References and Notes

1. D. Branton, *Phil. Trans. Roy. Soc. London Ser. B* **261**, 133 (1971).
2. ———, *Annu. Rev. Plant Physiol.* **20**, 209 (1969).
3. N. S. McNutt and R. S. Weinstein, *J. Cell Biol.* **47**, 666 (1970).
4. S. Bullivant and A. Ames, *ibid.* **29**, 435 (1966).
5. S. Bullivant, *Micron* **1**, 46 (1969).
6. H. Moor, K. Mühlethaler, H. Waldner, A. Frey-Wyssling, *J. Biophys. Biochem. Cytol.* **10**, 1 (1961).
7. H. H. R. Friederici, *J. Ultrastruct. Res.* **23**, 444 (1968); *Lab. Invest.* **21**, 459 (1969).
8. D. W. Deamer, R. Leonard, A. Tardieu, D. Branton, *Biochim. Biophys. Acta* **219**, 47 (1970); S. J. Singer and G. L. Nicolson, *Science* **175**, 720 (1972).
9. D. D. Sabatini, K. Bensch, R. J. Barrnett, *J. Cell Biol.* **17**, 19 (1963).
10. S. Bullivant, D. G. Rayns, W. S. Bertaud, J. P. Chalcroft, G. F. Grayston, *ibid.* **55**, 520 (1972).
11. R. S. Weinstein, N. S. McNutt, S. I. Nielsen, V. W. Pinn, in *Proceedings of the Electron Microscopy Society of America* (Claitor's, Baton Rouge, La., 1970), pp. 108–109; L. A. Staehelin, T. M. Mukherjee, A. Wynn Williams, *Protoplasma* **67**, 165 (1969).
12. N. E. Flower, *J. Cell Sci.* **10**, 683 (1972); N. B. Gilula, *J. Ultrastruct. Res.* **38**, 215 (1972).
13. J. P. Chalcroft and S. Bullivant, *J. Cell Biol.* **47**, 49 (1970).
14. Personal communications from N. Flower and N. B. Gilula.

15. We thank J. B. Gavin and R. E. F. Matthews for discussion of the manuscript. Supported in part by a grant from the Medical Research Council of New Zealand.

4 August 1972; revised 10 October 1972 ■

Mechanism of Action of Vitamin K: Demonstration of a Liver Precursor of Prothrombin

Abstract. *Extracts of sonicated liver microsomes that are prepared from rats deficient in vitamin K or from rats given vitamin K antagonists contain a factor that liberates a thrombin-like activity when it is incubated with venom from* Echis carinatus. *The amount of this factor is low in control rats and in hypoprothrombinemic rats given vitamin K 1 hour before they were killed. These data indicate that this factor is a protein precursor of prothrombin, which is synthesized in the liver.*

The only generally accepted function of vitamin K in higher animals is that of regulating the synthesis of prothrombin and the other plasma clotting factors dependent on vitamin K (factors VII, IX, and X). The vitamin regulates the rate of synthesis of prothrombin after transcription but the nature of the control site is still undetermined. Although it has been suggested (*1*) that the vitamin regulates the de novo synthesis of prothrombin, observations from a number of different laboratories (*2*) indicate that protein synthesis is not required for the step sensitive to vitamin K in the production of prothrombin; we have recently presented indirect evidence (*3*) for the conversion of a precursor protein to prothrombin in the rat.

Stenflo (*4*) has reported the presence of an antigenically active, but biologically inactive, prothrombin in the plasma of cows treated with Dicumarol. The venom from *Echis carinatus* (saw-scaled viper) (*5*) can cause generation of thrombin from purified preparations of this abnormal prothrombin (*6*). This observation, and that of Josso *et al.* (*7*) suggested that the postulated prothrombin precursor from the liver might also be activated by such non-physiological means.

Male Holtzman rats (200 g) were made hypoprothrombinemic by treating them with the vitamin K antagonists warfarin or 2-chloro-3-phytyl-1,4-naphthoquinone (chloro-K) (*8*), or by feeding them a diet deficient in vitamin K for 7 days, while they were in cages that prevented coprophagy (*9*). Blood was drawn by cardiac puncture from starved rats anesthetized with ether; a liver microsomal extract was also prepared from these animals (*10*, *11*). Prothrombin concentrations were measured in plasma by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (*12*), and in the microsomal extracts by methods previously described (*11*).

