Biochemical Pharmacology, 1965, Vol. 14, pp. 579–587. Pergamon Press Ltd., Printed in Great Britain.

STUDIES OF THE MODE OF ACTION OF DICOUMARIN*

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(*Received* 21 *August* 1964; *accepted* 18 *November* 1964)

Abstract—From chemical, chromatographic and immunological results, it appears likely that the qualitative nature of the synthesis of prothrombin remains intact during therapy with dicoumarin (Dicumarol), and what small amount of prothrombin is produced is structurally normal.

Certain problems concerned with the isolation of prothrombin and the apparent heterogeneity of the available preparations have been discussed as they relate to the problem of the mode of action of dicoumarin.

STUDIES of the pharmacological effects of the administration of coumarin compounds have been generally limited to those demonstrable on the clotting systems in bovine, human, or various small-animal plasmas. It would appear, with the assays employed, that multiple coagulation factors are altered in some fashion by these compounds, namely, factors II (prothrombin), VII (proconvertin), IX (PTC), and X (Stuart factor). Seegers *et al.*[1, 2] however, have presented evidence to show that these are functions not of specific protein entities but of prothrombin itself. The response to the administration of dicoumarin and similar agents has been measured by a number of assays that show wide variations in relative sensitivity to the clotting factors involved.[3, 4] We have attempted in the present study to obtain both a chemical and an immunological characterization of prothrombin, isolated during the course of therapy, as a direct means of investigating possible modes of action of dicoumarin. It was felt that such a study would provide direct evidence against the presence of an inhibitor in response to the substance and allow a decision as to whether therapy resulted in the synthesis of a defective protein or simply prevented production at some stage of synthesis.

MATERIALS AND METHODS

Animals and schedule of dicoumarin administration. Two healthy cows were given daily oral doses of dicoumarin (Dicumarol; Abbott Laboratories) over a period of 14 days. A total amount of 26·7 g was given to the cow weighing 1,100 pounds and 28·2 to the cow weighing 1,150 pounds. The daily amounts administered and the variation in the level of prothrombin as measured by the two-stage method of Ware and Seegers[5] can be seen in Fig. 1. A 1·5-liter sample of blood was obtained from

* A preliminary report presented at the 36th annual meeting, Central Society for Clinical Research, November 1963, and at the 12th Annual Symposium on Blood, Rochester, Minn., January 1964. These investigations were supported in part by research grants from the Minnesota Chapter of the American Heart Association, the Minnesota Medical Foundation, and the Graduate School Research Fund of the University of Minnesota.

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each cow before treatment, on the fourth day, and on the final (14th) day of treatment. Another sample was obtained 11 days after the administration of dicoumarin had been discontinued. All samples were collected in 0·1 M sodium citrate (150 ml/1·5 liters whole blood).

Isolation of prothrombin. Prothrombin was adsorbed from the plasma with barium citrate formed by the addition of 1·0 M barium chloride (87 ml/liter plasma) to the citrated (0·02 M) plasma, as described by Miller and McGarrahan.[6] All solutions were maintained at approximately 4° and the operations carried out in a cold room maintained at 4°. The precipitate was collected by centrifugation at 500 *g* for 30 min. The precipitate was then suspended in 500 ml distilled water/liter original plasma and dissolved by the addition of dry Amberlite CG-50-Na^+ resin, 200–400 mesh size, to a pH of 8·7 to 9·15. The precipitation and dissolution were repeated once. After removal of the resin by filtration, the solution was titrated to a pH of 4·7 by the addition of 0·1 N HCl. Cold 95% ethanol was added slowly to a final concentration of 15% (v.v). The precipitate was collected by centrifugation and dissolved in 0·1 M phosphate buffer of pH 5·95 (approximately 4 ml/500 ml original plasma). A small amount of CG-50-Na^+ was added to obtain maximal dissolution of the precipitate. Portions (1–3 ml) of this preparation were chromatographed on Amberlite XE-64 columns, 0·9 × 30 cm, equilibrated with 0·1 M phosphate buffer of pH 5·95. The sample was mixed with approximately 20 ml of a thick slurry of equilibrated resin and applied to the top of the column. The prothrombin was eluted with 0·1 M phosphate buffer, pH 5·95.

