CORRECTION OF DEFECTS IN CLOTTING ACCELERATOR ACTIVITY BY ADMINISTRATION OF METHIONINE AND VITAMIN K AND OF A NEW SULFHYDRYL-SUBSTITUTED METHYLNAPHTHOQUINONE, VITAMIN K-S(II) [1, 2]

By JOHN R. CARTER and E. D. WARNER

(From the Department of Pathology, State University of Iowa, Iowa City, Iowa)

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Recent studies on the role of sulfur-containing linkages in blood coagulation have contributed to the growing fund of knowledge of the biochemistry of prothrombin, its derivatives and activation (1–5). It is the purpose of this paper to present the salient data of a related series of investigations, extending over a nine-year period, which have resulted in 1) the discovery of a synergistic effect of DL-methionine and vitamin K on accelerator activity in the transformation of prothrombin to thrombin in both normal and pathological states; 2) the design, preparation, experimental and therapeutic trial of S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid, designated as vitamin K-S(II), a compound comparable in its effect on accelerator activity to the concomitant administration of methionine and vitamin K; and 3) the complete correction of both factor V and factor VII acquired deficiencies in two human cases and of the hemorrhagic manifestations associated with them by these compounds.

To minimize confusion, we have chosen to employ terminology and concepts in current usage with respect to the presumed separate identities of factor V and factor VII. Lewis and Ware (6) have challenged this assumption, presenting evidence that probably there is only one fundamental accelerator system in human plasma rather than two, and that changes in accelerator activity may result from alterations of the prothrombin molecule. The studies of Seegers, Alkjaersig, and Johnson (7), indicating that factor VII is a derivative of prothrombin (autoprothrombin I), and our own quantitative studies on –S–S– bonds common to purified bovine prothrombin and its derivatives, support the hypothesis that chemical alterations of a single complex glycoprotein molecule may exhibit prothrombin, thrombin or accelerator activity (4). The term "accelerator," as used in this paper, will refer to the total clotting accelerator activity of the plasma as measured by the method indicated below.

METHODS

Venous blood was collected in 1.85 per cent potassium oxalate in standard glassware using the two-syringe technique. The oxalated plasma was quick frozen and stored at −30° C. for at least one hour before being used in clotting studies. The thromboplastin used in all assays, except the prothrombin time, was a stable, potent, heat treated, centrifuged saline extract of beef lung which was free from prothrombin, thrombin and accelerator activity (8). Prothrombin concentration was estimated quantitatively by the modified two-stage method (9). 'Prothrombin activity' was determined by a one-stage method which consisted of incubating 0.1 ml. crude saline extract of beef lung thromboplastin, 0.1 ml. plasma and 0.1 ml. 0.025 M $CaCl_2$ solution at 37° C. Fibrinogen was determined by a semimicro-Kjeldahl method on the washed fibrin clot resulting from the addition of thrombin to the plasma.

Clotting accelerator activity was estimated quantitatively by our two-stage method (8). This method, which we have employed since 1950, utilizes three variables: onset of thrombin formation, conversion rate and thrombin yield. During the early years of this study, plasma treated with $HgCl_2$ was used as a source of accelerator-free plasma (10) and no distinction between factor V (Ac-globulin, labile factor, proaccelerin) and factor VII (stable factor, proconvertin, serum prothrombin conversion accelerator) was made. For the past five years, defibrinated oxalated human plasma, to which an additional amount of potassium oxalate is added (2 Gm. per 100 ml.) and which is aged 12 to 14 days at room temperature (11) or 5 to 6 days at 37° C., has been used for this purpose. Appropriately diluted in our test system, this preparation has contained 150 to 200 Iowa units per ml. of prothrombin and neither factor V nor factor VII activity has been detected. The results of accelerator assays using these two different substrates have been strictly comparable. Factor V and factor VII, specifically, were estimated quantitatively by this method also.

[1] Aided by a grant from Parke, Davis and Co.

[2] Presented before the Thirtieth Annual Meeting of the Central Society for Clinical Research, Chicago, November 1 and 2, 1957.

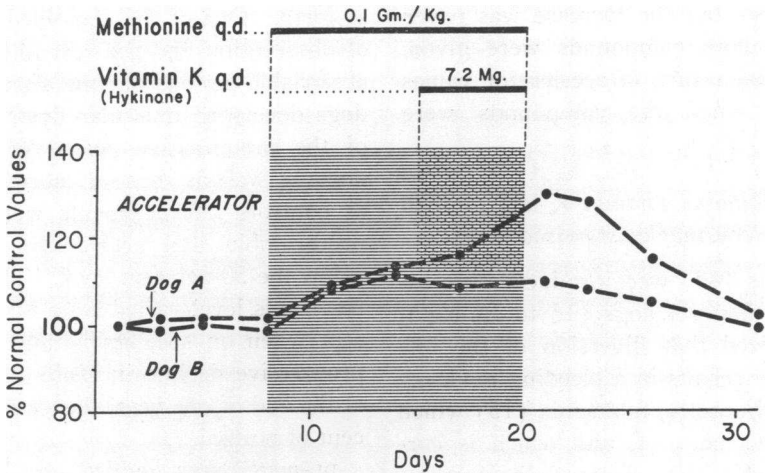

FIG. 1. DATA FROM NORMAL DOGS GIVEN DL-METHIONINE (DOG B) AND DL-METHIONINE PLUS VITAMIN K (DOG A)

Neither aged serum nor $BaSO_4$-adsorbed plasma when added separately to accelerator-free, prothrombin-rich plasma will elicit an accelerator response, but when both are added to the system, accelerator activity occurs equivalent to that of native whole plasma. Thus, both factor V and factor VII must be present to demonstrate accelerator activity by this method. This has been shown previously in test systems using highly purified clotting reagents (4). Prothrombin and factor VII-free plasma are prepared by mixing $BaSO_4$ with the oxalated plasma (100 mg. per ml.) constantly for 30 minutes at room temperature. Factor V-free serum is obtained by aging 48 hours (for human) and not less than 75 hours (for dog, rat) at room temperature. In all accelerator assays, accelerator-free substrate has been of human origin, but factor V and factor VII-free reagents have been species specific.

The standard stock diet for all dogs was Purina dog chow.

ANIMAL EXPERIMENTS

I. Historical

Preliminary experiments, begun in 1947,[3] were designed to test the hypothesis that the rate of regeneration of prothrombin following liver injury might be increased by the concomitant administration of vitamin K and DL-methionine. This was suggested by existing knowledge of the prothrombin-vitamin K relationship, the role of the quinones in oxidation-reduction systems, and especially the protective effect of methionine and cystine against chloroform injury in the protein depleted dog (12).

[3] U. S. Naval Medical School, Bethesda, Maryland.

It was observed that the rate of recovery of prothrombin activity (one-stage) in mature guinea-pigs, rendered hypoprothrombinemic by chloroform, was increased somewhat by the administration of either DL-methionine or vitamin K, but was decidedly more rapid when both methionine and vitamin K were given concomitantly. The prothrombin activity exceeded that of the control animals by as much as 70 per cent at the end of the recovery period. This was greatly in excess of the slight overcompensation generally observed in the normal (untreated) recovery period.

II. Effect of DL-*methionine and vitamin K administration in normal dogs*

Four normal healthy mongrel dogs (18 to 27 Kg.) were fed 0.1 Gm. per Kg. DL-methionine by mouth daily for from 12 to 14 days in addition to the stock diet. During the second half of this two-week period, they were given 7.2 mg. vitamin K (Hykinone®) by vein daily. A typical experiment is shown in Figure 1 (Dog A). The results were qualitatively similar in all four dogs. Prothrombin, fibrinogen and hematocrit values remained essentially unchanged. With the feeding of methionine, accelerator values gradually increased. When vitamin K was given concomitantly with the methionine, the increase became even greater. In three other dogs (*e.g.,* Figure 1, Dog B), in which methionine but not vitamin K was given, there was a gradual, mild rise in ac-

celerator values also, but the increase was never as great as when both compounds were given. There was a gradual return of accelerator values to normal levels when the compounds were stopped.