The microsomal extract from rats treated with warfarin for 18 hours before they are killed has previously been shown (*11*) to contain no detectable prothrombin activity. However, when the thrombin-generating step of the two-stage assay was replaced by an incubation with *Echis carinatus* venom, a thrombin-like activity that would clot the fibrinogen mixture in less than 15 seconds was generated in extracts from the rats treated with warfarin. When the mixture containing fibrinogen was incubated with venom alone, or with microsomal extract alone, no clot was formed in 120 seconds. The clotting activity, generated from some factor other than prothrombin in the microsomal extract, will be subsequently referred to as precursor activity.

The amount of precursor activity in the liver microsomal extract is inversely related to prothrombin concentrations (Table 1). The data show that two different vitamin K antagonists, as well as a nutritional deficiency of the vitamin, all result in a decrease in prothrombin concentrations in the plasma and

Table 1. Activities of prothrombin and precursor in microsomal extracts of rat liver. Prothrombin in plasma and microsomal extracts was assayed, and results were expressed as Iowa units. To measure precursor activity, I incubated 0.5 ml of microsomal suspension with 0.1 ml of *Echis carinatus* venom (1 mg/ml) at 37°C. After 15 minutes, 0.1 ml of the treated suspension was added to a mixture of 0.1 ml of fibrinogen (1 percent clottable protein in 25 m*M* imidazole (*p*H 7.4) and 150 m*M* NaCl), and 0.3 ml of a buffered acacia solution containing 50 m*M* imidazole (*p*H 7.4), 120 m*M* NaCl, 5 m*M* $CaCl_2$, and 3 percent acacia. The mixture was tipped gently in a small tube until a visible clot formed. These clotting times were converted to thrombin units by comparison to a standard curve prepared by dilution of NIH standard thrombin. Sodium warfarin (5 mg/kg, intraperitoneal) or chloro-K (5 mg/kg, intracardial) was given 18 hours before the experiments, and vitamin K (5 mg/kg) was given 1 hour before the animals were killed. The vitamin was given intramuscularly to rats deficient in vitamin K, and intravenously to rats treated with warfarin. Cycloheximide (5 mg/kg, intraperitoneal) was given 30 minutes before the vitamin. Results are expressed as means ± standard errors. The number of animals used in each experiment are given after the treatment in column 1.

Treatment	Prothrombin concentration (unit/ml) in:		Precursor activity	
	Plasma	Microsomal extract	Clotting time* (seconds)	Thrombin equivalent in microsomal extract (unit/ml)
Control (7)	218 ± 12	2.1 ± 0.1	30 (22–46)	1.9 ± 0.3
Warfarin (10)	32 ± 6	<1	12 (11–14)	8.7 ± 0.6
Chloro-K (4)	34 ± 4	<1	11 (10–11)	11.8 ± 0.3
Deficient in K (6)	81 ± 20	<1	20 (18–24)	3.2 ± 0.2
Deficient in K + K (6)	175 ± 27	2.2 ± 0.1	77 (65–120)	0.7 ± 0.1
Warfarin + K (4)	136 ± 3	1.9 ± 0.1	97 (96–120)	0.6 ± 0.1
Warfarin + K + cycloheximide (4)	104 ± 3	2.0 ± 0.1	52 (34–62)	1.1 ± 0.2

* Expressed as median with range in parentheses.

liver and an increase in the precursor concentration in the liver. Administration of the vitamin to hypoprothrombinemic rats caused a decrease in the amount of precursor in the liver, and an increase in prothrombin concentration in the liver and plasma. In agreement with previous observations (*2, 3, 11*), cycloheximide treatment failed to block the increase in prothrombin in the plasma induced by the vitamin, and also failed to block the decrease in precursor activity in the liver. The concentration of precursor in the liver increased rapidly when prothrombin synthesis was blocked by warfarin, and more slowly during development of a vitamin K deficiency (Fig. 1).

Prothrombin and the other plasma clotting factors dependent on vitamin K are strongly adsorbed to barium salts, as is the prothrombin in a liver microsomal extract (*11*). In contrast, the abnormal prothrombin found in the plasma of cows treated with Dicumarol is not adsorbed by barium salts (*4, 5*). The microsomal extract from 12 rats treated with warfarin was mixed with $BaSO_4$ (35 mg/ml) for 1 hour at 4°C. The mixture was centrifuged, and the supernatant was assayed with the venom; it retained 97 ± 4 percent of the activity it possessed before being adsorbed. When extracts from control rats were treated in a similar manner, 58 ± 5 percent of the activity remained in the supernatant, a suggestion that, in this case, about half the activity was generated from prothrombin rather than from precursor.

Although the data presented are based on clotting activities generated after 15 minutes of incubation with venom, the maximum activity was usually generated within a few minutes, and was not decreased by prolonged incubation (> 60 minutes) with the venom. When lower concentrations of the venom were used, the activity was generated more slowly and was less stable. The venom from *Dispholidus typus* also generated clotting activity from the microsomal extract, but not to as great an extent.