Analytical methods. Quantitative determinations of prothrombin activity were made by the two-stage method of Ware and Seegers.[5] Protein determinations were made by measuring the absorbancy at either 280 mμ or 210 mμ in a Zeiss PMQII spectrophotometer. The absorbancy index ($A^{1\%}_{1\,cm}$) at 210 mμ was taken as 205.[7] The micro-immunoelectrophoretic method of Scheidegger[8] has been employed essentially as described by this author. The antisera used were prepared by immunization of rabbits with the relatively crude unchromatographed preparations of prothrombin. The rabbits were initially given intradermal injections of antigen emulsified in complete Freunds' adjuvant (Difco), and were subsequently given multiple intravenous courses of the antigen. Each course consisted of injections three times a week for three weeks. The total amount of protein administered to each rabbit in each course was approximately 470 mg. Certain of the antisera obtained were absorbed with barium sulfate-adsorbed bovine plasma or with bovine serum from a dicoumarinized animal. The antibody golubulins were isolated from the supernatant by precipitation with 50% ammonium sulfate. The isolated globulins were dissolved in water and dialyzed against 0·15 M NaCl until free from ammonium sulfate.

An antibody neutralization inhibition assay was developed to measure prothrombin. The mixture of a purified prothrombin preparation and the antiserum against prothrombin, which resulted in 85–90% neutralization, was shown in preliminary experiments to be the proper area of the precipitin curve for the assay. With the addition of varied amounts of prothrombin to this mixture, a plot of the units added against the units recovered gave a linear response over a range of about 1,000 units of prothrombin. In the actual assay the standard prothrombin preparation was first mixed with the unknown preparation, brought to a total volume of 0·5 ml, and then mixed with 0·5 ml of the antiserum. The mixtures were incubated at 37° for 1 hr and

then at 4° for 18 hr. The precipitates were removed by centrifugation, and the supernatants were assayed for the total prothrombin activity. The appropriate relative concentrations of the known prothrombin preparation and antiserum varied with the preparations employed and were determined in preliminary experiments.

RESULTS

Response to dicoumarin administration. The response to therapy is presented graphically in Fig. 1. There was an apparent immediate cessation of prothrombin

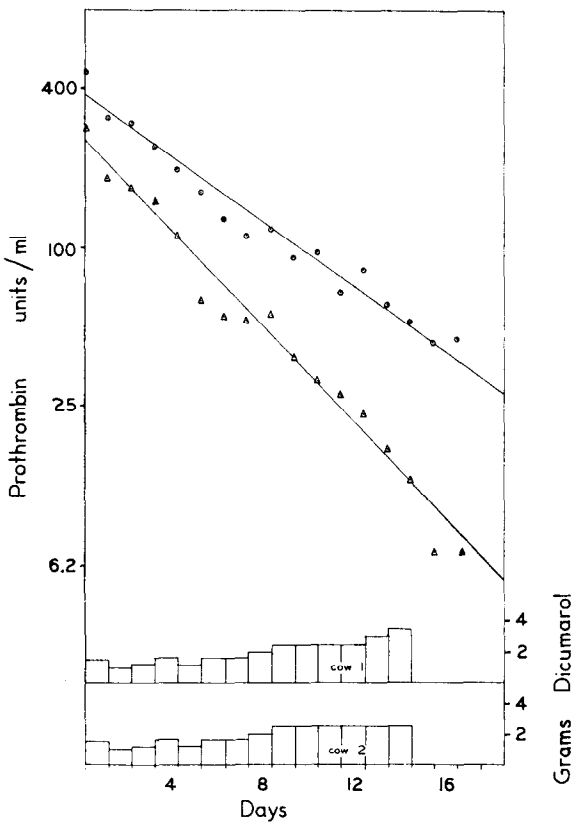

FIG. 1. Response to Dicumarol administration. Units of prothrombin, as measured by the two-stage method of Ware and Seegers,[5] are plotted for each day. Cow 1, ○; cow 2, △. The grams of Dicumarol as given in a single oral daily dose are represented by the bar diagrams.

production as shown by the first-order disappearance of the prothrombin from the plasma. The half-life of the prothrombin activity, as estimated graphically, was 4·2 days in one cow and 3·1 days in the other. Since a steady state was not reached during the treatment period, and since there was not a deviation from a first-order decay, it is not possible to decide whether or not there was any residual synthesis of a functional molecule. At the time dicoumarin administration was stopped there were 28 units/ml in one cow and 14 units/ml in the other. This level was approximately 5% of the pretreatment value. The prothrombin levels remained low for the first few days after the dicoumarin had been discontinued, but were approaching the pretreatment levels when the cows were again bled 11 and 30 days later.

