ARYLSULPHATASES A AND B OF HUMAN LEUCOCYTES : SPECIFIC INHIBITORS AND ELECTROPHORETIC CHARACTERIZATION[1]

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Summary. In human leucocytes, a rapid method of disc polyacrylamide electrophoresis at acidic pH allows the detection of two arylsulphatasic activities as well as their specific inhibition by ions. These results, in association with other investigations, have enabled characterization of the activities of arylsulphatases A and B.

According to studies in animal tissues, three arylsulphatases (EC 3.1.6.1) have been identified (1,2,12). One is microsomal (A.S.C.), the two others are soluble (A.S.A. and A.S.B.) ; recent studies seem to indicate that A.S.A. is lysosomal and perhaps also A.S.B. (14). These enzymes have been found in human liver (2), brain (4), serum (3), urine (3) and leucocytes (5). In the latter, it has been shown (13) that activity is localized in granulocytes only. An assay has been established (5) for A.S.A. and A.S.B. based on results observed (6) in urine. To shed more light on the properties of these enzymes in white blood cells, electrophoretic identification of A.S.A. and A.S.B., as well as their characterization by means of specific inhibitors, have been undertaken.

MATERIALS AND METHODS

Blood was a generous gift from "la Banque du Sang" of the hospital. Leucocytes were prepared by the following method (8):450ml of fresh blood (from normal controls or patients with hemochromatosis) including 50 ml of 3% glucose and 2% Na citrate aqueous solution are poured into a 500 ml squibb funnel containing 50 ml of 10% gelatine (Merck) solution in water. The suspension is left to sediment 30 min at 37°C. The red lower phase is slowly allowed to flow away and dis-

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carded. The clear upper phase is collected into two 250 ml bottles and centrifuged 5 min at 1000 g in a Sorvall centrifuge RC_2B. The supernatant is discarded. All other operations are performed at +4°C. Purification of leucocytes is carried out according to Cohn and Hirsch(9) ; 120 ml of water are used to lyse the red blood cells and 40 ml of NaCl 3.6% are necessary to reestablish isotonicity. After another centrifugation, a white pellet is obtained which is resuspended in 5 ml of distilled water and sonicated 15 sec by means of a Branson B_{12} sonifier. Protein concentration, measured according to Lowry (10) with bovine serum albumin as standard, is about 20 mg/ml.

Arylsulphatasic determinations have been performed according to Roy (12) ; volumes of TCA and NaOH have been reduced to sensitize the method. Each incubation (total volume : 1 ml) is carried out in a medium containing N.C.S. (p. nitrocatechol sulphate, Sigma) 5 m M, Na acetate-acetic acid buffer 0.45M, and 1 mg proteins for 30 min at 37°C. To stop the reaction, the tubes are cooled in ice water and the proteins are precipitated with 0.2 ml TCA 50%. After a rapid centrifugation at room temperature, in the International Clinical Centrifuge, each supernatant is pipetted into 0.3 ml of 30% NaOH. Optical densities are read at 515 nm in a spectrophotometer Zeiss PMQ II against a reagent blank without enzyme.

To study inhibitors (11), 1 volume of an inhibitor solution 20 times more concentrated than described in the figures 3, 4, or 5, is mixed with 1 volume of leucocyte solution. The mixture is left 10 min at room temperature and from it, 100 µl is added to the incubation medium.

For acid polyacrylamide gel electrophoresis (Hoefer Scientific Co. apparatus), the final concentrations in the gels are : acrylamide 7.5% ; N N' bis acrylamide (Merck) 0.1% ; Na acetate-acetic acid buffer pH 2.7, 0.6M ; Triton X-100 2% ; riboflavin 0.85 mg% ; NNN'N' tetramethyl-ethylenediamine (Eastman Kodak) 200 µl%. The gels are made with 1.3 ml of this mixture on which 0.2 ml of diluted buffer is layered; photopolymerization is allowed under U.V. light for 30 min. The buffered solutions in the upper and lower reservoirs are Na acetate-acetic acid buffer 5M, pH 2.7. 150 µl of a solution containing leucocytes, Triton X-100 25%, sucrose 40% v/v/v are deposited on each tube (1 mg protein/tube). Methyl green is used as tracking dye. Electrophoresis is conducted at +4°C for four hours with the proteins migrating towards the cathode, using a current of 3 mA/tube. To detect enzymatic activity, the gels are removed from the tubes and immersed 15 min in a Na acetate-

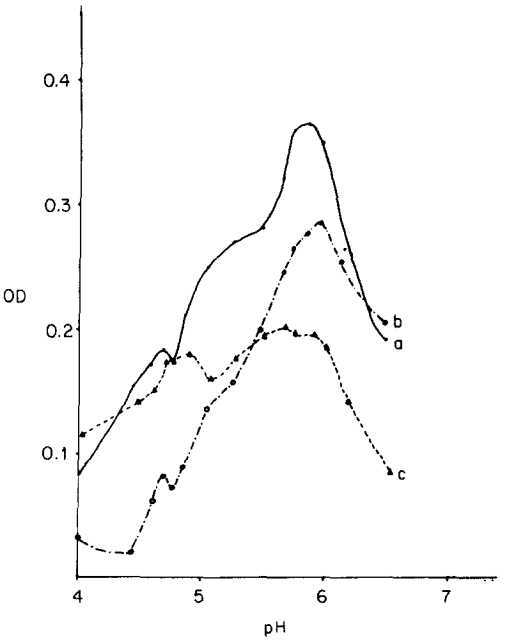

Fig. 1.