III. Effect of methionine vitamin K and vitamin K-S(II) in cholecystnephrostomized dogs

Bile-renal fistulas (13) were produced successfully in six adult mongrel dogs (15 to 16 Kg.). It is well recognized that diversion of the bile through the kidney results in a bleeding tendency (14) caused by hypoprothrombinemia (15) which results from a deficiency of, and which is corrected by, vitamin K (16). Except where otherwise indicated, the dogs were maintained on the stock diet. Approximately every six weeks, vitamins A and D were given parenterally. A variety of experiments was performed on each of these dogs in duplicate or triplicate and the period of study in each dog covered not less than one year. During this period, the dogs gradually lost weight and became anemic. Some spontaneously hemorrhaged and several developed decubitus ulcers. These complications were treated symptomatically by transfusions, antibiotics, and so forth. One of the dogs died during the period of observation and the rest ultimately succumbed at variable periods of time after one year. Three dogs developed muscular dystrophy. The values of the preoperative period of the experimental dogs as well as those of normal dogs were used as controls. Experiments were begun after the prothrombin and accelerator values had fallen below the 50 per cent level. The time required to reach this level varied from 80 to 130 days. Except when on specific therapy, there was a slow progressive decline in these clotting values until, at the end of one year, they were from 5 to 20 per cent of normal.

DL-*methionine added to protein-free diet.* Three bile-renal fistula dogs, maintained on the stock diet for approximately 175 days, were placed on the following protein-free diet: 327 Gm. sucrose, 450 Gm. starch, 20 Gm. salt mixture, 200 Gm. Crisco, 1.5 Gm. L-tryptophan, 1.5 Gm. L-cystine, and 2 tablespoons polyvitamin dispersion; total, 1,000 Gm. The dogs were offered 500 Gm. per day for 21 days and consumed an average of 85 per cent of the diet per day. The results were similar and those of one of the dogs are shown in Figure 2. There was a pronounced

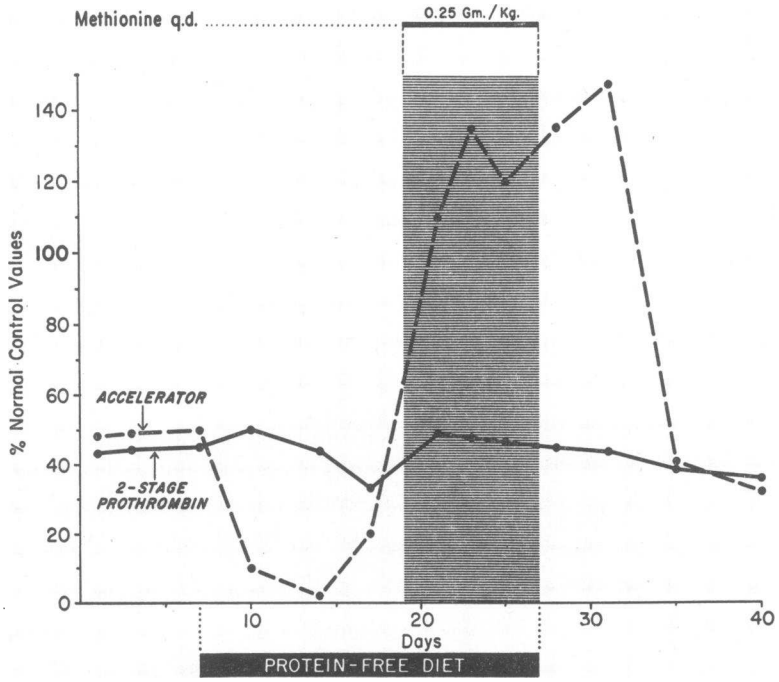

FIG. 2. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS ON PROTEIN-FREE DIET AND PROTEIN-FREE DIET SUPPLEMENTED WITH DL-METHIONINE

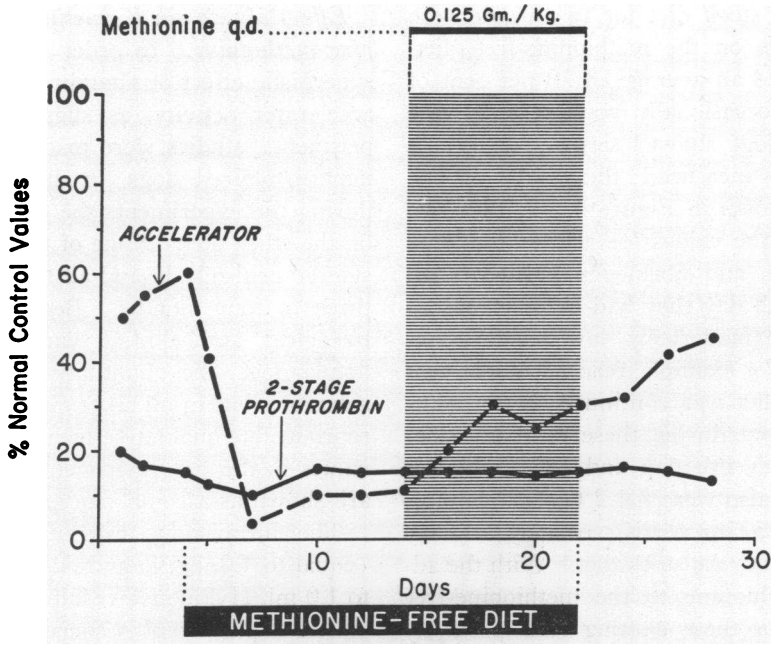

FIG. 3. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS ON DL-METHIONINE-FREE DIET AND DL-METHIONINE-FREE DIET SUPPLEMENTED WITH DL-METHIONINE

decrease in accelerator values on this diet. Two-stage prothrombin and fibrinogen values remained essentially unchanged. With the addition of DL-methionine to the diet (4 Gm. per day for nine days; 0.25 Gm. per Kg.), accelerator values increased rapidly and exceeded those of the control animals. There was a concomitant mild increase of fibrinogen of questionable significance, but again prothrombin values were not affected. With the return of the dogs to the stock diet, accelerator values dropped to the pretreatment level by the eighth postmethionine feeding day. The elevated accelerator activity, which lasted for at least four days after the methionine feeding was stopped, suggested a limited degree of storage. No significant change in hematocrit levels was noted. One-stage prothrombin values tended to parallel those of the accelerator, but the magnitude of change was considerably less.

DL-methionine added to methionine-free diet. After a period of 50 days on the stock diet, two of the same three dogs were placed on the following diet: 24 Gm. methionine-free casein hydrolysate (1,000 ml.), 0.75 Gm. L-cystine, 0.75 Gm. DL-tryptophan, 139 Gm. sucrose, 225 Gm. starch, 10 Gm. salt mixture, 100 Gm. Crisco, and 1 tablespoon polyvitamin dispersion. The dogs were offered 500 Gm. per day for 19 days and consumed an average of 95 per cent of the diet per day. The casein hydrolysate was rendered free from methionine and tryptophan by the method of Albanese (17). The results were similar, and those of one of the dogs are shown in Figure 3. By the fourth day on this diet, accelerator values had fallen from a level of from 50 to 60 per cent to from 3 to 10 per cent of normal, and they remained at this low level for the next eight days. Fibrinogen remained at essentially normal levels; one- and two-stage prothrombin values remained low throughout the test period. With the addition of 0.125 Gm. per Kg. DL-methionine to the diet for nine days, half as much as that given in the previous experiment, there was a moderate, gradual increase of about 20 per cent in accelerator values. Fibrinogen and one- and two-stage prothrombin values remained essentially unaltered. After the return to the stock diet, accelerator values continued to rise slowly. Hematocrit levels were not affected.