Isolation of prothrombin during dicoumarin treatment. Table 1 summarizes some of the characterizing data for the prothrombin preparations from the plasma obtained during the course of therapy. The total number of units of prothrombin isolated from each of the plasmas is consistent with the number of units present in the starting sample. The total amount of protein recovered, however, was fairly constant in all

TABLE 1. ISOLATION OF PROTHROMBIN FROM BOVINE PLASMAS

Sample		Plasma prothrombin (units/ml plasma)	Prothrombin isolated (units/liter plasma)	Prothrombin units recovered (%)	Protein isolated (mg/liter plasma)	Specific activity isolated prothrombin (units/mg protein)
Pretreatment	Cow 1	424	144,000	34	220	507
	Cow 2	315	135,000	42	219	447
Dicoumarin, 4th day of treatment	Cow 1	165	108,000	66	257	316
	Cow 2	105	71,000	68	306	224
Dicoumarin, 14th day of treatment	Cow 1	28	24,200	83	204	104
	Cow 2	14	14,000	102	189	41
Day 11 of recovery	Cow 1	187	80,500	43	225	357
	Cow 2	150	77,000	51	166	464

preparations, thus resulting in decreased specific activities as the course of treatment progressed. Such a result would be obtained if a relatively large amount of the protein isolated in these preparations were a nonprothrombin contaminating protein. That this seems likely is suggested by the much higher specific activities obtained with these preparations after further purification by chromatography (Table 2). The low

TABLE 2. CHROMATOGRAPHY OF PROTHROMBIN ISOLATED FROM BOVINE PLASMA

Sample	Specific activity	
	Before chromat.	After chromat.
Pretreatment	507	1,050
Dicoumarin, 4th day of treatment	316	1,070
Dicoumarin, 14th day of treatment	104	427
Recovery, 11th day	357	1,080

specific activity of the chromatographed prothrombin from the 14th day of treatment can be explained by the gross heterogenity of all these preparations evident in the chromatograms. In Fig. 2, the series of chromatograms of the prothrombin preparations from one cow provides evidence of the heterogeneity of the preparations. Even though the specimens with the high specific activities indicate relatively pure preparations, neither the pretreatment nor the subsequent samples show coincidence between the protein peak and the peak of prothrombin activity. From the amount of inactive protein present in the 14-day sample after chromatography, it appears that the contribution of prothrombin to the total protein of all the samples must be relatively small.

