A Chemical Method for the Quantitative Determination of Male Sperm Antagglutin

P. E. LINDAHL, J.-E. KIHLESTRÖM and S. ROSS

Institute of Zoophysiology, Uppsala, Sweden

A chemical method for the quantitative determination of male sperm antagglutin has been worked out, allowing determinations in the range of 0.5 to 3.5 μg antagglutin.

Since it had been understood that the agglutination of spermatozoa in mammals is prevented by certain substances, named sperm antagglutins, and produced in the male and female genitalia¹⁻⁵, the quantitative determination of these substances has been of great interest. For the male antagglutin a biological test method was elaborated², giving within certain limits and under favourable conditions certain rough quantitative values. Some variable and unknown factor, located probably in the spermatozoa used as test objects, limits, however, this method to be as yet a mere qualitative one. Still, it has the great advantage of permitting tests on very small samples (about 0.1 ml of normal sperm plasma), and making possible a differentiation between antagglutin in the reduced, biologically active and the oxidized, biologically inactive state. Because of the above mentioned disadvantages we have tried to develop a method, based only on chemical reactions and physical measurements, and rendering possible the determination of 2.0 μg of antagglutin with a reasonable error. Such a method would *e. g.*, allow a study of the physiological variation of the antagglutin content in sperm plasma, using only 0.1 ml samples.

The male sperm antagglutin is a complex, made up by protein, sugar, sulphuric acid residues, and a carbocyclic substance. After attempts to use colour reactions on protein or sugar for the quantitative determination of antagglutin in spots obtained by chromatography on different kinds of threads we intended to convert the antagglutin into a compound with a sufficiently high molar extinction to allow spectrophotometric determinations. It was found that heating of the antagglutin to 220°C with potassium bisulphate (melting point 210°C) results in the formation of a compound which is soluble in ethanol but not in ethyl ether, and which by oxidation with manganese dioxide is transformed into a substance with a high molar extinction in ultra-

Fig. 1. Micro reaction tube. Note thickening of the edge supporting the tube in the centrifuge.

violet. The solubility of the intermediary product in ethanol but not in ethyl ether makes possible the removal of ether-soluble breakdown products which otherwise disturb the spectrophotometric determination. The extinction maximum disappears at further oxidation, *i. e.* aeration or prolonged standing in contact with air. This process, being slower in ethanol than in water, is, however, retarded by manganese ions. As the extinction of the end-product is much higher in water than in ethanol solution, the former solvent is preferred for the determination of the absorption.

Procedure. Of the sample to be analyzed * 0.1 ml is put into a pointed micro reaction tube (Fig. 1) of pyrex fitting into the micro-comb centrifuge (Laboratoriemaskiner Ltd, Stockholm). By addition of 0.03 ml saturated solution of potassium sulphate the antagglutinin is precipitated, and spun down at 7 000 *g* for 5 min. The precipitate is dissolved in 0.1 ml distilled water, and the salting out repeated. The supernatant is removed, and the precipitate dissolved in 0.2 ml distilled water, occupying about two thirds of the height of the conical part of the tube. The water is evaporated at 40°C under vacuum whereby the antagglutinin is deposited as a very thin film at the wall of the tube. The latter is then filled up with dry, powdered potassium bisulphate ** till slightly above the edge of the film, the powder pressed down tightly with a small glass pestle, and the filling completed if necessary. The reaction tube is then placed in a big test-tube (18 cm length) with a layer of glass beads at the bottom to keep the reaction tube upright, and the test tube immersed for one hour in an oil-bath of 220°C. Under these conditions the potassium bisulphate melts within 5 min.