DL-methionine added to methionine-free diet in normal dogs. As a corollary experiment to that just preceding, two normal dogs which had been

maintained on the stock diet for two years were placed for 14 days on the methionine-free diet. The dogs consumed an average of 70 per cent of the diet per day, occasionally vomited and were force fed. Both dogs showed similar changes in the clotting factors measured; the results on one of the dogs are shown in Figure 4. Within two days on this diet, the values for accelerator, one-stage prothrombin, and fibrinogen began to fall and by the eleventh day they had become reasonably stabilized at moderately low levels. Accelerator activity, for example, reached 40 per cent of normal. The diet was continued for an additional four days, but during these four days, beginning on the eleventh day and continuing for 13 days, the dogs also were fed 2 Gm. DL-methionine per day (0.125 Gm. per Kg. per day). They then were returned to the stock diet. With the addition of the methionine to the methionine-free diet, the values for these clotting tests promptly returned to essentially normal levels and so remained throughout the methionine supplemented stock diet feeding period. Again, the accelerator response was greatest, the values exceeding those of the normal by about 10 per cent during the period on the methionine supplemented stock diet. Throughout the test period, the two-stage prothrombin and hematocrit values remained unaltered.

Effect of vitamin K, methionine, and vitamin K plus methionine. In order to better evaluate the synergistic effect of vitamin K and methionine on accelerator activity, as suggested by earlier experiments, studies were made on three cholecyst-nephrostomized dogs, in duplicate. Before and during the experiments the dogs were maintained on the stock diet. None of the animals were considered critically ill and all lived for an additional three to four months. Because of the inherent biological and clinical variations in bile-renal fistula dogs, the quantitative results varied considerably, but in all six experiments, a significant reproducible qualitative trend was observed. The values in the six experiments were averaged, and are shown as a composite graph in Figure 5.

The intravenous injection of from 0.17 ml. (equal to 1.0 mg. anhydrous menadione bisulfite) to 1.0 ml. Hykinone® resulted in the expected elevation of prothrombin concentration in all dogs to normal levels. This vitamin K effect often lasted from four to six days, and the subsequent decline of prothrombin was slowly progressive. The accelerator response to vitamin K was slight, seldom greater than a 20 per cent increase, but again rather long-lasting in a few of the experiments. Methionine (4 Gm. per day; 0.25 Gm. per Kg. per day) was then fed for eight days prior to the second injection of vitamin K. This resulted in

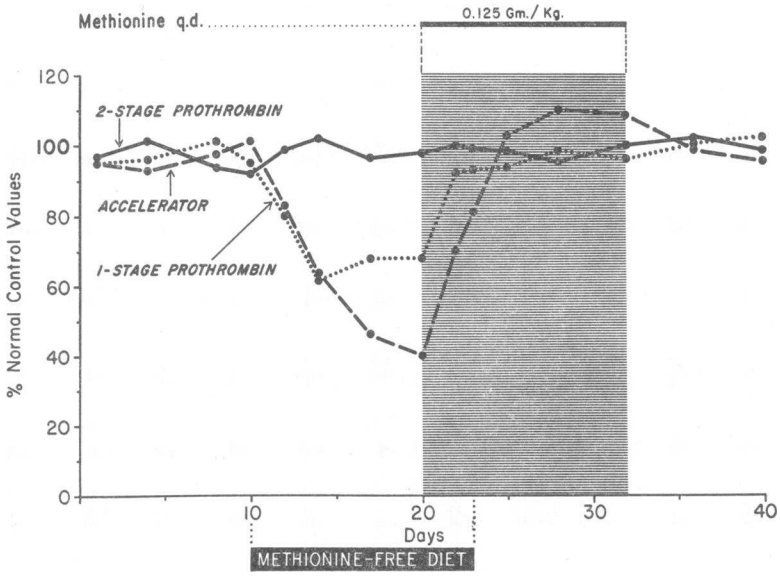

FIG. 4. DATA FROM NORMAL DOGS ON DL-METHIONINE-FREE DIET, DL-METHIONINE-FREE DIET SUPPLEMENTED WITH DL-METHIONINE AND STOCK DIET SUPPLEMENTED WITH DL-METHIONINE

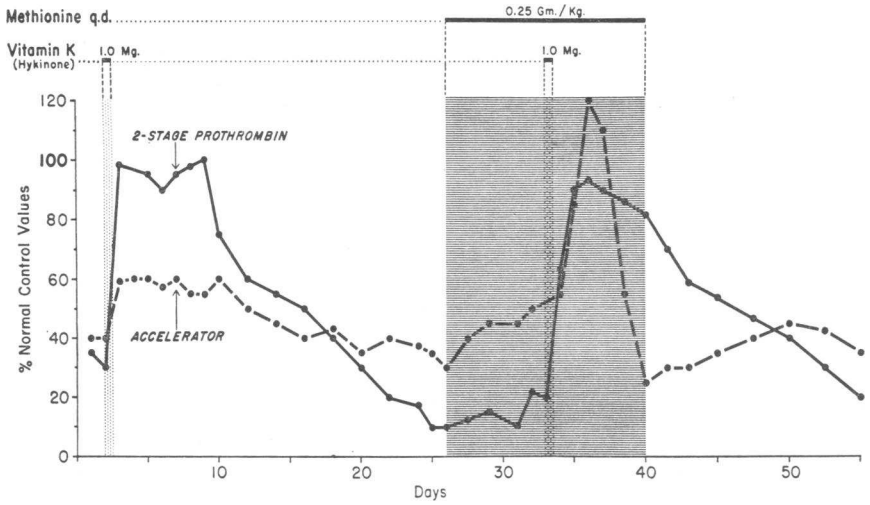

FIG. 5. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS GIVEN VITAMIN K, DL-METHIONINE AND VITAMIN K PLUS DL-METHIONINE

an average increase of 20 per cent in accelerator activity but an essentially unaltered prothrombin concentration. The methionine feeding was continued for an additional eight days. On the eighth day of the 16-day methionine feeding period, Hykinone® again was given intravenously in the same dosage as before. Prothrombin levels again rose and were comparable to those produced by the first injection. Accelerator values in all dogs, in contrast to those obtained when vitamin K alone was given, promptly returned to normal or above normal levels. In spite of continued methionine feeding, the levels were not sustained when the vitamin K was no longer operative. Thus, the biological synergistic effect of DL-methionine and vitamin K on accelerator activity again was demonstrated.

Effect of vitamin K-S(II). On the basis of the preceding experiments, it seemed reasonable to suppose that a sulfhydryl-substituted methylnaphthoquinone might exert a similar effect on accelerator activity. S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid, designated as vitamin K-S(II), was prepared.[4] Although slightly modified by one of us (J.R.C.) to realize better yields, the method of preparation of vitamin K-S(II) has been described by Hanna (18). The compound was dissolved in 1.0 per cent $NaHCO_3$ (1 mg. per ml. concentration), adjusted to pH 7.4, passed through a sintered glass filter and administered intravenously to 4 bile-renal fistula dogs on 10 comparable occasions. The animals were maintained on the stock diet for at least one month prior to each test period of administration of the drug. The results were similar in all

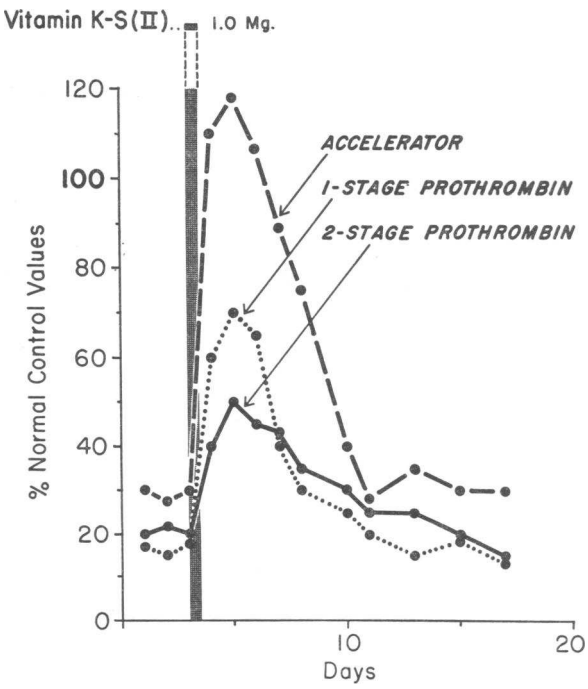

FIG. 6. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS GIVEN A SINGLE DOSE OF VITAMIN K-S(II)

[4] We are indebted to Dr. Calvin Hanna, formerly of the Department of Pharmacology, for the original preparation of vitamin K-S(II).

