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The 1964 recipient of the Meltzer Award was Dr. I. Bernard Weinstein of the Department of Medicine, College of Physicians and Surgeons, Columbia University. The selection was made by a Committee appointed by the President, consisting of Doctors Dwight J. Ingle, Lewis Thomas, and Maurice B. Visscher.

SECTION MEETINGS

CLEVELAND	
Lakeside Hospital	March 17, 1964
JOINT DISTRICT OF COLUMBIA - MARYLAND SECTIONS	
University of Maryland	April 4, 1964
MARYLAND SECTION	
University of Maryland	January 23, 1964
Johns Hopkins University	February 27, 1964
MINNESOTA	
University of Minnesota	March 19, 1964

Intracellular Localization of Prothrombin.* (29142)

GORDON F. ANDERSON AND MARION I. BARNHART
Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Mich.

Prothrombin formation and storage are clearly functions of liver parenchymal cells according to information obtained with the fluorescent antibody technique(1,2). The present report extends the information on prothrombin localization to the intracellular level in dogs and cattle.

Materials and methods. Isolated parenchymal cell concentrates were obtained with the technique of St. George(3) on Dowex 50 W resin columns. Evaluation of the homogeneity of the fractions was made by dif-

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ferential cell counts on Leishman stained smears. A typical cell concentrate contained 96.5% parenchymal cells, 3% free nuclei and 0.5% red blood cells. In cell counts up to 600 cells neither Kupffer cells nor platelets were found.

Subcellular fractions were prepared in 0.25 M sucrose by the procedures of Schneider and Hogeboom(4) and de Duve and associates (5). The following fractions were studied: total cell homogenate (TCH), cytoplasmic extract (CE), nuclei (N), mitochondria (M), lysosomes (L), microsomes (Mic) and soluble fraction (S). Further purification of the nuclear fraction was achieved by centrifugation at 345 *g* for 5 minutes to sediment the associated whole cells and other subcellular particulates. The resulting supernatant containing nuclei was recentrifuged at 750 *g* for 5 minutes. The nuclei in this precipitate were then resuspended in 0.25 M sucrose and washed twice. All fractions were finally suspended in sucrose so that the stock solutions were 10% by volume.

Antiprothrombin sera were produced in rabbits using purified prothrombin(6,7) injected with $Al(OH)_3$ as a single intramuscular injection according to our described procedure(8). Each antiserum was characterized by immunoelectrophoresis. Only those antisera were selected that formed precipitin bands in the alpha-2 region during immunoelectrophoresis. Further studies were made in agar gel diffusion systems with Ouchterlony type plates as modified by Wilson and Pringle (9,10). Such antisera formed a precipitin band when reacted with purified prothrombin. Furthermore these antisera lost their reactivity when adsorbed with purified prothrombin. It was clearly established that these antisera did not react with either fibrinogen or albumin also synthesized in the liver.

Fluorescent antiprothrombin was prepared from a gamma globulin concentrate of a specific antiprothrombin serum. Lissamine rhodamine B 200 was used as the fluorescent dye and the conjugation procedure followed our previously described procedure(8).

Fluorometric determination of prothrombin content in sub-cellular fractions. Aliquots of sub-cellular fractions were mixed with various concentrations of rhodamine antiprothrombin. These mixtures were incubated for 30 minutes at 25°C. The unadsorbed rhodamine antiprothrombin was recovered in the supernatant after centrifugation at 4°C. The following centrifugation schedule was used with a Clay Adams Centrifuge for nuclei, a Serval Centrifuge for mitochondria and lysosomes and a Spinco Centrifuge for microsomes and soluble fractions: Nuclei 790 *g* for 10 minutes; Mitochondria, 7700 *g* for 10 minutes; Lysosomes 20500 *g* for 10 minutes; Microsomes and Soluble Fractions 107000 *g* for 45 minutes. A Turner Fluorometer was used to measure the fluorescent solution before and the supernatant after centrifugation. The primary filter was 546 mμ and the secondary filter was 570 mμ. A sensitivity setting of 3$\times$ gave satisfactory results. The amount of fluorescent antiprothrombin adsorbed by the cellular fraction was considered an index of the prothrombin localization. For control purposes rhodamine anti gamma globulin was employed in similar fashion as a check for nonspecific adsorption. Also incubation of the fraction with unlabeled antiprothrombin prior to treatment with rhodamine antiprothrombin blocked the uptake of fluorescent antiprothrombin.

