# Dicumarol-induced Prothrombin in Bovine Plasma

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The regulatory function of vitamin K on prothrombin synthesis is not exerted before the ribosomal level.[1,2] It is, however, still controversal whether vitamin K deficient animals have a reduced *de novo* synthesis of prothrombin or produce some biologically inactive prothrombin precursor.[3,4] An abnormal prothrombin has recently been demonstrated in plasma from patients receiving the anticoagulant dicumarol,[5,6] and in some patients with a bleeding tendency due to advanced biliary stasis.[7] Isolation and characterisation of such an "abnormal" prothrombin would facilitate understanding of the mode of action of vitamin K and dicumarol. But human plasma is less suitable as a starting material owing to the large amount of plasma necessary for purification of the abnormal prothrombin. This communication reports the isolation and some properties of a dicumarol-induced prothrombin, in bovine plasma.

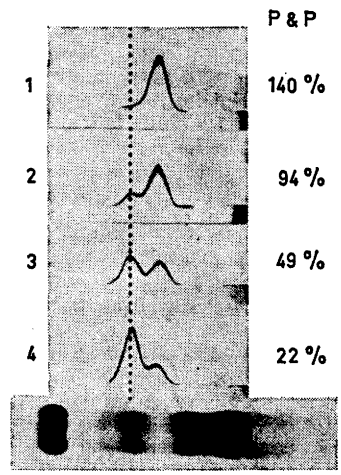


*Fig. 1.* L-electrophoresis of bovine plasma in agarose gel with 0.075 M barbital buffer 2.0 mM in calcium lactate using rabbit anti-bovine prothrombin as antiserum. The electropherogram of bovine plasma shows the migration rate of the two prothrombins. The prothrombin activity relative to a reference plasma is given to the right. 1, precipitate obtained before administration of dicumarol; 2, 3, and 4, precipitates obtained with samples drawn 2, 4, and 7 days after the beginning of treatment with dicumarol.

A 2 years old ox was given 0.25—0.5 g dicumarol a day in its fodder for 4—8 days. Citrated plasma from blood samples obtained before and at regular intervals after administration of dicumarol was analysed with "antigen-antibody crossed electrophoresis" according to Laurell [8] hereinafter called "L-electrophoresis". The prothrombin activity measured according to Owren and Aas [9] fell during dicumarol treatment. Judging from the estimated area of the surface under the precipitation arcs (Fig. 1) the fall was roughly proportional to the decrease in prothrombin determined immunochemically. The fall was accompanied by the appearance and gradual increase of an abnormal prothrombin with the antigenic determinant in common with that of normal prothrombin, but with a higher electrophoretic mobility.

"L-electrophoresis" revealed that unlike normal prothrombin the dicumarol-induced prothrombin was not adsorbed with barium citrate. When the electrophoretic separation was performed in a buffer where calcium lactate was substituted by 2 mM EDTA, the two prothrombins migrated like a single component indicating that the dicumarol induced prothrombin did not bind calcium ions. Furthermore in contrast to normal prothrombin, it persisted apparently unchanged in plasma samples coagulated by addition of calcium ions.

The dicumarol-induced prothrombin was purified from bovine plasma prepared from blood obtained when the prothrombin activity was about 20 % of a reference plasma. After removal of normal prothrombin by adsorption to barium citrate the plasma, diluted 1:2.5 with 0.9 % saline was fractionated with ammonium sulphate at pH 5.5. The precipitate obtained between 1.35 M and 1.85 M ammonium sulphate was recovered and subjected to displacement chromatography on a column of DEAE cellulose equilibrated with 0.01 M phosphate buffer pH 8.0. When about one tenth of the column was blue owing to the ceruloplasmin it was washed with the starting buffer. The fraction containing prothrombin was then eluted with 0.3 M $KH_2PO_4$. The next step in the purification was chromatography

on a column of DEAE-Sephadex A 50, which was eluted with a linear sodium chloride gradient (0.0–0.2 M in 7 column volumes) in 0.24 M Tris, HCl buffer, pH 8.0. The fractions containing the dicumarol-induced prothrombin were pooled and subjected to chromatography on a column of hydroxylapatite, which was eluted stepwise with phosphate buffers 0.1 M in KCl pH 6.8. Most of the contaminating proteins were eluted with 0.05 M buffer, whereas the dicumarol-induced prothrombin was eluted with 0.2 M buffer. The eluate was then purified by recycling chromatography on a 2.5 × 100 cm column of Sephadex G 100. After three cycles the peak corresponding to the dicumarol-induced prothrombin was completely separated from the rest of the proteins. During purification the abnormal prothrombin was assayed with a quantitative immunochemical technique using rabbit anti-bovine prothrombin as antiserum.[10] The overall yield was 13 %, corresponding to 17 mg of the dicumarol-induced prothrombin when the purification was started from 2.3 litres of barium citrate adsorbed plasma.

Judging from polyacrylamide disc electrophoresis the purified material was homogeneous. On "L-electrophoresis" in calcium ion containing buffer it behaved like the barium citrate adsorbed starting material indicating that there had been no transformation of the abnormal prothrombin into normal prothrombin during the purification procedure. Normal prothrombin and dicumarol-induced prothrombin had the same molecular size, as judged by Sephadex G-100 gel filtration. After incubation with neuraminidase the two prothrombins were subjected to "L electrophoresis" in calcium ion containing buffer. The migration rates of both proteins were reduced to the same extent indicating that they have roughly the same number of sialic acid residues. After trypsin digestion of the dicumarol-induced prothrombin reduced and aminoethylated in 7 M guanidine hydrochloride a peptide map showed 61 spots. It appeared to be identical with one prepared from normal prothrombin with the possible exception that one very faint spot in the dicumarol-induced prothrombin map could not be identified with certainity in the normal prothrombin map.

Prothrombin with a molecular weight of about 70 000 consists of only one polypeptide chain stabilized by 8 disulphide bonds. The present results suggest that vitamin K is necessary for the proper folding of this very long polypeptide chain.

Dicumarol strongly inhibits the flavoenzyme DT diaphorase, which catalyses the oxidation of reduced di- and triphospopyridin nucleotides with certain benzo- and naphthoquinones as hydrogen acceptors.[11] The enzyme is probably identical with "vitamin K reductase"[11] described by Martius and Strufe in 1954.[12,13] It has been suggested by Ernster *et al.* that one role of the enzyme might be to bring about the reduction of 2-methylnaphthoquinone necessary for the condensation with poly-isoprenoid alcohols to form the natural forms of vitamin K. A diminished supply of metabolically active vitamin K, possibly in the reduced form, after dicumarol administration might lead to synthesis of a kinetically stable conformational variant of prothrombin [14] without biological activity.

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