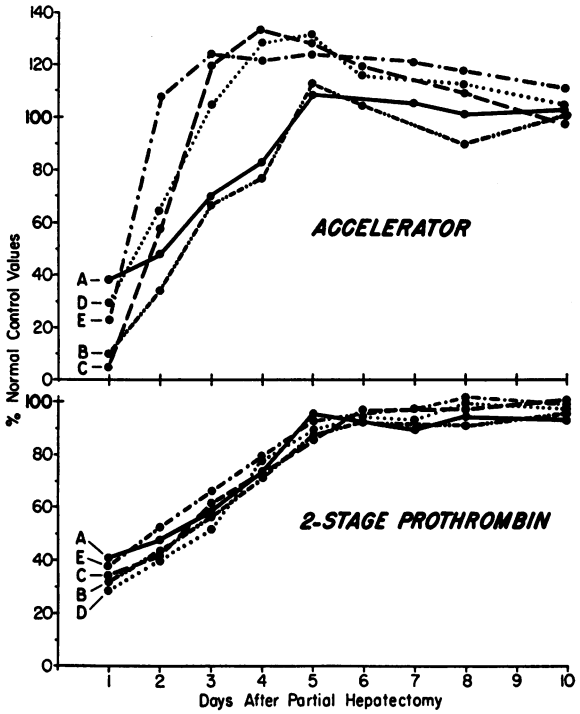

FIG. 7. DATA FROM PARTIALLY HEPATECTOMIZED RATS ON FIVE DIETARY REGIMENS

Curves: A, Controls; B, Vitamin K; C, DL-Methionine; D, Vitamin K plus DL-Methionine; E, Vitamin K-S(II).

10 tests. The values in the 10 experiments were averaged, and are shown as a composite graph in Figure 6. In control experiments with $NaHCO_3$ alone, no detectable alterations of the clotting factors were observed. With a single 1 mg. dose (0.07 mg. per Kg.), accelerator values, previously 30 per cent of normal, promptly returned to normal levels or above, thus equalling or exceeding the response effected by the same dosage of Hykinone® plus methionine. The effect usually lasted two to three days. Prothrombin concentration increased more slowly and never exceeded a 30 per cent rise. The one-stage prothrombin levels reflected the combined accelerator and prothrombin response. Fibrinogen levels were not altered significantly. When 5 mg. quantities of the compound were administered, accelerator activity increased to even higher levels (150 to 160 per cent of normal), but the prothrombin concentration was not increased appreciably above the level effected by the 1.0 mg. dosage. Accelerator and prothrombin values, 10 to 15 per cent of normal, in two dogs in near moribund condition, failed to respond to vitamin K-S(II). Six weeks previously, these clotting factors in the same two dogs had responded to the compound in the manner indicated above.

IV. Effect of vitamin K and/or methionine, and of vitamin K-S(II) on partially hepatectomized rats

Twenty-two adult, partially hepatectomized white rats (Sprague-Dawley) were used in this study. An estimated 70 per cent of the liver was removed aseptically under intraperitoneal Nembutal® anesthesia from which they recovered in approximately 12 hours. None of the rats used developed obvious pulmonary, liver or wound infections. They were offered only water and 20 per cent glucose for the first 24 hours after operation. Beginning with the second 24 hours and continuing throughout the experimental period, they were offered water and the specific diets, as indicated below, which they consumed *ad libitum*. The animals were divided into five dietary groups, the constituent common to all of which was ground Rockland rat ration, adequate in vitamin K. This was supplemented as follows:

A. Controls, five rats; no supplement.
B. Vitamin K, four rats; 1 mg. menadione per Gm. of diet.
C. Methionine, five rats; 1 Gm. DL-methionine per 50 Gm. of diet (2 per cent).
D. Vitamin K plus methionine, three rats; 0.5 mg. menadione per Gm. of diet plus 1 Gm. DL-methionine per 50 Gm. of diet (2 per cent).
E. Vitamin K-S(II), five rats; 1 mg. vitamin K-S(II) per Gm. of diet (three rats); 1 mg. vitamin K-S(II) per Gm. of diet plus 1 ml. (1 mg. per ml. concentration, dissolved in 1 per cent $NaHCO_3$) vitamin K-S(II), daily, intraperitoneally (two rats).

The average values of two-stage prothrombin and accelerator determinations, respectively, in each group of rats during the 10 to 20 day experimental period are shown in Figure 7. With respect to accelerator activity, the recovery rates of the control (curve A) and vitamin K (curve B) groups of rats were similar, approximately 20 per cent of the normal activity per day. Normal values were not obtained until the fifth postopera-

tive day. The recovery rates of the methionine (curve C) and of the vitamin K plus methionine (curve D) groups also were similar, but on these diets the rates were approximately 45 per cent per day, the values returning to normal or above on the third postoperative day. Even more rapid and impressive was the recovery rate of the vitamin K-S(II) (curve E) group, approximately 85 per cent in one day with normal values or above achieved by the second postoperative day, the rats having been on the diet for only 24 hours. The results were essentially the same whether the vitamin K-S(II) was given orally or intraperitoneally and orally. Two of the rats of this group were given 50 mg. vitamin K-S(II) by stomach tube seven days after the accelerator values had returned to normal. There was no additional effect on either accelerator or prothrombin activity.

It is also apparent from Figure 7 that rats on the diets supplemented with methionine and vitamin K-S(II) exhibited rather pronounced hyperaccelerator activity following the recovery phase as compared with that of the control and menadione groups. The overcompensation persisted for 10 days or longer. Factor V and factor VII, specifically, were estimated routinely following partial hepatectomy and until recovery was complete. Barium sulfate-adsorbed plasma and aged serum added separately to the accelerator test system each resulted in only partial correction of the total accelerator activity. The aged serum, however, did effect a decidedly greater correction than did the $BaSO_4$-adsorbed plasma, thus indicating a greater deficiency of factor VII than of factor V. When both reagents were added together to the test system, complete correction was achieved.

The recovery rates of prothrombin concentration (two-stage) in all groups of rats were essentially similar, approximately 15 per cent per day for the first five postoperative days. At the end of this period, the levels were still only 90 per cent of normal, an additional 5 to 10 days elapsing before recovery was complete regardless of diet. Thus, the effect of the dietary supplements on prothrombin concentration, in striking contrast to that on accelerator, was nil. One-stage prothrombin values, routinely determined in all groups of rats throughout the experiment, closely paralleled accelerator rather than prothrombin values with respect to rate of recovery time required for complete restitution and overcompensation.

V. Toxicity studies

Eight healthy mongrel dogs (12 to 16 Kg.) were the recipients, on repeated occasions, of vitamin K-S(II) in doses varying from 100 to 1,000 mg. daily, by mouth, for from 7 to 30 days. The dogs remained clinically healthy at all times. Two dogs were sacrificed after courses of this drug. Grossly and microscopically, no significant anatomical abnormalities were found. Similar studies were made on six normal albino rats, none of which exhibited evidence of toxicity. Clinical evidence of toxicity has not been observed in human patients who have received vitamin K-S(II) orally in doses varying from 50 to 500 mg. daily for from 2 days to 1 year, or intravenously, 20 mg. daily for 10 days.

CLINICAL STUDIES

Results of experimental work had progressed sufficiently by 1950 to suggest application to a man critically ill with a severe hemorrhagic diathesis, refractory to all the usual therapeutic measures. On repeated occasions, low accelerator values were normalized promptly by the concomitant administration of methionine and vitamin K and later by vitamin K-S(II). Clinical improvement with cessation of bleeding was noted on each occasion in spite of a persistent severe hypoprothrombinemia. Because of the complexity of the hemorrhagic disorder which has been studied extensively for six years, only those features relevant to the preceding experimental study will be presented in detail at this time. A second patient is the third recorded example of hypoprothrombinemia and "hypoproconvertinemia" during pregnancy (19, 20), but she had, in addition, a deficiency of factor V. The respective factors were overcorrected by vitamin K-S(II).