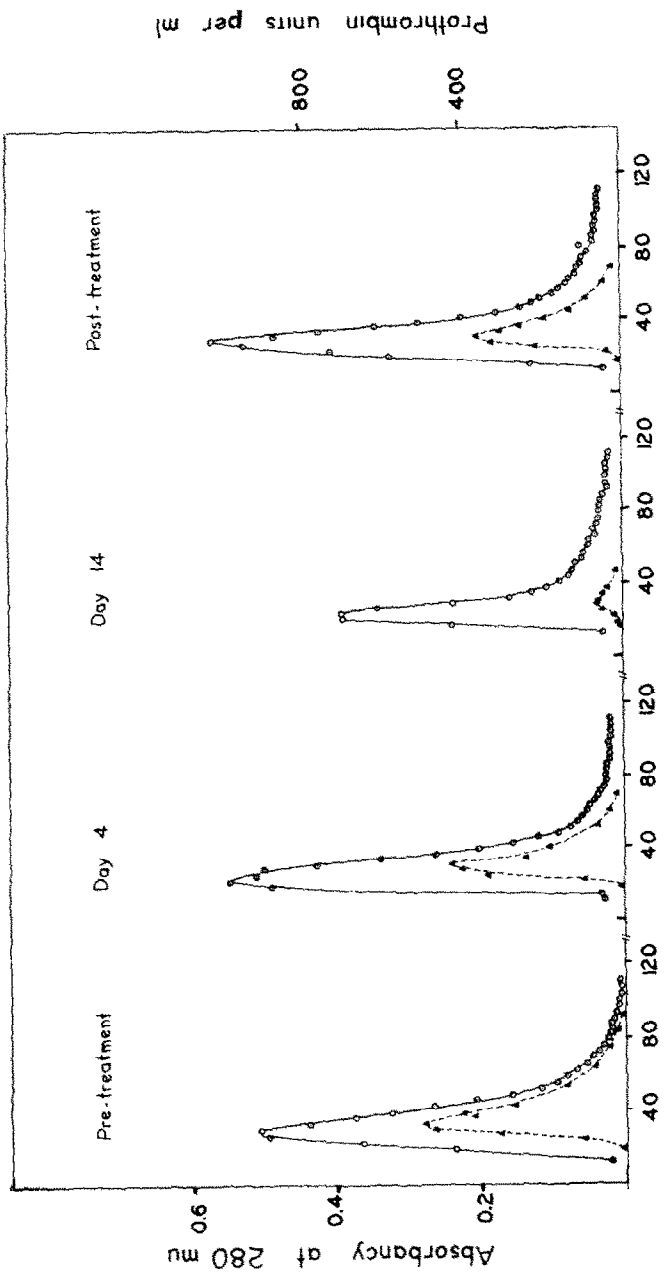

FIG. 2. Chromatograms of prothrombin isolated from cow 1 (left to right): pretreatment sample (plasma prothrombin 424 units/ml plasma), 15,100 units (23·2 mg protein) applied to column; day 4 of treatment (plasma prothrombin 165 units/ml plasma), 11,100 units (28·6 mg protein) applied to column; day 14 of treatment (plasma prothrombin 28 units/ml plasma); 3,550 units (28·5 mg protein) applied to column; post-treatment, 11th day of recovery (plasma prothrombin 187 units/ml plasma), 10,603 units (78·2 mg protein) applied to column. ○, protein determination as measured by the absorbancy at 280 mμ; △, units/ml of prothrombin.

Immunological characterization, The heterogeneity suggested by the chemical and chromatographic studies was confirmed by immunoelectrophoretic analysis. Multiple different precipitation bands of varied electrophoretic mobility occurred with all the preparations prior to chromatography. An example of these multiple components is shown in Fig. 3, together with the analysis of a preparation that had been chromatographed. An attempt to define these multiple bands was made, by means of the absorbed antisera described. With the antiserum that had been absorbed with $BaSO_4$-adsorbed plasma, two or three different bands are regularly present in all preparations of prothrombin (Fig. 4). These bands should represent proteins that are adsorbed from plasma by $BaSO_4$. In order to determine which, if any, of these bands represented a specific prothrombin precipitate, the same lot of antiserum was absorbed with serum from one of the cows at the peak of the response therapy. This absorption with prothrombin-free serum would be expected to remove antibodies directed against any antigenic determinant not removed by the process of coagulation, and should produce an antiserum specific for prothrombin. With this antiserum, only a single, relatively small precipitin band is demonstrable by immunoelectrophoresis (Fig. 5) and it is presumably, then, this band that represents the specific prothrombin precipitate.

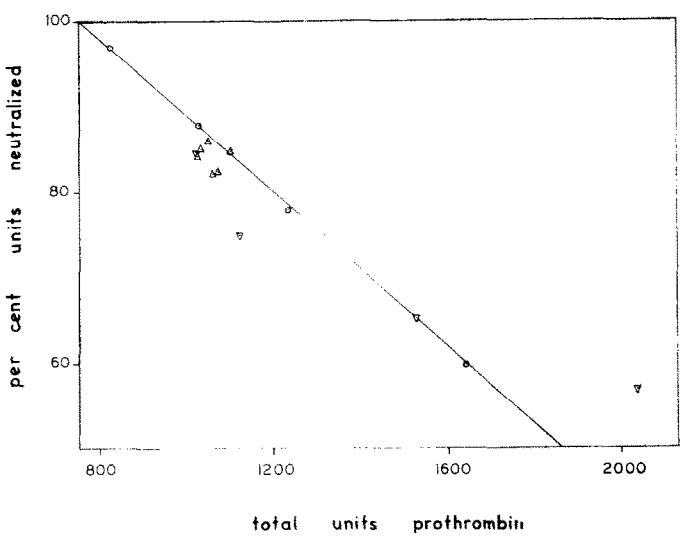

FIG. 6. Neutralization of prothrombin with antiprothrombin serum. ○, Normal prothrombin, experiment 1. ▽, Normal prothrombin, experiment 2. △, Normal prothrombin (1,020 units) plus increments of prothrombin isolated on 14th day of treatment, experiment 2.

The precipitin band, evident with all three antisera, is demonstrable in all preparations containing an adequate concentration of prothrombin, but below approximately 3,000 units/ml, it has not been demonstrated. Because of the relative insensitivity of the technique, this band has not been demonstrated in all the chromatographed preparations of prothrombin.

A more critical appraisal of the prothrombin present in these various preparations has been possible through application of the technique of neutralization inhibition.