The amount of precursor activity (assayed by the extraction procedure used to obtain these data) is a fraction of that in the microsomes themselves. Preliminary investigations (*13*) have shown that detergent treatment of liver microsomes from rats treated with warfarin can release clotting activity equivalent to 400 units of thrombin per liver. The amount of prothrombin which is insensitive to cycloheximide in

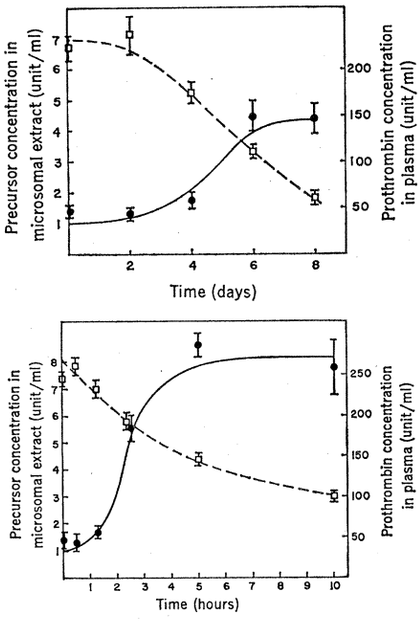

Fig. 1. Concentrations of prothrombin in plasma (dashed line), and of precursor in microsomal extracts of liver (solid line), in normal rats fed a diet deficient in vitamin K (top), or in normal rats after intraperitoneal administration of warfarin (5 mg/kg) (bottom). The data are plotted as the mean of four rats ± the standard error.

a hypoprothrombinemic rat (150 to 200 g) is about 100 units per milliliter of plasma in the first hour after administration of vitamin K (*2, 3*). As the yield of microsomes by the isolation procedure used (*10*) is probably no better than half of that in the cell, it appears that there is sufficient precursor present in hypoprothrombinemic animals to account for the rapid production of prothrombin seen after administration of vitamin K.

The data presented are consistent with the following mechanism of action of vitamin K. A precursor protein is produced in the liver, which is converted to prothrombin in a step requiring vitamin K. In the rat, at least, the absence of the vitamin, or the presence of antagonists of the vitamin, causes an increase in the amount of this precursor. In the cow (*4, 5, 14*) and human (*7, 15*) this precursor, or probably some further modification of it, is released into the plasma as an abnormal prothrombin; the situation in the rat is less clear. There is evidence to support the existence of such a protein (*16*), but other investigators (*17*) have failed to find an appreciable amount of an abnormal prothrombin that is antigenically active in the rat. The concentration of liver precursor prothrombin in species where an abnormal prothrombin is released into the plasma also is not known.

Normal and abnormal prothrombin differ in their electrophoretic mobility in the presence of calcium ions, and we have shown (*18*) that the major physical difference between the purified bovine abnormal prothrombin and bovine prothrombin is in the inability to bind calcium ions in solution, or to bind to insoluble barium salts. These observations suggest that the step sensitive to vitamin K involves the attachment of some unrecognized prosthetic group, or the modification of some amino acid residues, to form the metal binding sites on the precursor.