Case 1

C. M., a 57 year old male Caucasian house painter, was admitted to the medical service of the University Hospitals July 17, 1950, ***in extremis***. Fatigue, weakness and general malaise had been noted for three months. He had received seven transfusions for severe anemia at another hospital, but two days following the last transfusion, had developed hematomata of the neck and jaw, the right leg and knee and palm of the left hand. Pro-

thrombin was reported to have been 12 per cent; after 300 mg. Hykinone®, the value was 19 per cent. The first evidence of a hemorrhagic tendency had occurred two years before when he bled profusely for 135 hours following a tooth extraction and had noted increased ease of bruising. He was wounded in World War I and had had numerous traumatic episodes, including other tooth extractions, over the years without unusual blood loss. There was no history of any bleeding tendency in his parents, grandparents, siblings, children or other blood relatives. He had never been jaundiced.

One-fourth of the oropharynx was occupied by a bulging submucosal hematoma. Other large hematomata were present in the right peritonsillar area, beneath the right sternocleidomastoid muscle, in both antecubital spaces, on both the dorsal and ventral surfaces of the left hand, and in the subcutaneous tissues of the right thigh. All had occurred spontaneously.

Laboratory studies. The patient was hospitalized until November 8, 1950, a total of 115 hospital days. Throughout this period, on subsequent hospital admissions, and at numerous intervals over the six year period, laboratory studies were made repeatedly. The following were usually *abnormal:* platelets, 60,000 to 100,000 per cu. mm.; clot retraction, none to partial at four hours; coagulation time (Lee-White), 35 plus minutes; serum calcium, 8.0 to 10.6 mg. per 100 ml.; cephalin cholesterol flocculation, 2 plus to 4 plus at 24 and 48 hours. Kolmer, Kline and VDRL tests were positive in 1:2 dilution. He emphatically denied venereal disease, and in view of the low titer and the blood dyscrasia, these were considered false positive reactions.

The following analyses were performed repeatedly: one-stage prothrombin; two-stage prothrombin; clotting accelerator; factor V and factor VII; fibrinogen; circulating anticoagulant (21); heparin (22); protamine titration (23); fibrinolysin (24).

Hospital course, therapy and results. As indicated in Figure 8, large doses of vitamins K (Hykinone®) and K_1, given intravenously, were unassociated with sig-

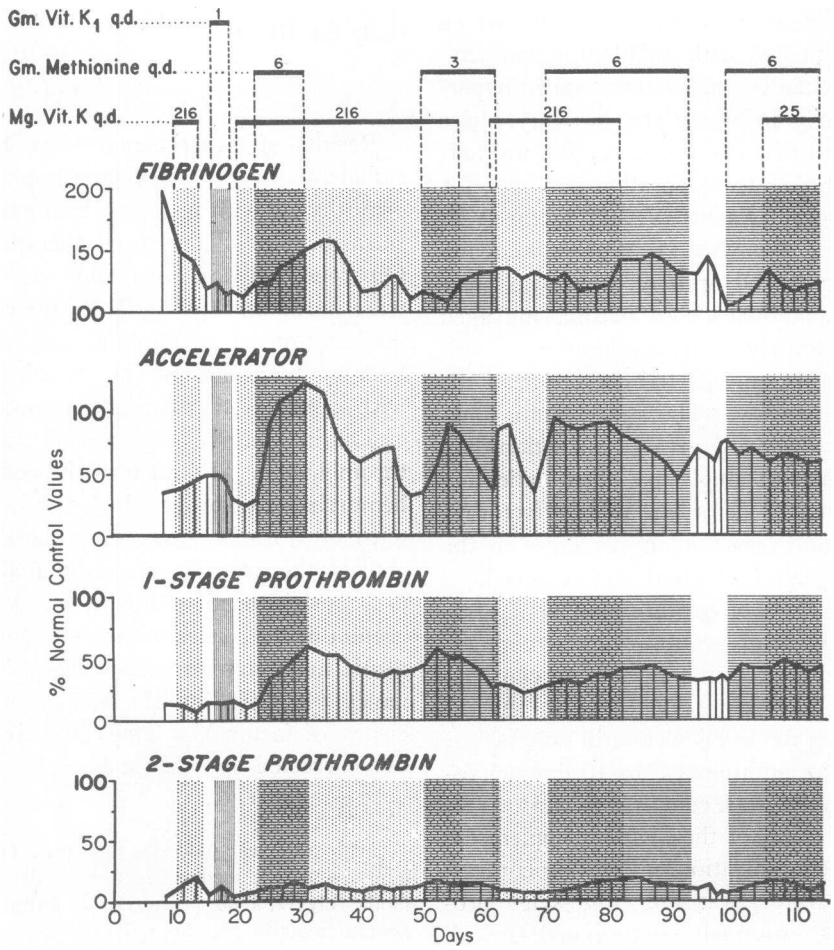

FIG. 8. DATA FROM PATIENT (CASE 1)

Note the accelerator response to the concomitant administration of DL-methionine plus vitamin K.

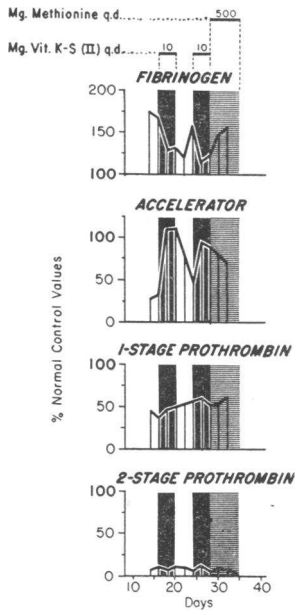

FIG. 9. DATA FROM PATIENT (CASE 1)

Note the accelerator response to intravenous administration of vitamin K-S(II).

nificant changes in prothrombin or accelerator concentration, and bleeding phenomena continued unabated. When, in addition to Hykinone®, DL-methionine was administered orally on the twenty-third day, *a prompt and spectacular rise in accelerator values and a significant elevation of one-stage prothrombin values* resulted. During the remainder of the patient's hospital stay, accelerator repeatedly responded to and could be maintained at near normal levels by the administration of vitamin K plus methionine, but neither compound alone was effective. Usually, a concomitant but decidedly less conspicuous response in one-stage prothrombin activity was obtained. The severe hypoprothrombinemia (two-stage) remained unaltered by the therapy. The initial apparent response of the hyperfibrinogenemia to the vitamin K compounds alone was not as evident in the later trials. Clinically, there was excellent correlation between absence of bleeding, resolution of hematomata and high accelerator levels. At no time, even when accelerator values were normal, was the coagulation time less than 35 minutes and clot retraction was never complete at four hours.

On the seventy-eighth and eighty-seventh days, 800 and 500 ml., respectively, of whole blood were given. These transfusions, some of which were with fresh blood, corrected the progressive hypochromic anemia but were without demonstrable effect on the clotting factors. Protamine (50 mg.) was given on the ninety-sixth day. No appreciable change in clotting factors was noted except that for the next four days fibrinogen values decreased appreciably. Ten days prior to discharge, he was placed on oral vitamin K (Synkayvite®) in addition to the methionine begun six days before. No significant alteration of clotting factors was observed. Lack of accelerator response may have been the result of too small a dose or lack of absorption or utilization of the orally administered vitamin K. He was discharged, greatly improved, on this regimen plus a high methionine diet (milk, meat and eggs).

Progress report. The patient was seen on five occasions at regular intervals from January, 1951, to June, 1952. Methionine and vitamin K were discontinued after January 21, 1951. Throughout this 17 month period, he had remained in good health and free from bleeding episodes. There was a progressive decline in accelerator levels to 30 per cent, with a concomitant rise in fibrinogen from 125 to 215 per cent. Other laboratory values were as stated initially.

Second hospital admission. The patient was readmitted on August 12, 1952, because of a large hematoma of the right elbow area. Several days previously he had noted gross hematuria and blood streaked stools. He appeared chronically ill, anemic, and had lost weight.

