STUDIES ON A HAEMOSENSITIN FROM *MYCOBACTERIUM TUBERCULOSIS* IN ZONE ELECTROPHORESIS

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If red cells are incubated with filtrates of cultures of *Mycobacterium tuberculosis*, and afterwards washed, they can be agglutinated by antisera against the bacillus (MIDDLEBROOK AND DUBOS[1]). One of the bacterial substances which becomes fixed onto the cells in this way is heat-stable and is present in fractions of tuberculin made from both heated and unheated culture filtrates, and has been named the α-haemosensitin (SORKIN AND BOYDEN[2]). Although this substance can be recognised serologically as a distinct entity, various attempts to purify and characterize it with certainty have met with little success, but it has been generally considered to be of polysaccharide nature.

In a recent paper (SORKIN, BOYDEN AND RHODES[3]) we described very active α-haemosensitin preparations containing about 90% of a heteropolysaccharide, which, however, was shown by boundary electrophoresis to be inhomogeneous. It was thought that this might be due to the presence of another substance with very similar properties. Further attempts were therefore undertaken to purify the preparation. Preparative zone electrophoresis (TISELIUS AND KUNKEL[4], KUNKEL[5]) appeared to be a convenient method. Glass beads (Topz ballotini 0.1 mm) were used as supporting medium and the buffer employed was veronal sodium/veronal pH 8.6, $\mu = 0.1$.

This paper presents the serological and chemical results of the electrophoresis experiments.

EXPERIMENTAL

Apparatus and materials for zone electrophoresis

A paste of glass beads, Topz ballotini 0.1 mm in veronal buffer pH 8.6, $\mu = 0.1$, was prepared and poured into a rectangular perspex container (70 cm × 4 cm × 1 cm). The excess liquid was removed from the paste by blotting with filter paper until the block was firm, but still moist.

A small slit at each end of the perspex mould permitted the passage of a filter paper which passed into the buffer solution in the cells. The paper was rolled up inside the mould and made contact with the glass beads. The mould was placed on the lip of the cells of the Tiselius type apparatus (TISELIUS AND FLODIN[6]) and the glass block was equilibrated for 2 hours with the buffer solution.

The α-haemosensitin preparation studied in electrophoresis corresponded to the one described by SORKIN, BOYDEN and RHODES[3]. It had been isolated by alcohol fractionation from heated culture filtrates of *M. tuberculosis* (humain strain E9656) and most of the protein impurities had been removed by treatment with ammonium sulphate and trichloracetic acid. The preparation (fraction 248) was active in sensitizing sheep red cells to agglutination by rabbit anti-tuberculous serum at a concentration of 1 γ/ml under the conditions previously described (SORKIN, BOYDEN AND RHODES[3]). Its main component was a heteropolysaccharide containing 61% arabinose, 25% hexose, and 1.3% hexosamine. In addition, about 1% fatty acids and 4% of a peptide component were present.

Electrophoretic procedure

200 mg of fraction 248 were dissolved in 0.1 ml veronal buffer and placed in a small trough which

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was cut out in the middle of the glass block (SORKIN, RHODES AND BOYDEN[7]). After laying a piece of dry filter paper under the lid, the latter was screwed down and the experiment run for 7 days at 260 volts, 16 mA, and at 4°C.

Elution of the separated material

The whole glass block was cut into 1 cm segments each of which was eluted with 5 ml of a 0.9% NaCl solution. The elution was performed on a glass funnel.

RESULTS

Determination of the electrophorogram

(a) *Chemical.* Since the starting material contained over 90% polysaccharide, the distribution of the material was studied according to MORRIS[8] by carbohydrate analysis with anthrone–sulfuric acid as reagent (see also SORKIN, BOYDEN AND RHODES[3]). It is seen in Fig. 1 that the bulk of carbohydrates migrated 20–28 cm towards the *cathode*, whereas only a small part remained at the start or moved towards the anode. This result was in accordance with some of our earlier experiments in which we could not recover the material when it was placed near the cathode end of the glass block; obviously it had migrated into the cells containing the electrodes.

(b) *Serological.* The fractions were titrated serologically for (1) sensitizing activity (this term is used to denote the capacities of the various eluates to sensitize sheep red cells to specific haemagglutination by rabbit anti-tuberculous serum), (2) inhibiting activity (meaning the capacity of a preparation to neutralize the antiserum's ability to agglutinate red cells coated with the α-haemosensitin (for details of the procedures used see SORKIN AND BOYDEN[2], SORKIN, BOYDEN AND RHODES[3]) (see Fig. 1).