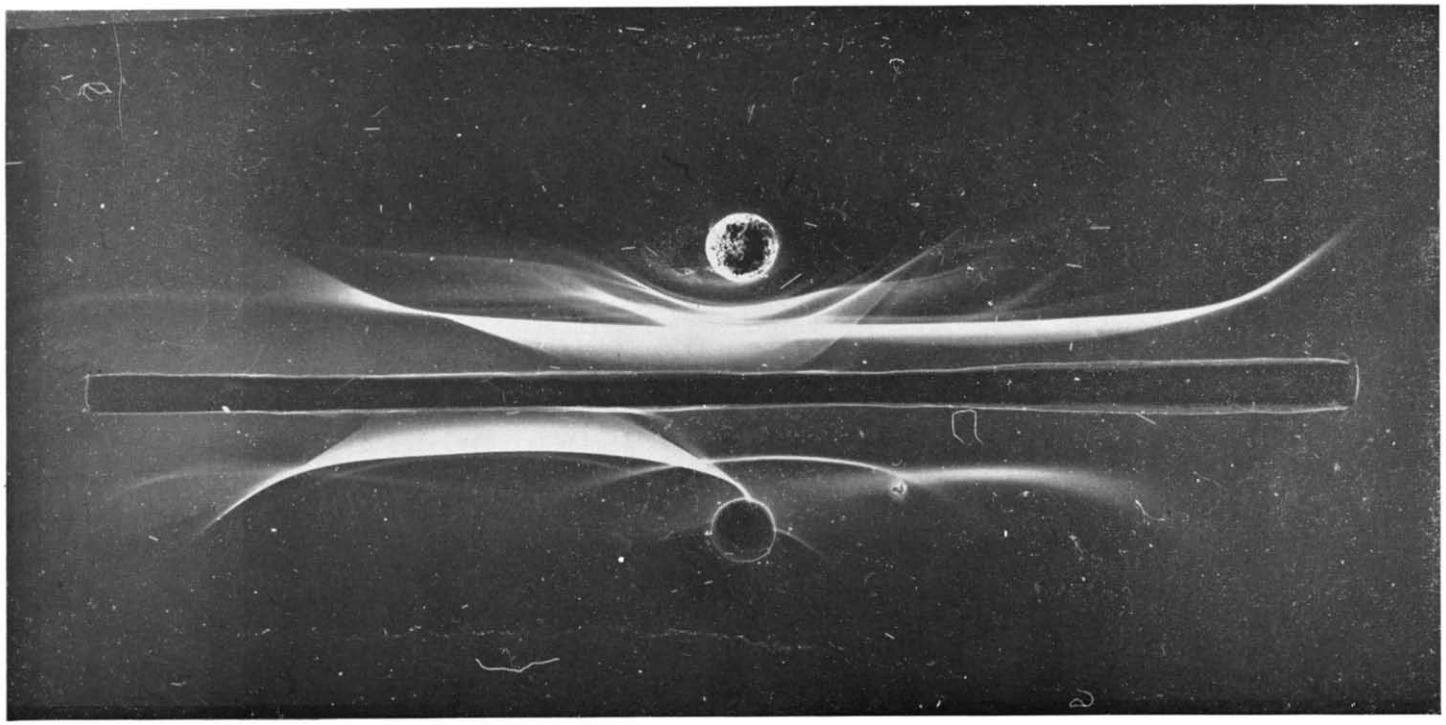

FIG. 3. Immunoelectrophoretic analysis. Untreated antiserum (center well), diffused against normal isolated prothrombin preparations: upper sample unchromatographed, lower sample chromatographed. Positive electrode on left.