Results. Subcellular localization of prothrombin as determined with a specific fluorescent antiprothrombin. The antibody binding capacity of the cellular fractions obtained from homogenized dog liver was measured with a fluorometer. Each cell fraction was placed in contact with a rhodamine antiprothrombin solution that had a dye/protein ratio of 26.4×10^{-3}. Time was permitted for the specific binding to occur; then the nonreactive fluorescent antibody was recovered and its fluorescence reassessed. Only the microsomal and soluble fractions removed significant amounts of rhodamine antiprothrombin (Table I). Controls run with appropriate concentrations of rhodamine normal gamma globulin or free rhodamine did not show significant adsorption to the subcellular fractions. Thus there was concentration of prothrombin or closely related molecules in the microsomes and soluble portion of liver cells.

TABLE I. Binding of Fluorescent Antiprothrombin by Subcellular Fractions of Dog Liver.

Fraction	Rhodamine antiprothrombin bound* Antibody dye (μg)	Antibody protein (μg)
Microsome	.15	5.6
	.49	18.4
	1.35†	51.0†
	.60	22.5
Soluble	.86	32.2
Lysosome	0	0
Mitochondria	0	0
Nuclei	0	0

* Binding per mg protein in the cell fraction.
† Equivalence zone.

Parenchymal cell localization of prothrombin. The previously described work with homogenates of total liver could not provide information on prothrombin content of the different types of cells that comprise the liver. Experiments therefore were undertaken on whole cell preparations which were separated on the basis of size and type with the aid of a Dowex 50 W resin column. The parenchymal cell fraction was essentially homogeneous. Another fraction contained reticuloendothelial (RE) cells with many blood cells. In addition, parenchymal cell fragments (*i.e.*, nuclei, cell membranes and debris) also were present making this fraction unsuitable for study. To study prothrombin localization agar gel diffusion systems were employed. Prothrombin content was assessed on the basis of a precipitin reaction formed between the cell concentrate and a specific antiprothrombin serum. This precipitin band identified completely with purified prothrombin. Parenchymal cells reacted with antiprothrombin and thus contained prothrombin and/or its close relatives (Fig. 1). The density of the precipitin reaction was increased by ultrasonic treatment of the parenchymal cell concentrate. Presumably rupture of cell membranes and structures released more prothrombin for the precipitin reaction. The parenchymal cell fractions were not contaminated with RE cells so that the prothrombin found was not from any other cell type of the liver. Both bovine and canine parenchymal cells contained prothrombin when reacted with their homologous antiprothrombin in our test system.

Prothrombin was concentrated in the microsomal and soluble fractions obtained from parenchymal cell preparations. The parenchymal cell preparations were disrupted and subcellular fractions were prepared. These were then reacted with the appropriate antiprothrombin in agar gel systems. Heavy precipitin bands formed between the antiprothrombin and each of the following fractions: total cell homogenate, cytoplasmic extract, microsomes and the soluble fraction (Fig. 2, 3A, 3B). A reaction was not observed with either mitochondria or lysosomes (Fig. 2). A weak precipitin reaction occurred with the standard nuclear fraction. However, this fraction was the most heterogeneous of any of the subcellular fractions used and contained some whole parenchymal cells and blood cells, as well as other sub-cellular structures. Further purification of these nuclei eliminated this precipitin reaction. It was, therefore, either the result of contaminating microsomes or adsorbed prothrombin. The precipitin reaction with microsomes was not significantly altered by several additional washes with sucrose. Prothrombin appeared to be rather firmly bound to microsomes. Especially good precipitin reactions were obtained when concentrated microsomes were used (Fig. 4). The precipitin band identified completely with purified prothrombin to provide an accurate intracellular localization of prothrombin. The reacting proteins in the microsomes and soluble fraction were antigenically similar (Fig. 2 and 3B). It seems reasonable that the prothrombin identified in the total cell homogenate (Fig. 3A) and cytoplasmic extracts was the consequence of the microsomes and soluble fraction which are part of these fractions.

