lindahl and Kihlström [1]). This substance which may be dissolved in ether only in a dry state has also appeared spontaneously on some occasions during the isolation of the antagglutin. In such cases it is found in the eluates obtained after the elution of the antagglutin was completed.

In some experiments besides $Fe^{3+}$ and $Cu^{2+}$, also $Mg^{2+}$, $Mn^{2+}$, and $Zn^{2+}$ were present during the incubation in concentrations of $10^{-4}$ M, and the treatment was shortened to 16 h. In these cases already the eluate showed a characteristic extinction curve (VI), yet with maxima identical with those of the quinone (II). There were, however, two further small peaks at 244 and 263 m$\mu$. Also this substance has occurred spontaneously in some instances (*cf.* above). We are, however, not able to state whether these two small peaks

may be demonstrated also in the extinctior curve of the free quinone or not, as our measurements were not sufficiently extensive at the relevant points.

The ultrafiltration was performed in the following way. The pH of the antagglutin solution was raised to 8.6 with NaOH, and the solution immediately sucked through a washed collodion membrane (Membranfiltergesellschaft, Sartoriuswerke, Göttingen), the pores of which permit the passage of particles corresponding to a max. molecular weight of about 4 000. The filtrate did not give a positive ninhydrin reaction but contained sugar (positive benzidine reaction). The absorption curve was identical with (V). Obtained in this way, the "active group" did not exhibit any antagglutinic activity.

With this method we have also been able to localize the incorporation of radioactive sulphur with the antagglutin molecule (*cf.* [8]) to the "active group". The occurrence of sulphuric acid residues in the "active group" was assumed earlier for different reasons [2,8]. This has now been verified by hydrolyzing the isolated protein-free material obtained from rabbits injected with $Na_2{}^{35}SO_4$ (*cf.* [8]) and following precipitation with $Ba^{2+}$. The precipitate contained about 95 % of the radioactivity registered in the "active group".

## DISCUSSION

The observations on the aromatic constituent of male sperm antagglutin recorded above suggest that we are dealing with a 6-hydroxy-chromane structure, possibly a tocopherol derivative. To decide whether this idea is right or not larger samples of the substance are necessary. The many dissimilarities between this substance and all the known tocopherols, *e.g.* the higher reactivity at oxidation and hydrogenation, the influence of hexane or impurities in the latter on the absorption spectrum, exclude, however, the identity with any known member of the latter group. It was further stated [1] that oxidation of this tocopherol-like substance with nitric acid according to Ungnade and Smith [9] yields a yellow colour instead of the red obtained for the $\alpha$-, $\beta$-, and $\gamma$-tocopherols. This is of great interest as it was shown by Smith *et al.*[10] that the presence of an ethylenic bond or a carbonyl group in the side chain of the quinone formed gives rise to a yellow colour instead of a red one. On the other hand also the substituents of the benzene ring in the chromane molecule influence this reaction as $\delta$-tocopherol gives an orange-yellow colour [4].

When the protein constituent has been removed the complex which has been called the "active group" represents the rest of the antagglutin molecule in a more or less denaturated state. Most probably this complex with a molecular weight below 4 000 in its native state has an absorption curve similar to that of the male antagglutin. A moderate change brings about the appearance of the absorption bands of the tocopherol-like constituent — although somewhat displaced towards shorter wavelengths — without impairing the antagglutinic activity. A more far-reaching change abolishes this activity without further altering the absorption spectrum. Oxidation then gives the complex an absorption curve very similar to that of the quinone described above. The occasional spontaneous appearance of the "active group" at the preparation of antagglutin must depend upon some change in the molecule of the latter,

having occurred probably already in the semen. This altered "active group" as well as the tocopherol-like substance extractable with ethyl ether from aqueous solutions must by looked upon as artefacts.

As long as the antagglutin molecule is not in some way denaturated the tocopherol-like constituent seems to be rather resistant against catalytic hydrogenation.

So far the "active group" has been shown to consist of the tocopherol-like substance, sugar, and sulphuric acid residues. The presence of the many hydrophilic groups in the two last mentioned residues causes the water solubility of the complex, and makes it dissolve in ethyl ether only when dry.

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