After the reaction tube has cooled down it is taken out of the test-tube, its outside is cleaned with ethyl ether, and the tube with its hard content powdered in a mortar. The whole material is transferred to a centrifuge tube with a ground stopper, the mortar being washed out with 3 ml absolute ethanol ("Spectroscopically pure", Vin- och Sprit-centralen Ltd) which is then used for extraction of the salt powder. About 5 mg of manganese dioxide is added, and the closed centrifuge tube shaken vigorously lengthwise for 12 h. The solids are then brought down by centrifugation, and the supernatant poured into a 10 ml flask. The solids in the centrifuge tube are washed out with another half ml ethanol, which is added to the extract in the flask. From this the ethanol is driven off

* If the sample is semen the biologically active part of the antagglutinin sticking to the surface of the spermatozoa (*cf.* Ref. *) is released by oxidation with 0.01 ml $8 \cdot 10^{-2}$ M hydrogen peroxide per 0.1 ml of semen, and the spermatozoa spun down.

** As commercial preparations of this salt often contain a surplus of sulphuric acid which is deleterious to the method, it is recommended to extract the potassium bisulphate for 12 h in a Soxhlet apparatus with dry freshly distilled ethyl ether.

Fig. 2. Absorption curves: analytical procedure applied to *a* antagglutin containing sample, *b* to equal quantity distilled water.

under vacuum at 50°C. As soon as the liquid has disappeared 3 ml freshly distilled dry ethyl ether are poured into the flask which is then shaken for a moment, and left for 5–10 min. The ether is drawn off, and the rest of it quickly evaporated. The remainder in the flask is dissolved in 3.0 ml redistilled water. The UV-absorption of this extract should be read within one h with distilled water as a blank. This determination was carried out by means of a Beckman spectrophotometer, Model DU.

The absorption maximum of the product, obtained with the described procedure is at 256–257, the minimum at 240–242 $m\mu$ (Fig. 2a). A blank treated in the same way gives the extinction curve *b* (Fig. 2). Here the extinction values at 240–242 and 256–257 $m\mu$ do not differ significantly. The difference between the extinction maximum and minimum is 0.012 per μg bull antagglutin in the range 0.5–3.5 μg (Fig. 3). Above 3.5 μg this difference is not proportional to the amount of antagglutin treated, the straight line in Fig. 3 becoming parallel to the abscissa.

The standard deviation (*s*) calculated from 20 duplicate determinations on bull semen in the range 0.5 to 3.5 μg (*cf.* Hald⁶, p. 244) is 0.103. The duplicate determinations with values below 1.0 μg give a somewhat higher standard deviation than those with values above 1.0 μg (*cf.* below).

Range μg antagglutin	Number of determinations	<i>s</i>
0.5–1.0	8	0.111
1.1–3.4	12	0.097

Fig. 3. Differences between extinction maximum at 256–257 $m\mu$ and extinction minimum at 240–242 $m\mu$ plotted against quantities of antagglutin.

It is essential that no other substances occurring in the sperm plasma give rise to an extinction maximum at 256—257 $m\mu$ when treated in the same way. This was controlled by performing the procedure of analysis on samples of sperm plasma from which the antagglutin had been removed by salting out. Further different appropriate quantities of antagglutin were added to equal samples of a sperm plasma with its original antagglutin, and the total antagglutin content analysed. The values obtained fell all on a straight line parallel with that in Fig. 3.

The reactions upon which the described method is based are being studied.

The financial support of the *Swedish Agricultural and Natural Science Research Councils*, the *Population Council*, Inc., New York, and *Pharmacia, Ltd.*, Uppsala, is gratefully acknowledged. We thank Mr. O. Heidenberger for valuable technical assistance.

REFERENCES

1. Lindahl, P. E. and Kihlström, J.-E. *Rep. 2nd Internal. Congr. Physiol. Pathol. Animal. Repr.* Copenhagen 1 (1952) 70.
2. Lindahl, P. E. and Kihlström, J.-E. *Fertility and Sterility* 5 (1954) 241.
3. Lindahl, P. E. and Kihlström, J.-E. *Nature* 174 (1954) 600.
4. Lindahl, P. E. and Nilsson, A. *Arkiv Zool. Ser. 2* 7 (1954) 223.
5. Lindahl, P. E., Ingelman-Sundberg, A., Furuhjelm, M. and Nilsson, A. *J. Obstet. Gynaecol. Brit. Empire* 63 (1956) 363.
6. Hald, A. *Statistiske Metoder*, Copenhagen, 1948.

Received September 13, 1956.