Hypoprothrombinemia reduced or eliminated microsomal prothrombin. Prothrombin deficiencies were produced in dogs following treatment with Coumadin (4 mg/kg first day and then 2 mg/kg second day). One or two days later subcellular fractions were obtained from the liver. Microsomes gave only faint precipitin reactions or no reactions when reacted with anti dog prothrombin (Fig. 3D). Prothrombin was present in all soluble frac-

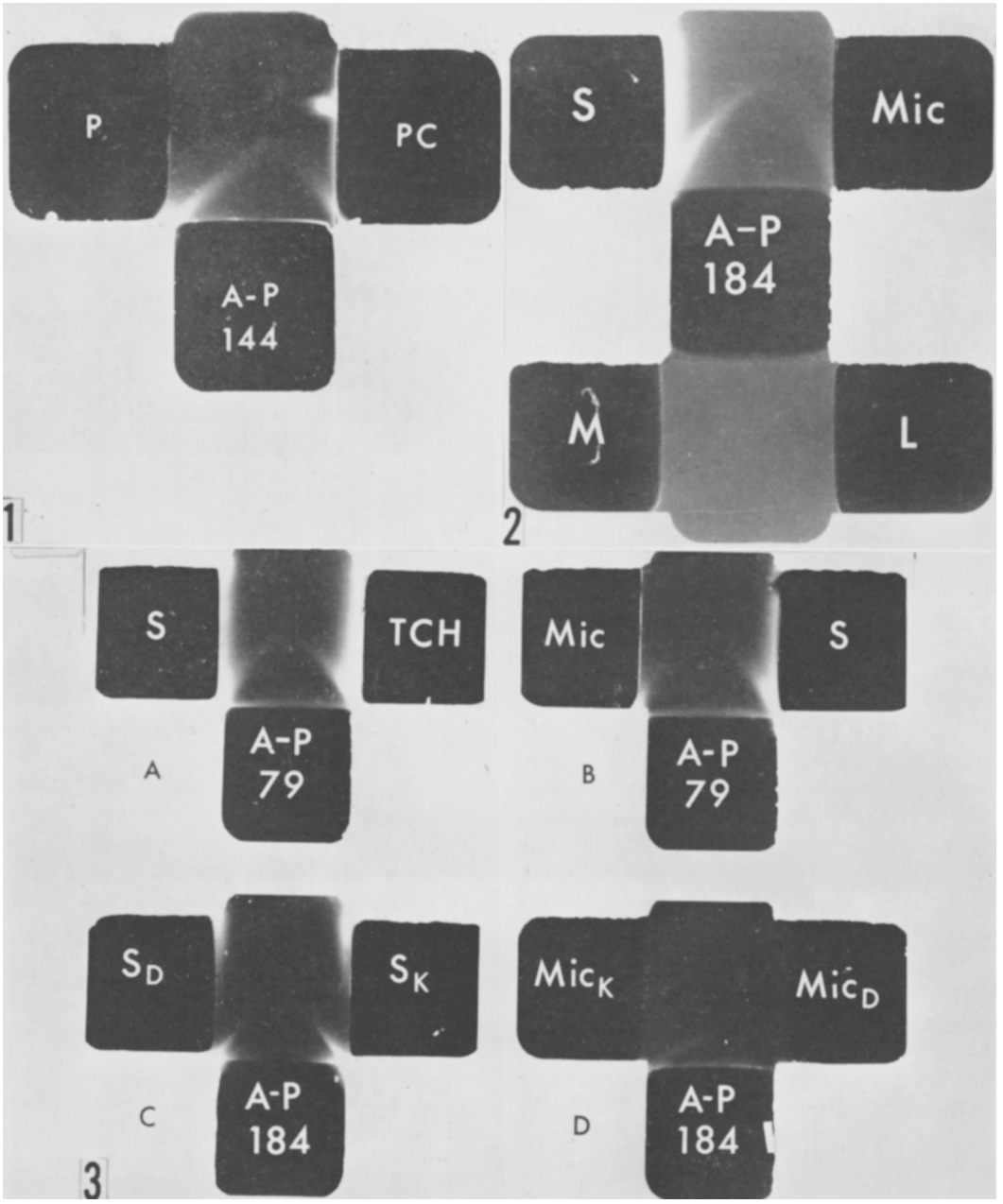

FIG. 1. Identification of prothrombin in bovine liver parenchymal cells. Note the band of identity between ultrasonically treated parenchymal cells (PC) and purified bovine prothrombin (P) when reacted against a specific anti-bovine prothrombin (A-P). The PC fraction was used as a 10% solution in 0.25 M sucrose.