J. W. SUTTIE

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison 53706

References and Notes

1. L. F. Li, R. K. Kipfer, R. E. Olson, *Arch. Biochem. Biophys.* **137**, 494 (1970); R. E. Olson, in *The Fat-Soluble Vitamins*, H. F. DeLuca and J. W. Suttie, Eds. (Univ. of Wisconsin Press, Madison, 1970), pp. 463–489; M. F. M. Johnson and R. E. Olson, *J. Biol. Chem.* **247**, 4001 (1972).
2. R. G. Bell and J. T. Matschiner, *Arch. Biochem. Biophys.* **135**, 152 (1969); R. B. Hill, S. Gaetani, A. M. Paolucci, P. B. Ramarao, R. Alden, G. S. Ranhotra, D. V. Shah, V. K. Shah, B. C. Johnson, *J. Biol. Chem.* **243**, 3930 (1968); J. W. Suttie, *Arch. Biochem. Biophys.* **141**, 571 (1970).
3. D. V. Shah and J. W. Suttie, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 1653 (1971).
4. J. Stenflo, *Acta Chem. Scand.* **24**, 3762 (1970).
5. A. Schieck, F. Kornalik, E. Habermann, *Naunyn-Schmiedebergs Arch. Pharmacol.* **272**, 402 (1972).
6. G. L. Nelsestuen and J. W. Suttie, *Fed. Proc.* **31**, 217 (abstr.) (1972); *J. Biol. Chem.*, in press.
7. F. Josso, J. M. Lavergne, M. Gouault, O. Prov-Wartele, J. P. Soulier, *Thromb. Diath. Haemorrhag.* **20**, 88 (1968).
8. J. Lowenthal, in *The Fat-Soluble Vitamins*, H. F. DeLuca and J. W. Suttie, Eds. (Univ. of Wisconsin Press, Madison, 1970), pp. 431–446.
9. V. C. Metta, L. Nash, B. C. Johnson, *J. Nutr.* **74**, 473 (1961); M. S. Mameesh and B. C. Johnson, *Proc. Soc. Exp. Biol. Med.* **101**, 467 (1959).
10. The livers were removed, minced, and homogenized in three volumes of 0.25*M* sucrose in a tight fitting glass-Teflon homogenizer for 30 seconds at 1500 rev/min. The homogenate was centrifuged for 10 minutes at 12,800*g*, and the supernatant was filtered through cheesecloth, and centrifuged for 60 minutes at 105,000*g*. The microsomal pellet from 12.5 ml of filtered supernatant was suspended in a Dounce homogenizer in 2 ml of nine parts of Krebs-Ringer-bicarbonate buffer, free of calcium, *p*H 6.9, and one part of 0.15*M* potassium oxalate. This suspension was sonicated for 2.5 minutes in a 20-kcycle Branson sonic oscillator, and was then centrifuged for 45 minutes at 105,000*g* to obtain the microsomal extract that was assayed. All steps were carried out at 0° to 4°C.
11. D. V. Shah and J. W. Suttie, *Arch. Biochem. Biophys.* **150**, 91 (1972).
12. S. S. Shapiro and D. F. Waugh, *Thromb. Diath. Haemorrhag.* **16**, 469 (1966).
13. D. V. Shah and J. W. Suttie, unpublished observations.
14. O. P. Malhotra, *Nature New Biol.* **239**, 59 (1972).
15. P. O. Ganrot and J. E. Nilehn, *Scand. J. Clin. Lab. Invest.* **22**, 23 (1968); H. C. Hemker, A. D. Muller, E. A. Loeliger, *Thromb. Diath. Haemorrhag.* **23**, 633 (1970); K. W.

E. Denson, *Brit. J. Haematol.* **20**, 643 (1971).
16. H. V. Johnson, J. Martinovic, B. C. Johnson, *Biochem. Biophys. Res. Commun.* **43**, 1040 (1971); H. V. Johnson, C. Boyd, J. Martinovic, G. Valkovich, B. C. Johnson, *Arch. Biochem. Biophys.* **148**, 431 (1972); M. Pereira and D. Couri, *Biochim. Biophys. Acta* **237**, 348 (1971).
17. J. J. Morrissey, R. K. Kipfer, R. E. Olson, *Fed. Proc.* **31**, 218 (abstr.) (1972); G. A. Grant and J. W. Suttie, unpublished observations.
18. G. L. Nelsestuen and J. W. Suttie, *Biochemistry*, in press.
19. *Echis carinatus* venom was obtained from Sigma, and *Dispholidus typus* venom was a gift from D. Aronson. The vitamin K was a colloidal solution of phylloquinone [Aqua-Mephton (Merck Sharp & Dohme)], and chloro-K was synthesized from 2-chloro-1,4-naphthoquinone provided by J. Lowenthal. It was emulsified in Tween-80 before being used. Supported by the College of Agricultural and Life Sciences, University of Wisconsin, and in part by NIH grant AM-14881.

5 July 1972; revised 25 October 1972

Water Excretion by Hydra

Abstract. *Hydra were cut so that regenerates consisting only of the central gastric region were formed. This region, which has no natural opening to the environment, is capable of osmoregulation and of removing excess fluid from the gut. The fluid is excreted through a break in the body wall created as a result of a strong contraction when the gut is distended with fluid. A normal hydra, therefore, must remove excess fluid by contracting and expelling it through its mouth.*

Previous experiments suggested that osmotic regulation and volume regulation in hydra involved the active transport of sodium from the external medium into the cells and into the gut in one or more steps (*1*, *2*). It was suggested that water followed passively. However, we were unable to demonstrate in any of these previous experiments how water leaves the animal. It was suggested that water leaves through the mouth, largely because histological studies have demonstrated neither pores nor contractile vacuoles in hydra, and there are no openings to the outside with the exception of the mouth and possibly the aboral pore. However, a regenerating section of an animal lacking a mouth regulates just as well as an intact animal with a mouth (*3*). There is a resting potential across the epithelium of a regenerating animal similar to that of normal animals, indicating that sodium transport is similar in intact and regenerating animals. Thus, hydra regenerates and intact animals are continually transporting sodium from the environment into the gut, and water passively follows.