Because of the beneficial effect of vitamin K-S(II) on accelerator activity in the cholecystnephrostomized dogs, this compound was dissolved in 1 per cent $NaHCO_3$ (1 mg. per ml. concentration), adjusted to pH 7.4, passed through a sintered glass filter and administered to the patient, intravenously, in dosages and with results shown in Figure 9. Prothrombin concentration was not altered. Ten mg. dosages, on two occasions, promptly normalized the accelerator values. One-stage prothrombin activity gradually increased; fibrinogen, reciprocal with accelerator activity, decreased on vitamin K-S(II) therapy. No toxic reaction was noted. Bleeding (skin, urine, stools) stopped after the administration of vitamin K-S(II). On the thirty-fourth day, he was transfused and discharged on 500 mg. DL-methionine per day and a high caloric diet. He returned one month later. He had felt well, gained weight and had had no bleeding. Laboratory tests were virtually the same as they had been at the time of discharge. He was continued on 500 mg. DL-methionine per day.

Third hospital admission. Two weeks prior to this admission on January 14, 1953, the patient noted increasing fatigability, dyspnea, palpitation, pale complexion, decreased appetite, hematuria and ecchymoses. He had remained on the methionine, but this was stopped on admission. Other than those of the blood and blood clotting tests, the values of repeated laboratory procedures were similar to those given previously. Two blood transfusions (1,000 ml.) which partially corrected the severe hypochromic anemia were given on the third day after admission. These did not alter the low levels of prothrombin and accelerator activity.

On four occasions, vitamin K-S(II) was given orally in dosages and with results indicated in Figure 10. During the administration of the drug, accelerator values were within normal limits; when it was stopped, they returned to the pretreatment range (25 to 35 per cent). The response was prompt and cessation of it equally so, the latter indicating no significant storage or accumulative effect. One-stage prothrombin activity tended to

parallel accelerator activity, but the magnitude of the fluctuations was minimal by comparison. The lack of a more striking response may be accounted for by the exceptionally low, consistently unaffected prothrombin concentration which probably was not the result of a vitamin K deficiency. Fibrinogen values were consistently above normal, but not as high as they had been. No inverse relationship with accelerator activity occurred as had before. During the week prior to discharge, the patient was given six transfusions (3,000 ml.) of whole blood. He appeared clinically improved and was discharged on the one hundred first day to receive 500 mg. vitamin K-S(II), orally, every other day.

Progress report. The patient returned in May, June, and September, 1953. He had felt entirely well and had had no bleeding. Physical examinations were essentially negative. The red cell count and hemoglobin fell very slowly over this period. Other laboratory tests remained essentially unchanged. Accelerator activity remained normal or above; all other clotting factors were as before. Vitamin K-S(II), orally (500 mg. every other day), was continued.

Fourth hospital admission. The patient was readmitted on March 4, 1954, complaining of dyspnea, easy fatigability and weakness of legs which had begun insidiously three weeks before. Prior to this he had felt well and had had no bleeding. He had been taking vitamin K-S(II) as directed until one week prior to admission at which time his supply was exhausted. Physical examination was negative. Moderately severe hypochromic anemia was demonstrated. Accelerator activity was 30 per cent of normal. All other laboratory tests were similar to what they had been. He was started on 500 mg. vitamin K-S(II), orally, every other day. Accelerator values returned to normal in 24 hours, and remained normal; no other clotting factors were affected. The anemia was corrected with transfusions and he was discharged on 100 mg. vitamin K-S(II) every third day.

Fifth hospital admission. The patient remained in good health and without complaints until three weeks prior to admission on December 29, 1954, when he again noted signs and symptoms of anemia. He had experienced no bleeding and had tolerated the drug well. Physical examination was again essentially negative. Again, a moderately severe hypochromic anemia was demonstrated, but all other laboratory tests showed the same abnormalities as before. There was no blood in stools or urine and clinically there was no evidence of bleeding. The patient had been taking 100 mg. vitamin K-S(II) every third day as directed, and his admission accelerator level was 34 per cent. When the dosage was increased to 500 mg. per day, accelerator activity promptly rose to normal levels (95 to 110 per cent) and

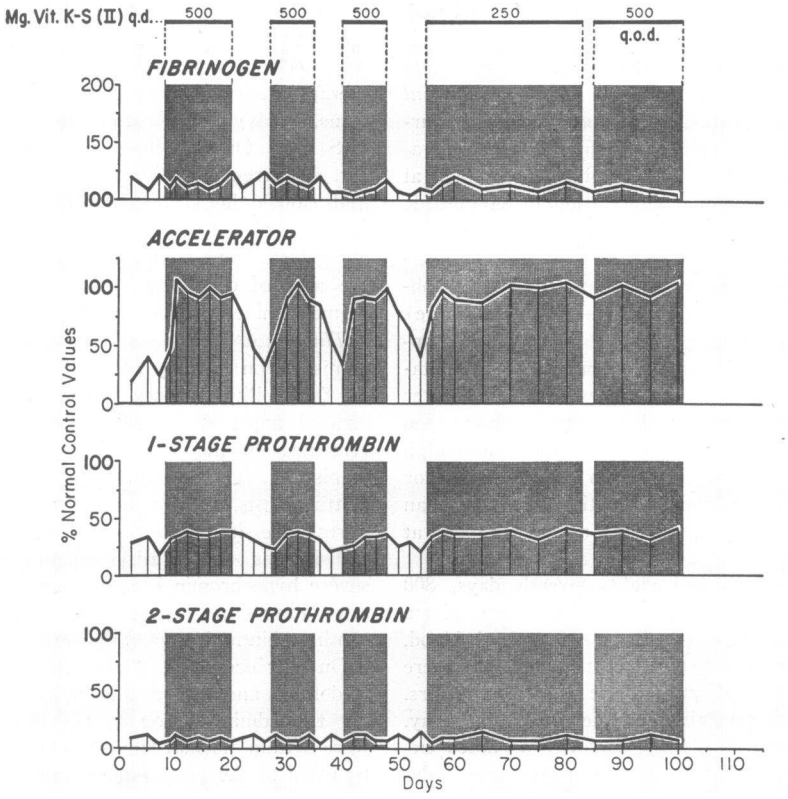

FIG. 10. DATA FROM PATIENT (CASE 1)
Note the accelerator response to oral administration of vitamin K-S(II).

there remained until the drug was stopped one month later (February 4, 1955). Other clotting factors remained at abnormal levels as before.

Progress report. The patient was checked nine times between February, 1955, and December, 1956. He had remained symptomatically well, had had no bleeding and was on no medication except iron salts. Accelerator values ranged from 80 to 100 per cent. He had transfusions in April, 1955, and in August, 1956, to correct his anemia. All other laboratory tests remained essentially the same as on previous admissions. He was seen again in April, 1957, with manifestations of anemia but with no history of recent bleeding episodes. On two separate occasions, low accelerator values (30 and 40 per cent) returned to normal levels following the oral administration of 300 mg. vitamin K-S(II) per day for two days.

Throughout the periods of therapeutic trial with vitamin K-S(II), the dosage varied from 10 mg. intravenously, to 500 mg. orally, daily, both quantities of which resulted in complete correction of low accelerator levels. The minimum effective oral dosage in this patient has appeared to be 300 mg. daily. The difference in magnitude between the oral and intravenous administration may have many explanations, one of which may be poor absorption of the drug from the gastrointestinal tract.

Factor V and factor VII activity. Numerous samples of the patient's plasma, both fresh and frozen (− 30° C.) throughout the six year period, were studied for factor V and factor VII activity. Low accelerator values always could be normalized by the addition of 0.03 ml. normal human $BaSO_4$-adsorbed plasma to the clotting system, whereas a similar quantity of normal 48 hour old human serum never corrected the deficiency, even partially. These results, therefore, indicate a deficiency of factor V, not factor VII, and further indicate that the corrective effect of vitamin K-S(II), as well as of methionine and vitamin K, was on factor V.