FIG. 2. Intracellular localization of prothrombin in subcellular fractions obtained from normal dog liver. Precipitin bands formed when either microsomes (Mic) or soluble (S) fraction were reacted with anti-dog prothrombin (A-P). There was no indication of prothrombin content in either mitochondria (M) or lysosomes (L). All cell fractions were 10% suspensions in 0.25 M sucrose.

FIG. 3. Subcellular localizations of prothrombin in dog liver parenchymal cells. Anti-dog prothrombin (A-P) was employed with material from normal, hypoprothrombinemic and prothrombin stimulated livers. A. Note common antigenic component in total cell homogenate

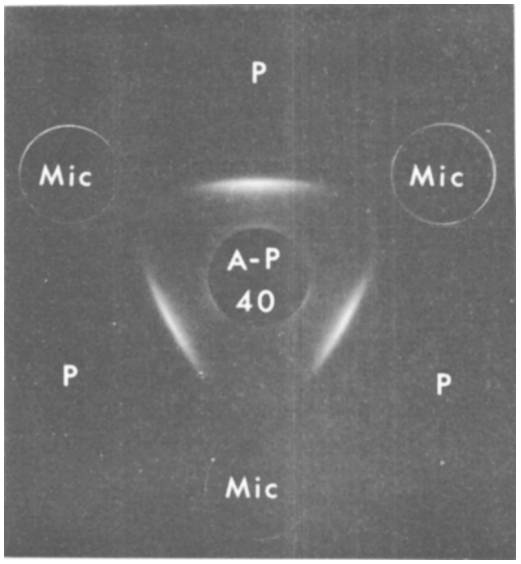

FIG. 4. Identification of prothrombin (P) in bovine microsomes (Mic). Note central continuous precipitin of prothrombin-antiprothrombin complex. Center well contained anti bovine prothrombin (A-P) which was a 2 component reagent that reacted with both prothrombin and associated accelerator globulin present in test prothrombin. Bottom well contained a 10% suspension of microsomes while the other wells were 20% microsomal suspensions. Prothrombin was a 0.5% solution.

tions studied (Fig. 3C). Details of a typical experiment follow. One prothrombin deficient animal had a circulating prothrombin of 4 U/ml as determined by the method of Ware and Seegers(11). Prothrombin time was 3 minutes. Fibrinogen was in the normal range. The study of intracellular fibrinogen with antifibrinogen serum showed fibrinogen present in both microsomal and soluble fractions. But only the soluble fraction reacted with antiprothrombin (Fig. 3C). Prothrombin could not be detected in the microsomes of this animal with the immunologic procedure employed (Fig. 3D). Such results as these encourage the view that microsomes are the intracellular sites for prothrombin synthesis. Coumadin then could block prothrombin production at this point.

Restitution of microsomal prothrombin synthesis by treatment with vitamin K_1. Vit. K_1 was given to several dogs including some that were severely depleted of prothrombin. Liver was taken by surgical biopsy 3-5 hours later and subcellular fractions prepared. Prothrombin was identified in both microsomal and soluble fractions (Fig. 3C, D). No other fractions gave precipitin reactions under these circumstances. These data offer strong support for the microsomes being the intracellular sites for prothrombin production.

Discussion. Most studies of prothrombin synthesis have employed or regarded liver as if it were a homogeneous organ with respect to cell type except for the work of Barnhart and associates(1,2,12). They recently showed with the fluorescent antibody technique that only liver parenchymal cells and not reticuloendothelial cells synthesize prothrombin. Further evidence on the parenchymal cell's content of prothrombin is provided by the present report.

One major objective of this work was to investigate the distribution of prothrombin within the cell. Prothrombin was found in the microsomal and soluble fractions obtained from isolated liver parenchymal cells of both canine and bovine origin. Although all cell fractions obtained in a fractionation were assayed, no other subcellular fractions such as nuclei, mitochondria or lysosomes contained firmly bound prothrombin. Recently, preliminary reports from 3 other laboratories have appeared which describe the microsomal localization of prothrombin activity in rats (13-15). In all cases entire liver was used for the cellular fractions and disruption of structure improved the amount of prothrombin that could be detected. There would appear little doubt considering these reports and our study that prothrombin is concentrated in the microsomes of at least 3 different distantly related animals (rats, dogs, cattle).