It was suggested that water enters a hydra by diffusive flow but leaves by bulk flow through the mouth (*1*, *4*). Indeed, it is well known that hydra eliminate waste material after digesting prey by contracting and expelling it out of their mouths (*5*). However, the question remains as to what happens in a regenerate which has no obvious route for bulk flow of water. To answer this question, we began a series of experiments in which various dyes were electrophoretically injected into the enteron of *Hydra pseudoligactus* and *H. oligactus* regenerates, reared as described previously (*1*), 24 hours after cutting.

In virtually every experiment in which dye was injected into the enteron of an animal, the animal was observed to burst. After continual failure, it became apparent that we were observing a normal mechanism.

Twenty-four hours after the animal is cut, the regenerate is turgid and quite transparent due to distention of the body wall. By careful observation of regenerates we noted that, 24 to 28 hours after cutting, the animals would contract repeatedly. Eventually a sufficiently strong contraction caused the body wall to burst, and this was accompanied by an ejection of cellular debris and fluid from the enteron (Fig. 1). Upon relaxation, the turgid appearance was gone and the enteron was reduced in volume. The area where the ejection of solids and liquids occurred as a result of bursting was often at the tip of the animal, but occasionally bursting occurred on the side. In 48 to 72 hours the regenerates had formed normal small hydra, which were able to expel fluid through their mouths. If gastric region regenerates were placed in distilled water 24 hours after cutting similar results were observed (Fig. 2). The contraction and bursting of the animal were very rapid after it was placed in distilled water because of the rapid influx of water with the increased osmotic gradient. In addition, when the animals were placed in distilled water, body cells became notice-

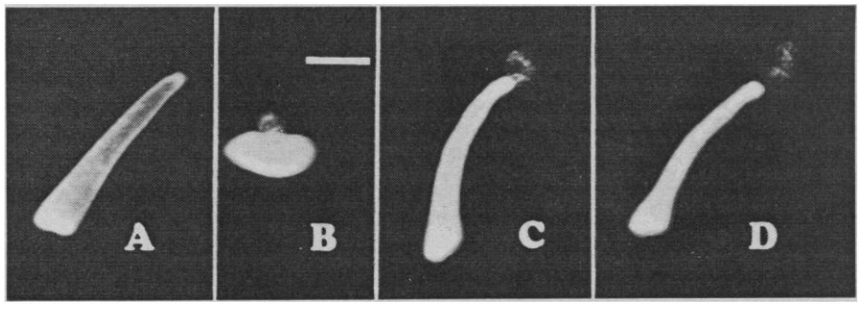

Fig. 1. *Hydra pseudoligactus* regenerate voiding fluid in normal culture water 24 hours after cutting. The turgid, fluid-filled animal (A) contracts until a sufficiently strong contraction (B) causes the body wall to burst. Cellular debris which left the regenerate along with excess fluid can be seen adjacent to the animal. Upon relaxation (C, D) the enteron has returned to a normal volume. The four photographs represent a time span of 15 minutes. Scale line, 1 mm.

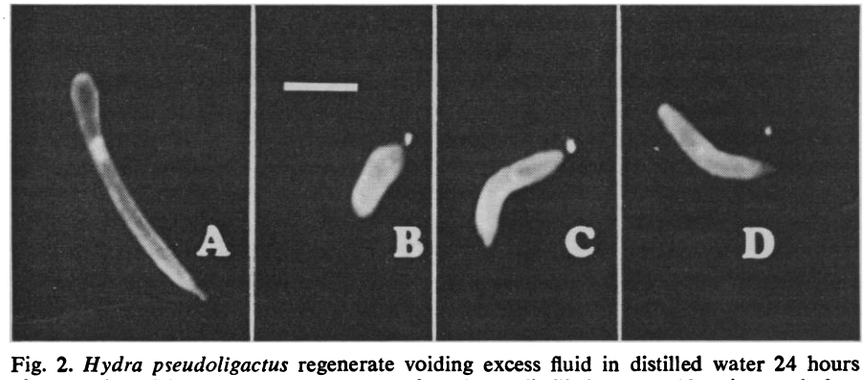

Fig. 2. *Hydra pseudoligactus* regenerate voiding excess fluid in distilled water 24 hours after cutting. The regenerate was transferred to distilled water 10 minutes before it burst (B). The photographs are in temporal order and show the same events as Fig. 1. Scale line, 1 mm.