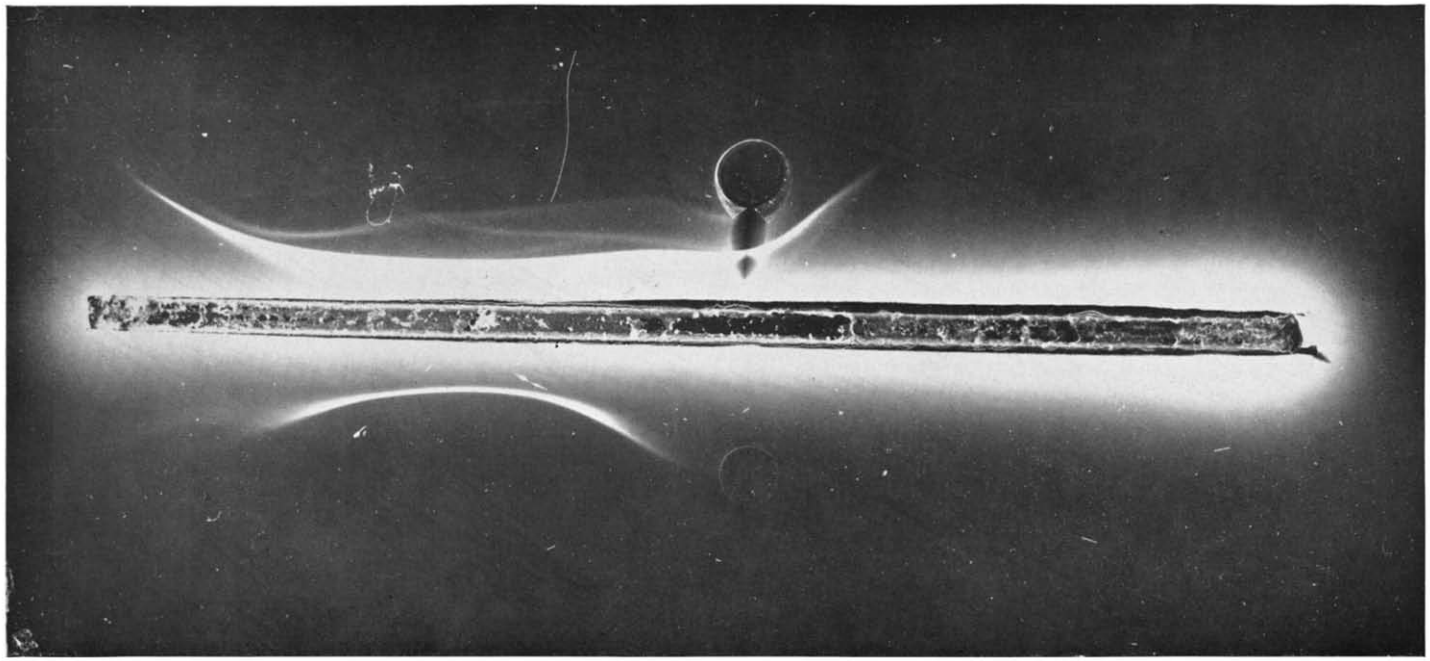

FIG. 4. Imunoelectrophoretic analysis. Antiserum absorbed with $BaSO_4$-adsorbed bovine plasma (center well), diffused against normal isolated prothrombin preparations: upper sample unchromatographed, lower sample chromatographed. Positive electrode on left. The concentrations of protein and prothrombin in the chromatographed preparation were relatively low, and the band presumed to be due to prothrombin is not visible.