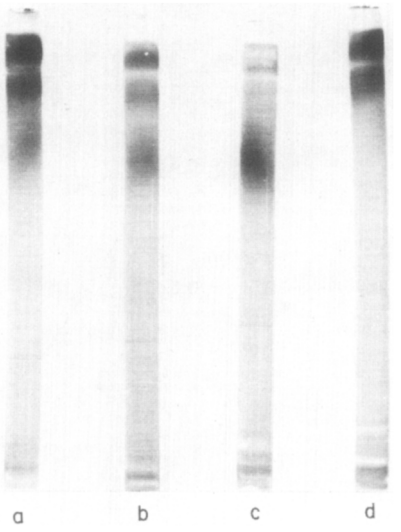

Fig. 2.

Fig. 1. Arylsulphatasic activity with varying pH of incubation mixture : a- without salt addition ; b- with 0.25 mM $AgNO_3$ - c- with 0.17 M NaCl.

Fig. 2. Disc electrophoresis of leucocyte solution : a- incubation at pH 4.6 ; b- incubation at pH 5.8 ; c- as b, plus 0.25 mM $AgNO_3$; d- as b, plus 0.17 M NaCl.

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acetic acid buffer 0.5M pH 5.8 (or 4.6) at room temperature, then 20 min at 37°C in a 20mM N.C.S. solution in the same buffer. At last, the gels are transferred into NaOH 2N. Red bands of arylsulphatasic activity appear after several minutes. The gels are rapidly photographied with Agfa Copex Ortho film.

RESULTS

Fig. 1 a is the resultant of several experiments made with different leucocyte preparations. We always found three peaks of activity with only a variation in the amplitudes of the peaks. pH optimums are 4.6, 5.2, 5.8. The electrophoretic method shows (fig. 2 b) three bands of enzymatic activity.

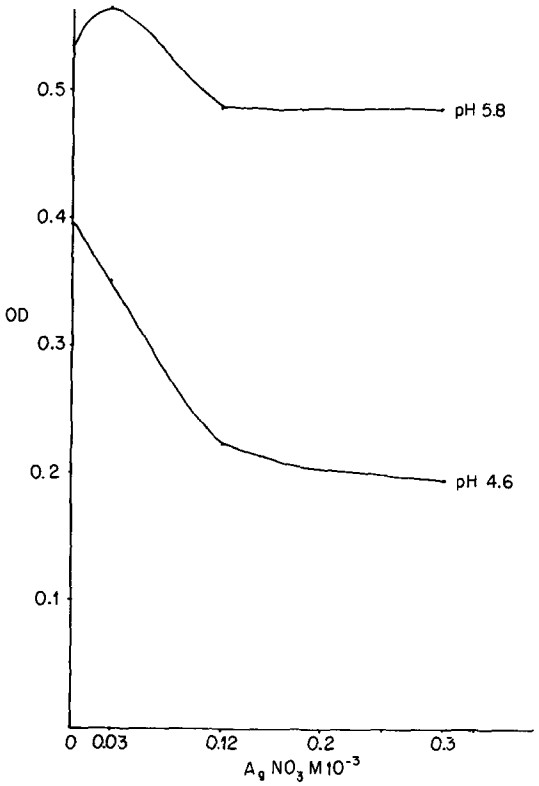

Fig. 3. Arylsulphatasic activities with varying concentrations of $AgNO_3$ in the incubation mixture.

The influences of $AgNO_3$, specific inhibitor of A.S.A. (11) and of NaCl, specific inhibitor of A.S.B. (6) have been studied. Fig. 3 and 4 confirm these inhibitions, but show that about 50% activity remains in presence of high concentrations of these salts. Variations of pH in the presence of these salts (fig. 1 b and c) show that with $AgNO_3$, at pH 4.6 and 5.2, activities are strongly decreased, contrary to pH 5.8 ; with NaCl, the first peak at pH 4.6 is not affected, the second at pH 5.2 disappears, and the third at pH 5.8 is decreased. By the electrophoretic method, the two upper bands disappear with $AgNO_3$ (fig. 2 c) ; with NaCl, only the lower band is abolished (fig. 2 d).