Additional studies. Details of the following features of this case will be published separately, but are summarized briefly here. The hypochromic anemia, most marked during the third and fifth admissions, was found to be associated with a moderately severe hemolytic mechanism on both occasions. The hemolytic anemia was considered to be another facet of his hemorrhagic disorder and not related to the therapeutic agents employed. Platelet agglutination studies in February, 1956, suggested the presence of both iso- and autoagglutinins.

An anticoagulant in plasma, but not in aged serum, could be demonstrated easily and in high titer for months at a time, but for other equally long periods none was detected or it was of such low titer as to be equivocal. No correlation between anticoagulant titer and other specific clotting factors, clinical manifestations, diet and therapy could be demonstrated. Using two-stage systems, neither the patient's plasma nor serum altered the prothrombin or accelerator concentration of normal plasma, nor did it alter the reaction rates in these assays. Antithrombin levels were always within normal limits, and no fibrinolysins were ever detected. No evidence could be obtained which would suggest a species specific antithromboplastin in the patient's plasma. Plasma heparin levels were within normal range and protamine titrations indicated no excess endogenous heparin or heparin-like substances in the patient's blood.

Case 2.

M.S., a 22 year old white married woman, one month pregnant, was admitted to the otolaryngology service on February 4, 1955, because of severe epistaxes. Numerous bleeding points were found in both nostrils, and, in spite of the application of both anterior and posterior nasal packs, the bleeding continued. Except for occasional epistaxes at age 11, the patient had never experienced abnormal bleeding or bruising previously. Menstrual blood loss always had been normal. The birth of her first child in 1952 was uneventful; the delivery had been accompanied by a total loss of 325 ml. of blood. There was no familial history of a bleeding tendency. The patient had been in excellent health and there had been no injury to the nose. Initial laboratory studies revealed the following: erythrocytes, 2.88 millions per cu. mm.; hemoglobin, 8.8 Gm. per 100 ml.; hematocrit, 23 per cent; coagulation time, 8 minutes; bleeding time, 6 minutes; platelets, 274,000 per cu. mm.; normal clot retraction; urinalysis, normal. A direct Coombs test was negative and serum bilirubin was 0.3 mg. per 100 ml.

From February 4 to 7, the patient received seven transfusions (3,500 ml. of banked blood) but the bleeding continued, at times profusely. Subsequently, massive nasal hemorrhage occurred, necessitating ligation of the right external carotid artery. On February 7 (fourth hospital day), blood clotting studies were requested; the significant abnormalities are shown in Figure 11. Accelerator, one-stage and two-stage prothrombin values were 10, 30 and 70 per cent, respectively, in spite of the numerous transfusions the patient had received. Prothrombin utilization was decreased. Fibrinogen, antithrombin, clotting time and clot retraction values were normal. There was no evidence of a circulating anticoagulant.

Vitamin K-S(II) (440 mg.) was given by mouth. The following day, after blood had been obtained for clotting tests, an additional 550 mg. was given. The compound was administered in divided dosages and was well tolerated. No transfusions or other drugs were given at this time. The effect was prompt and dramatic; all values had returned to normal levels within a few hours after the last dose of vitamin K-S(II). There was a rather pronounced and sustained hyperaccelerator activity after the drug was discontinued. A similar, but transient and less marked elevation of prothrombin concentration occurred. Prothrombin utilization returned to normal and all other clotting tests remained so. Nasal bleeding stopped completely and never recurred, even when the nasal packs were removed on the seventh hospital day. The patient was seen on six occasions from the time of discharge until May 17, 1955. Clotting tests were normal on each occasion. In December, 1955, she was delivered of her second child without excessive

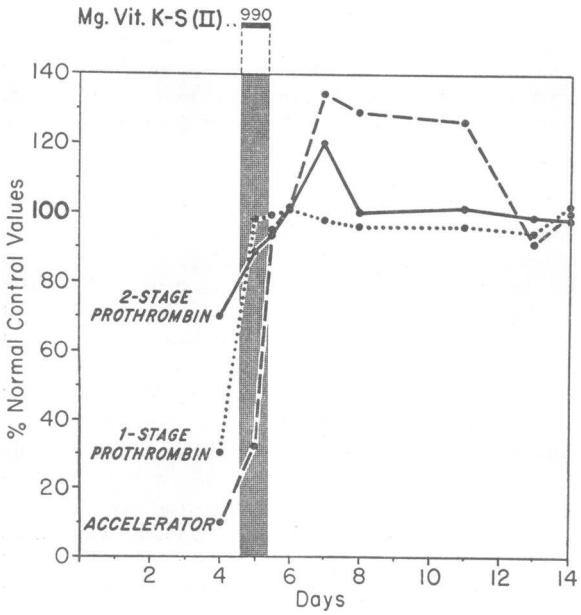

FIG. 11. DATA FROM PATIENT (CASE 2)

Note the accelerator response to oral administration of vitamin K-S(II).

bleeding, and remained in excellent health and free from abnormal bleeding through December, 1956.

Factor V and factor VII activity. Prior to treatment, the accelerator value was 10 per cent of normal. When 0.03 ml. of normal 48 hour old human serum was added to the system, there was a correction to 79 per cent. When 0.03 ml. of normal $BaSO_4$-adsorbed human plasma was added, there was a correction to 48 per cent. When both were added, the result was 96 per cent, indicating that the patient's plasma was noninhibitory to either heterologous factor V or factor VII. Thus, in this patient, a deficiency of both factor V and factor VII, predominantly the latter, existed. Both deficiencies presumably were corrected by vitamin K-S(II)—presumably, because spontaneous recovery, although unlikely, cannot be excluded.

DISCUSSION

The experimental data, accumulated from different animal species and by a variety of techniques, provide convincing evidence that accelerator levels may be increased by the synergistic action of DL-methionine and vitamin K and by vitamin K-S(II). Certain apparent incongruities regarding the accelerator response to methionine in the cholecystnephrostomized dogs deserve comment, however. The accelerator values fell equally when the dogs were on the protein-free (Figure 2) and the methionine-free (Figure 3) diets, but the response to the administered methionine was much more dramatic in the dogs on the protein-free diet. This could have resulted from the difference in the quantity of methionine fed or from the poorer physical condition of the dogs. The accelerator response to methionine alone, prior to the administration of vitamin K (Figure 5), also was by no means comparable to that observed in dogs on the protein-free diet (Figure 2). Since the quantity of methionine administered was the same in these experiments, an explanation for the difference might be the different diets. The stress impact of cholecystnephrostomy with its attendant complications is obviously unphysiological and cannot be ignored in attempting to evaluate these observations. Factor V and factor VII, specifically, were not determined in our bile-renal fistula series of experiments since factor VII was not sufficiently characterized at the time they were conducted. From the work of others (25, 26), we assume that the low accelerator levels in our cholecystnephrostomized dogs were a reflection of factor VII deficiency and that factor V values were presumably normal.

In contrast to the striking response of accelerator, neither fibrinogen nor prothrombin responded to methionine, alone or in combination with vitamin K, in any of the animal experiments except, of course, for the expected prothrombin response to vitamin K in the cholecystnephrostomized dogs. Prothrombin and fibrinogen were unaffected by either an excess or a deficiency of methionine. This suggests that, for short periods of time, exogenous sources of this amino acid are not essential to maintain prothrombin and fibrinogen levels in spite of the fact that amino acid analyses reveal significant quantities of methionine in both (27, 28). One-stage prothrombin activity tended to parallel that of accelerator except when the two-stage prothrombin level was particularly low, in which case the one-stage prothrombin activity usually was depressed.

It is apparent from the clinical data that the simultaneous administration of vitamin K and DL-methionine, and of vitamin K-S(II), had a sustained beneficial effect on accelerator activity. The first case, in addition to the therapeutic aspects, is, in itself, enigmatic and multifacetted. After six years of study, the etiology and pathogenesis are still obscure. Although no blood relatives were available for study, the disorder ap-

parently was acquired and unrelated to obstructive jaundice, severe liver disease, antibiotics, drug sensitivity, lead poisoning, sprue or celiac disease. In parenchymatous liver disease and vitamin K deficiency, both prothrombin and factor VII are decreased, indicating that both substances are formed in the liver and that both require vitamin K for synthesis (29). In this case, factor VII values have been consistently normal and neither vitamins K or K_1 in large doses corrected the clotting abnormalities. The dietary intake was always satisfactory.