The first of these procedures gives a measure of the content in the fractions of serologically specific material capable of becoming fixed to the red cell surface. The second measures material of the same serological specificity as the red cell sensitizing component, irrespective of its red cell sensitizing potentialities.

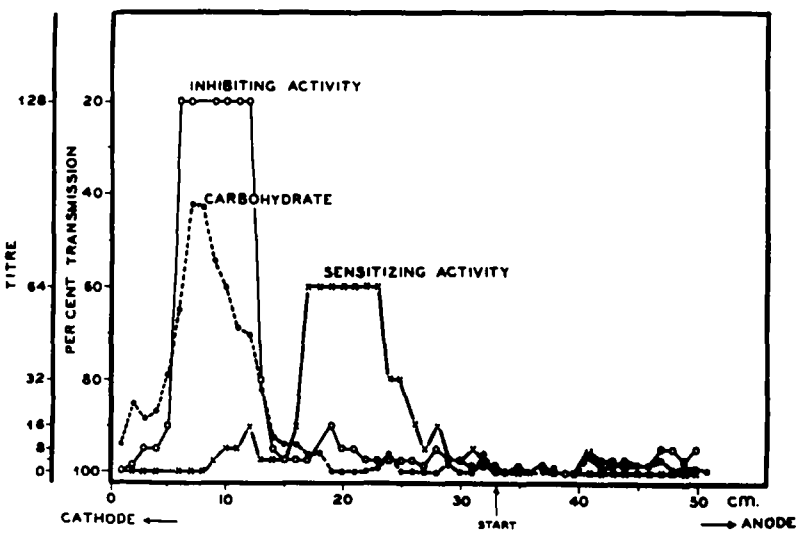

Fig. 1. Zone electrophoresis in glass of haemosensitin in veronal buffer pH 8.6, μ = 0.1. Duration: 7 days at +4°C. The eluates from 1 cm segments were assayed for sensitizing and inhibiting activity and for carbohydrate.

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The data in Fig. 1 show that the sensitizing activity has moved slightly towards the cathode and resides mainly in one small section of minimal carbohydrate content, but is also present to some extent where the bulk of the carbohydrate positive material is found. In contrast, however, the main inhibiting activity is localized further towards the cathode and corresponds with the position of greatest carbohydrate content. This and similar experiments suggest therefore that even purified fractions contained only a small amount of sensitizing material together with larger amounts of inhibiting substances of the same serological specificity.

DISCUSSION

In the experiments described results were presented which showed why α-haemosensitin as a chemical entity has probably never been isolated. It is most likely that all workers have been dealing with varying mixtures of the α-haemosensitin proper and a chemically related substance possessing the same serological specificity but unable to sensitize red cells. The close relationship of the two substances has up to now prevented any clear separation, although MIDDLEBROOK[9] has mentioned that he succeeded in removing all sensitizing activity from a preparation by repeated adsorption with red cells, leaving behind only inhibiting activity.

In what way the inhibiting and the sensitizing material, which occurs in much smaller amounts, differ chemically is at present under investigation. As stated earlier (SORKIN, BOYDEN AND RHODES[3]), both contain polysaccharide, mycolic acid(s) and probably a biuret-positive component. The fact that only very small amounts of sensitizing material are available has not yet permitted a further detailed study.

Finally, it is of some interest to ask why both the inhibiting and sensitizing activity move in glass towards the cathode, whereas in paper and free electrophoresis they migrate slightly towards the anode. It is possible that the migration seen in glass after 7 days is due to electroosmosis rather than to electrophoresis. Such an effect was observed by KUNKEL AND TISELIUS[4] for the polysaccharide dextran in paper electrophoresis and it was used as index substance for mobility determinations.

The serological titrations were carried out by Miss MERETE LARSEN and Miss BIRGITTE ENGELBRECHT KRISTENSEN.

SUMMARY

1. The fractionation in glass by zone electrophoresis of a highly purified preparation containing α-haemosensitin from *M. tuberculosis* active in sensitizing red cells in the Middlebrook-Dubos haemagglutination test is described.

2. The bulk of the material moved towards the cathode, and contained polysaccharide which did not sensitize red cells to agglutination by anti-tuberculous serum, but which was of the same serological specificity as the sensitizing component. The sensitizing component was found nearer the starting point and represented only a small fraction of total material.

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Received March 16th, 1957