Likewise, we have studied the influence of $BaCH_3COO^-$; neither inhibition nor activation have been found with the enzymatic assay or the electrophoretic method. With $Na_4P_2O_7$, both activities are strongly decreased (fig. 5). At pH 4.6 (fig. 6), activity becomes

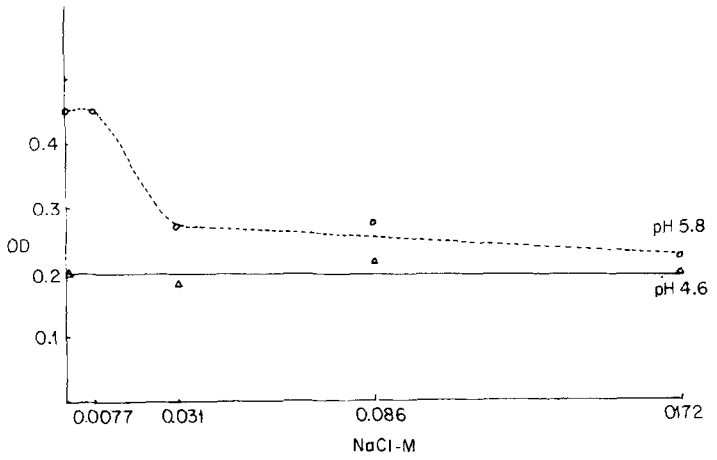

Fig. 4.

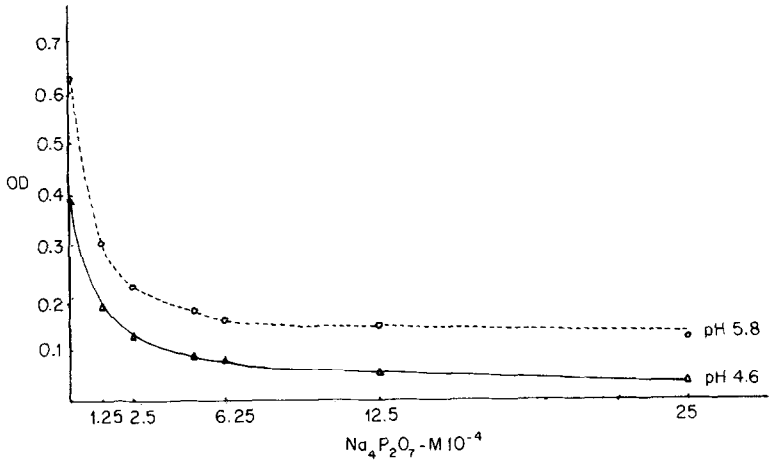

Fig. 5.

Fig. 4. Arylsulphatasic activities with varying concentration of NaCl in the incubation mixture.

Fig. 5. Arylsulphatasic activities with varying concentrations of $Na_4P_2O_7$ in the incubation mixture.

linear as a function of time after a lag period, but remains very low.

DISCUSSION

Acid polyacrylamide disc electrophoresis is a reliable method to detect arylsulphatasic activity in leucocytes without further

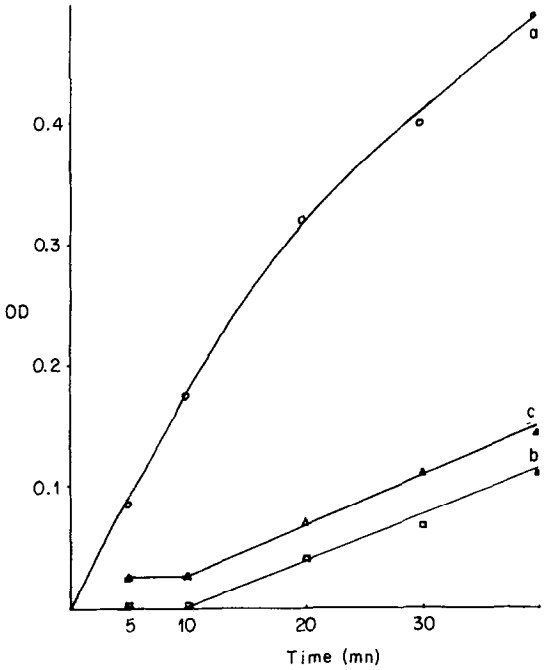

Fig. 6. Arylsulphatasic activity at pH 4.6 : a- without inhibitors ; b- with 1.10^{-3} M $Na_4P_2O_7$ as described in methods; c- enzyme added to an incubation mixture containing 5.10^{-4} M $Na_4P_2O_7$

purification. Three bands of activity have been identified, as well as their specific inhibition by ions. None corresponds to A.S.C., which does not react with N.C.S. The effect of $BaCH_3COO^-$ and $Na_4P_2O_7$ are different from other results (15,6). pH studies seem confirm the presence of three arylsulphatasic activities at maximum pH 4.6, 5.2, 5.8. Many similarities exist between enzymatic activity at pH 5.8, the electrophoretic lower band, -which decreases at pH 4.6 (fig. 2 a)- , and A.S.B. as described by Dodgson et al. (2). The two other activities resemble the A.S.A. described by Neuvelt et al. (16), and Baum et al. (15) using purified enzyme from human liver. It is noteworthy that NaCl decreases pH 5.2 activity but does not affect the middle electrophoretic band. Further studies are in progress to see whether this activity corresponds to an intermediary form between A.S.A. and A.S.B. -as it is known that A.S.B. may derive from A.S.A. (17)- or whether it is an isoenzyme.

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