The discrepancy between the prolongation of the clotting time of normal plasma by the patient's plasma, and the inability to demonstrate inhibition of prothrombin and accelerator activity by two-stage systems might be explained by the marked difference in plasma dilutions in the tests. Low accelerator values could be normalized repeatedly by normal human $BaSO_4$-adsorbed plasma but never by normal human 48 hour old serum. This not only indicated a deficiency of factor V, instead of factor VII, but also that an inhibitor was not operative against homologous factor V. Obviously, dilution was not a significant variable here. It is pertinent that Hörder has described a case of factor V deficiency in which a congenital familial inhibitor of this factor was demonstrated against purified factor V and factor V activity of normal human and $BaSO_4$-treated cow plasma (30). The patient never exhibited manifestations of hypersensitivity and consistently had a positive cephalin cholesterol flocculation, false positive complement fixation test for syphilis and a circulating anticoagulant. This triad, or just the circulating anticoagulant, not infrequently has been present in cases of systemic "collagen-vascular disease," particularly disseminated lupus erythematosus (31–33).

There have been few reports on the effect of methionine in blood coagulation, and many of these have been contradictory (34–38). The study most closely relevant to our work is that of Shin (39), who showed that the administration of L- and DL-methionine, orally or parenterally, to CCl_4- and $CHCl_3$-treated rabbits resulted in an increase in one-stage prothrombin activity [now generally considered to be a function of factors V and VII as well as of prothrombin (40)]. Vitamin B_{12}, sodium thiolactate and vitamin K had a similar effect. Vitamin K, administered concomitantly with methionine, was not reported, however.

The methionine intake in the normal diet is relatively large (approximately 4 Gm. per day), but doubling or tripling the intake in the form of chemically pure methionine effects a reduction of prothrombin and accelerator values (38). On the other hand, increasing the methionine to only 0.5 Gm. above the normal daily intake, in conjunction with vitamin K, results in an elevation of accelerator values. At first glance, it seems surprising to expect any effect from a 10 to 15 per cent increase of methionine intake above that present in a normal, or especially in a high protein or high methionine diet (Case 1) when digestion and absorption presumably are unimpaired. Methionine, for example, has been shown to have an insignificant nitrogen-sparing effect when the diet already contains large amounts of protein of good methionine content (41). A reasonable explanation for the effect of methionine supplementation is the assumption that the metabolism and end products of the administered methionine, unencumbered by proteins and other amino acids, are different from those of methionine present in natural foods. The relatively slow transformation from whole protein to amino acids and variation in absorption and mass action rates might well be factors which would determine a specific pathway different in the administered methionine from that present in a complex protein moiety.

The mode of action of the combined methionine and vitamin K and of vitamin K-S(II) effect on accelerator activity is unknown. The results obtained with vitamin K-S(II) do, however, substantiate our original hypothesis. It was postulated that since methionine and vitamin K acted synergistically, the mercapto-propyl radical substituted in the vitamin K molecule might be equally effective. Attention is directed to the structural similarity between this radical (SCH_2 CH_2 COOH) and a product of methionine catabolism. Although the mercapto-propyl radical is not one of the recognized cleavage products, recent studies have substantiated the formation of propionic acid in the catabolism of the methionine carbon chain in the rat (42). It might well be that certain true end products of methionine degradation such as

methyl mercaptan (43) might be equally effective or that methionine administered parenterally with vitamin K might be ineffective.

Although proof is lacking, our working hypothesis has been that the organic sulfur in the three-position of vitamin K-S(II) is important for its biological activity, and may account for that manifested by the methionine-vitamin K synergism also. It has been shown that in rats treated with ethionine, there is a marked depression of prothrombin, factor V, factor VII, antithrombin, to a lesser extent fibrinogen (44) and of antihemophilic globulin (45). Since ethionine, the ethyl analog of methionine, is a metabolic blocking agent which interferes with the synthesis of certain proteins, it seems reasonable to assume that this compound may act by competitively inhibiting methionine presumably essential for manufacture or activity of these clotting factors. With respect to accelerator activity at least, in both the experimental and clinical studies, the influence of methionine is apparent and would tend to substantiate the implications deduced from the ethionine experiments. Thus, to speculate one step further, it is possible that vitamin K-S(II) exerts its effect by contributing an essential mercapto-propyl radical as well as by its oxidizing propensity.

The ease with which sulfhydryl compounds may be added to naphthoquinones has been known for many years. Fieser and Turner (46) have prepared many thiol derivatives of 2-methyl-1, 4-naphthoquinone. The thioglycolic acid derivative was said to possess half the antihemorrhagic activity of vitamin K_1. In Case 1, prothrombin was refractory to all forms of therapy. In Case 2, both prothrombin and accelerator responded promptly to vitamin K-S(II). Although likely, it is not known whether these factors in this second case would have responded just as effectively to vitamin K_1. The etiology of the probable avitaminosis K in Case 2 is unknown. In any event, from both our experimental and clinical studies, it is apparent that vitamin K-S(II) does have some beneficial effect on prothrombin in vitamin K deficiency states.

SUMMARY

1. The accelerator response in blood coagulation to a new methyl naphthoquinone in cholecystnephrostomized dogs and in partially hepatectomized rats was striking. This compound, designated vitamin K-S(II), is S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid.

2. Administration of DL-methionine to normal dogs and rats effected a consistent but mild elevation of accelerator levels, but DL-methionine plus vitamin K always evoked an even greater response.

3. DL-methionine and vitamin K, administered separately to cholecystnephrostomized dogs, resulted in a moderate rise of accelerator levels; when given concomitantly, hypernormal levels were achieved.

4. Accelerator levels in cholecystnephrostomized dogs on protein-free and methionine-free diets became almost negligible. With the addition of DL-methionine to the diets, they promptly returned to normal on the former and gradually increased on the latter. A similar qualitative response was observed in normal dogs on the methionine-free diet.

5. In partially hepatectomized rats, the rate of recovery of accelerator activity was decidedly faster, and the time required to attain normal levels or above, two days less on methionine-supplemented diets than on the stock or menadione-supplemented diet.

6. A patient with a multifacetted hemorrhagic disorder, studied for six years, is presented. The etiology and pathogenesis remain obscure. The principal features included severe hemorrhagic manifestations, prolonged clotting times, hypoprothrombinemia, accelerator (factor V) deficiency, thrombocytopenia, hyperfibrinogenemia, a circulating anticoagulant, transient hypocalcemia, hemolytic anemia, elevated cephalin flocculation and false positive complement fixation tests. DL-methionine and vitamin K, administered concomitantly, repeatedly exerted a corrective effect on accelerator levels in this patient whereas fresh and banked blood and massive doses of vitamins K and K_1 had no effect. The synergistic effect was sustained so long as both methionine and vitamin K were given; neither one alone was capable of eliciting this response, nor once achieved, of maintaining it for long periods.

7. Vitamin K-S(II) similarly exerted a corrective effect on accelerator activity in this same patient.

8. The third recorded case of hypoprothrombinemia and "hypoproconvertinemia" in pregnancy is presented. This patient, in addition, had a factor V deficiency. All of these deficiencies were corrected by vitamin K-S(II).

9. Hemorrhagic manifestations disappeared completely when the patients were treated with the above compounds, and the correlation of this therapy with normal accelerator levels and freedom from bleeding in Case 1 was impressive.

10. In contrast to the effect of vitamin K-S(II) on accelerator activity, that on prothrombin was negligible except in vitamin K deficiency states.

11. Both methionine and vitamin K, and vitamin K-S(II) were well tolerated and no toxic manifestations were encountered.

CONCLUSIONS

Analysis of the experimental and clinical data indicates that accelerator activity in blood coagulation is influenced by the concomitant administration of methionine and vitamin K and by vitamin K-S(II). These compounds would appear to be useful, at times even specific, therapeutic measures in the treatment and management of accelerator deficiencies. Determination of the extent of applicability of these measures must await further clinical trial.

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