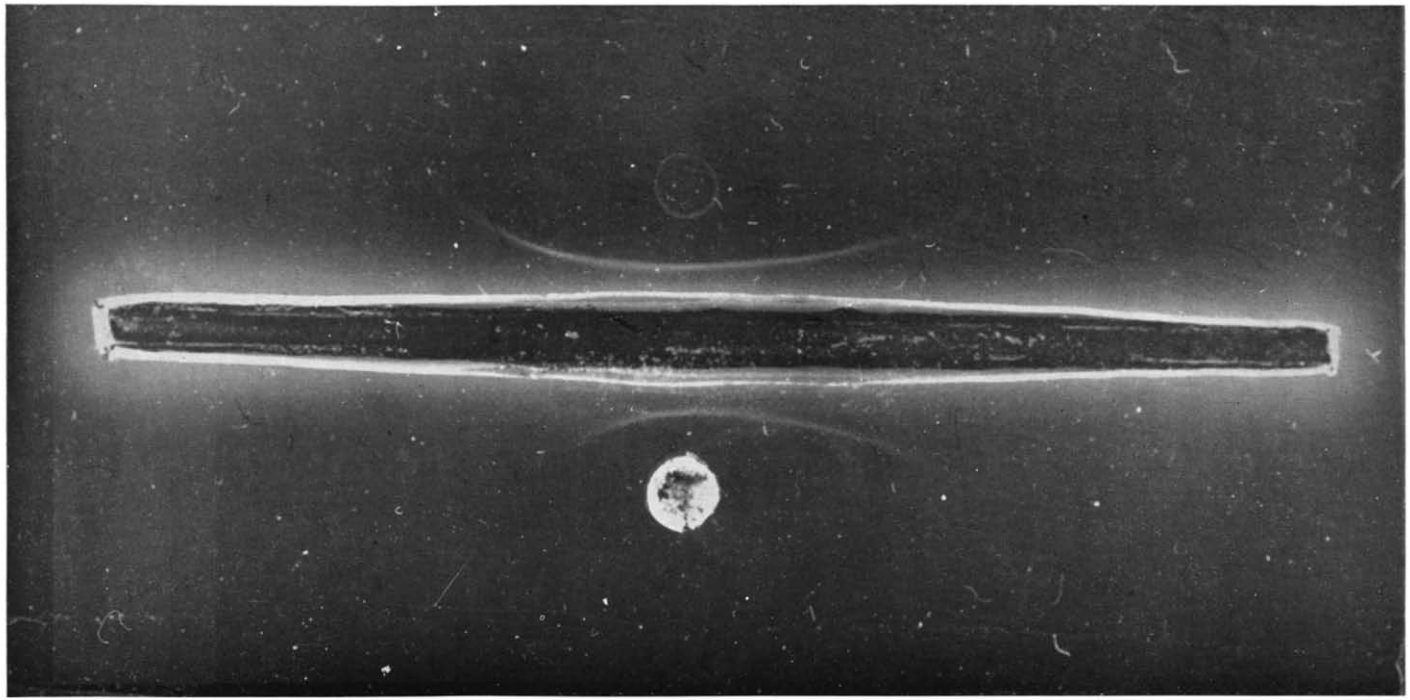

FIG. 5. Imunoelectrophoretic analysis. Antiserum absorbed with serum from a dicoumarinized cow (center well), diffused against normal isolated prothrombin: upper sample unchromatographed, lower sample chromatographed. Positive electrode on left.

In the zone of antigen excess the fractional neutralization of prothrombin was inversely proportional to the total units of activity present, with all preparations of prothrombin employed. The single line derived from the three sets of data presented in Fig. 6 was used to calculate the number of units of a constant test preparation of normal prothrombin neutralized in the presence of increasing amounts of prothrombin isolated from a normal and from a treated animal (Fig. 7). The increments of

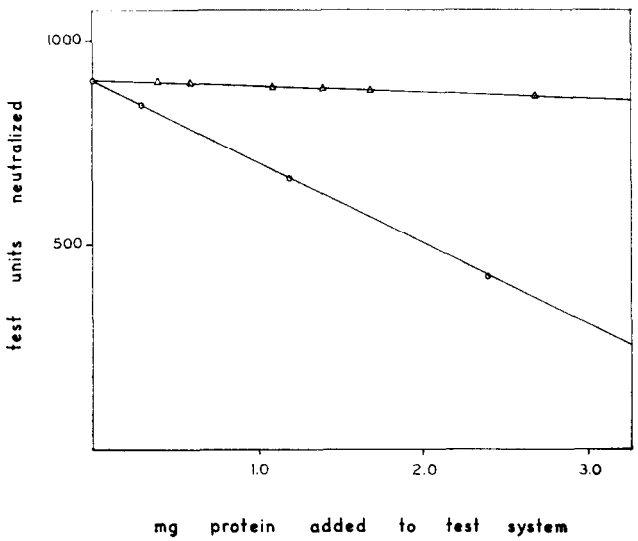

FIG. 7. Prothrombin–antiprothrombin neutralization inhibition (data from experiment 2, Fig. 6). ○, Normal prothrombin (1,020 test units, 2·3 mg protein) plus increments of normal prothrombin (control); △, normal prothrombin (1,020 test units, 2·3 mg protein) plus increments of prothrombin isolated on 14th day of treatment.

protein were similar for the two added preparations. The slight increase in inhibition of neutralization of the test prothrombin in the presence of the dicoumarinized preparation reflects the small additional number of units contained in this preparation. If, in the preparation from the treated animal, there had been a form of prothrombin antigenically intact but inactive as a zymogen, it should have caused a decrease in the fraction of the test units neutralized in a manner similar to that of the control set. From these experiments, it is evident in terms of the number of units of active protein that there is no difference in the immunologic character during dicoumarin administration. In terms of the amount of protein of each of these preparations required to obtain similar degrees of inhibition, it is also evident that a relatively large part of the protein present in some of these preparations represents antigenically unrelated contaminants.

The absence of an agent causing a degradation of prothrombin activity in the preparations isolated from a treated animal is indicated by the data presented in, Table 3. The mixture of normal prothrombin with dicoumarinized prothrombin, in a system identical with the antigen–antibody neutralization system but without the antiserum, resulted in a full recovery of prothrombin activity.