In any study involving disruption of cellular structures in a foreign medium the pos-

(TCH) and soluble (S) fraction from a normal dog. B. Identical antigenic substances exist in microsomes (Mic) and soluble (S) fraction of normal dogs. C. Note the precipitin band indicative of prothrombin localization in soluble fraction of dicumarolized dogs (S_D) as well as in soluble fraction from Vit. K_1 stimulated (S_K) animals. D. Microsomes obtained from a dicumarolized dog (Mic_D) did not react with antiprothrombin. After stimulation of prothrombin synthesis by treatment with Vit. K_1 the microsomes (Mic_K) formed a precipitin reaction indicating the intracellular site of prothrombin synthesis.

sibility exists that the localizations observed are artifacts of the technique(16). Possibilities are good for loosely bound or soluble constituents to be relocated, or even be lost by the washing procedures. The persistence of a substance in the cell structure after repeated washing is generally considered a good indication of its presence in an intact cell. Prothrombin localization in subcellular fractions should be considered with these points in mind. Clearly there is prothrombin firmly associated with the fine structure of the cell known as the microsomes or fragmented endoplasmic reticulum. Whether the prothrombin found in the soluble fraction was present in the cell sap normally or was eluted from structural sites of synthesis and storage cannot be accurately evaluated. It seems reasonable, however, to consider that some portion of the soluble prothrombin represents stored prothrombin.

Once prothrombin was localized intracellularly the question of whether this concentration represents an area for storage or synthesis could be reasonably considered. To distinguish between a stored or newly synthesized protein it is necessary to study a dynamic physiologic state. Several chemical agents are known to either suppress or stimulate prothrombin production. Dicumarol-like compounds (Coumadin) are well known for their capacity to reduce circulating prothrombin. Vitamin K and its relatives are equally competent in correcting this hypoprothrombinemia by the stimulation of prothrombin synthesis.

The microsomal localization of prothrombin identifies the intracellular sites for synthesis. This statement has its basis in the facts that prothrombin deficient animals lacked microsomal prothrombin while Vit. K_1 stimulated, as well as normal animals exhibited microsomal prothrombin. Such data are consistent, too, with the current views that microsomes are involved in synthesis of specific proteins(17,18).

1. Barnhart, M. I., *Am. J. Physiol.,* 1960, v199, 360.
2. Barnhart, M. I., Anderson, G. F., *Biochem. Pharm.,* 1962, v9, 23.
3. St. George, S., in *Reticuloendothelial Structure and Function,* Ronald Press, New York, 1960, p449.
4. Schneider, W. C., Hogeboom, G. H., *J. Biol. Chem.,* 1950, v183, 123.
5. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., Applmans, F., *Biochem. J.,* 1955, v60, 604.
6. Seegers, W. H., *Record Chem. Prog.,* 1952, v13, 143.
7. Anderson, G. F., Barnhart, M. I., *Am. J. Physiol.,* 1963, in press.
8. Barnhart, M. I., Anderson, G. F., Baker, W. J., *Thrombos. Diathes. haemorrh.,* 1962, v8, 21.
9. Wilson, M. W., Pringle, B. H., *J. Immunol.,* 1954, v73, 232.
10. Wilson, M. W., *Experimental Records, Medical Llibrary,* Wayne State Univ., Detroit, 1957-1958.
11. Ware, A. G., Seegers, W. H., *Am. J. Clin. Path.,* 1949, v19, 471.
12. Baker, W. J., Barnhart, M. I., *Physiologist,* 1960, v3, 14.
13. Munro, H. N., Goswami, P., *Biochim. Biophys. Acta,* 1952, v55, 410.
14. Helgeland, L., Laland, S., *ibid.,* 1962, v62, 200.
15. Hill, R. B., Gaetani, S., Johnson, B. C., *Fed. Proc.,* 1963, v22, 620.
16. Alfrey, V., in *The Cell,* Academic Press, New York, 1959, v1, 193.
17. Peters, T., *J. Hist. Cytochem.,* 1959, v7, 224.
18. Seikevitz, P., Palade, G. E., *J. Biophy. Biochem. Cytol.,* 1958, v4, 309.

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