TABLE 3. EFFECT OF PROTHROMBIN ISOLATED FROM DICOUMARINIZED COW ON ACTIVITY OF NORMAL BOVINE PROTHROMBIN

Normal prothrombin units (calc.)	Pretreatment prothrombin units (calc.)	Dicoumarin prothrombin units (calc.)	Total prothrombin activity in units (calc.)	Total activity recovered units (determined)
357	0	0	357	420
357	675	0	1,032	1,005
357	0	51	408	436

Reaction mixture as in antigen–antibody neutralization system: volume 1·0 ml; 37°, 1 hr; 4°, 18 hr.

DISCUSSION

Since the isolation of a protein of decreased specific activity suggests a functional alteration, we felt that it was important to provide as much data on this point as possible. The preparations of prothrombin that have been isolated before treatment with dicoumarin and during treatment when the plasma level was approximately 40% of the initial value, show, when highly purified, specific activities of the same order. This provides direct evidence for the normal character of what small amount of active protein remains during therapy. The chromatograms themselves, however, show that these preparations are still grossly heterogeneous. Such heterogeneity presumably is the cause of the rather low specific activity in the sample from the 14th day of treatment. The lack of coincidence between the protein and activity peaks has also occurred when similar preparations of human prothrombin are analyzed by density gradient centrifugation and Sephadex G-200 chromatography,[9, 10]

In the chromatographed preparations where the peak of protein eluted consists of two or more antigenic species, the major immunoelectrophoretic band presumably accounts for much of the protein present. This band appears antigenically unrelated to prothrombin from the experiment using the antiserum absorbed with serum from the dicoumarinized cow. The other regularly occurring band with these same antisera has been presumptively identified as prothrombin.

The mechanistic distinction sought for in the experiments reported can be best made from the results obtained with the technique of neutralization inhibition. With our antiserum only an extremely small amount of the total antibody is directed toward the portion of the molecule possessing thrombin activity after activation.* Presumably the remainder of the neutralizing antibody is directed against portions of the prothrombin molecule either split off as peptides during activation or specific secondary and tertiary protein conformations that must certainly be greatly changed by activation. These multiple determinants make highly probable the demonstration of a protein species altered in some prosthetic group. The probability of a specific chemical alteration simultaneously destroying the reactivity of all determinants seems most unlikely. It is clear that unit for unit, and only by this dimension, prothrombin is antigenically identical throughout the period of dicoumarin response studied. Since the prothrombin that remains during the course of therapy is indistinguishable from that synthesized in the normal state, it must be concluded that dicoumarin exerts its action at some stage concerned with the formation of the primary structure or the subsequent conjugation of subunits required to form the

* Unpublished observations

released active protein. The experiments reported by Barnhart and Anderson[11] provided evidence that this action is not simply a problem of the release of an otherwise structurally intact molecular species. Such results also offer proof against any theory of increased utilization or degradation.

REFERENCES

1. W. H. SEEGERS, N. ALKJAERSIG and S. A. JOHNSON, *Amer. J. Physiol.* **181**, 589 (1955).
2. W. H. SEEGERS and S. A. JOHNSON, *Amer. J. Physiol.* **184**, 259 (1956).
3. P. A. OWREN, *Arch. Intern. Med.* **111**, 248 (1963).
4. L. POLLER, *Acta haemat. (Basel)* **28**, 168 (1962).
5. A. G. WARE and W. H. SEEGERS, *Amer. J. clin. Path.* **19**, 471 (1949).
6. K. D. MILLER and J. MCGARRAHAN, *N.Y. State Dept. Health Ann. Report. Div. Lab. & Res.*, p. 69 (1958).
7. M. P. TOMBS, F. SOUTER and N. F. MACLAGAN, *Biochem. J.* **73**, 167 (1959).
8. J. J. SCHEIDEGGER, *Int. Arch. Allergy* **7**, 103 (1955).
9. G. F. LANCHANTIN, J. A. FRIEDMANN, J. DEGROOT and J. W. MEHL, *J. biol. Chem.* **238**, 238 (1963).
10. G. F. LANCHANTIN and J. A. FRIEDMANN, *Fed. Proc.* **22**, 242 (1963).
11. M. I. BARNHART and G. F. ANDERSON, *Proceedings, 8th Congr. Europ. Soc. Haemat., Vienna*, 1961, Part II, p. 400. Karger, Basel (1962).