

Prothrombin Activity in Rats with Mammary Tumors*

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(Received for publication October 4, 1947)

The status of hepatic function can be altered by diverse influences. Multiple functional processes are measurably affected by several extrinsic toxic agents (typhoid toxin, chloroform, yellow phosphorus) as well as by a large number of pathological disorders. After exposure to toxins, in obstructive jaundice, in cirrhotic processes, in intrahepatic tumors and the like, either anatomical disruption or functional impairment may first be observed, but generally the two are inseparable.

We have recently reported that only after extensive replacement of normal liver tissue by large hepatic tumors induced in rats by *p*-dimethylaminoazobenzene is the normal capacity of the liver to elaborate prothrombin reduced (4). This condition is not corrected by vitamin K. Furthermore, the anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin),¹ caused a more severe and persistent hypoprothrombinemia in rats with primary hepatic tumors than in normal rats. The purpose of the present report is to indicate that a gross increase in normal hepatic tissue has been observed in rats with spontaneous mammary tumors and that this condition is associated with a greater functional reserve as evidenced by increased plasma prothrombin activity and a reduced hypoprothrombinemic response to a standard dose of 3,3'-methylenebis (4-hydroxycoumarin).

METHODS

The basic technics employed as well as the rationale of handling the animals are given in pre-

*Published with the approval of the director of the Wisconsin Agricultural Experiment Station. This work was supported through special grants from the Wisconsin Alumni Research Foundation.

This paper represents a phase of the studies on the anticoagulant 3,3'-methylenebis (4-hydroxycoumarin) carried out in the laboratory of Prof. Karl Paul Link (see the Harvey Lectures Series XXXIX, 162-216. 1943-1944. The author wishes to acknowledge his indebtedness to Professors Link and C. A. Baumann for their counsel and guidance.

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¹Available to the clinician under the trademark *Dicumarol*.

vious publications (4, 11). The rats were fed the semi-synthetic diet for at least 6 days prior to the initial determination of prothrombin activity. They were fasted for 12 hours and fed 2.5 mgm. of 3,3'-methylenebis (4-hydroxycoumarin) contained in 2 gm. of food. Twenty-four hours after the anticoagulant was consumed, blood samples were taken by heart puncture under light ether anesthesia. The clotting time of 12.5 per cent plasma was determined by our standard procedure (1). Determinations of prothrombin activity were routinely made on whole plasma. However, since any changes in prothrombin activity² are readily detected and reproducibly determined by the diluted plasma technic, and are frequently obscured in the whole plasma determination by the inherent relatively large technical variation, the presentation of data is restricted to results obtained with 12.5 per cent of plasma.

Tumors.—Healthy rats with grossly visible spontaneous mammary tumors in various stages of development were provided in generous numbers from the stock colonies of Sprague-Dawley, Inc., Madison. Some confirmatory trials and analyses were performed on rats of the Wistar strain with spontaneous mammary tumors raised in the stock colony of the Rochester laboratories. In every case the tumor was examined by gross dissection, and sections stained with hematoxylin-eosin were classified by microscopic survey. The tumors were single or multiple, and in some rats the multiple tumors arose from widely separated mammae. They were discrete, well encapsulated, and varied in diameter from 2 to 8 cm. Characteristically, they showed no evidence of penetration into subcutaneous tissues. Predominantly, they were of the dense fibroadenoma variety with a minimum of duct and acinar proliferation amongst a diffuse connective tissue

²The effectiveness of the diluted plasma technic in revealing increased prothrombin activity (hyperprothrombinemia) has been discussed in Field, J. B., Larsen, E. G., Spero, L. and Link, K. P. Studies on the Hemorrhagic Sweet Clover Disease XIV. Hyperprothrombinemia Induced by Methylxanthines and its Effect on the Action of 3,3'-Methylenebis (4-hydroxycoumarin). *J. Biol. Chem.* 156, 725-737 (1944).

matrix. There was no histological evidence of malignancy, degeneration, or edema.

EXPERIMENTAL

Prothrombin activity of plasma from normal and tumor-bearing rats.—The prothrombin time of 12.5 per cent plasma from normal rats ranged from 34 to 46 seconds, average 39 (Table I). Only 4 of 360 samples (0.9 per cent) clotted in less than 35 seconds. The prothrombin time of the plasma from rats with mammary tumors ranged from 31 to 41 seconds, average 35. Of 38 samples from tumorous rats, 17 (44 per cent) clotted in less than 35 seconds. It has been repeatedly emphasized that this decrease in average clotting time probably represents an appreciable increase in prothrombin since in this particular time range of the plasma (prothrombin) dilution curve, a large change in prothrombin concentration produces a relatively small change in the clotting time³ (1).

TABLE I: PROTHROMBIN TIME OF 12.5 PER CENT PLASMA FROM NORMAL RATS AND RATS BEARING MAMMARY TUMORS

	Prothrombin time Average Range seconds	No. of rats	Animals below normal, per cent*
Normal rats	39 34-46	360	0.9
Rats bearing spontaneous mammary tumors	35 31-41	38	44.0

* This lower limit of "normal" plasma was arbitrarily considered to be 35 seconds.

The mere presence of a neoplasm in the animal does not of itself affect the prothrombin levels (4).

Induced hypoprothrombinemia.—Twenty-four hours after the administration of 2.5 mgm. of the anticoagulant a definite and readily measured hypoprothrombinemia was observed in all normal rats (Table II). However, the hypoprothrombinemia

TABLE II: INDUCED HYPOPROTHROMBINEMIA IN NORMAL RATS AND RATS BEARING MAMMARY TUMORS
Prothrombin time of 12.5 per cent plasma 24 hours after the administration of 2.5 mgm. of 3, 3'-methylenebis (4-hydroxycoumarin).

	Prothrombin time Average Range seconds	No. of rats	Animals below normal, per cent*
Normal rats	116 78-215	463	4.8
Rats bearing spontaneous mammary tumors	80 50-135	29	72.4

* The lower limit for "normal animals" receiving this dosage of anticoagulant was arbitrarily considered to be 85 seconds.

induced by the anticoagulant in rats with mammary tumors was neither extensive nor consistent. The average prothrombin time of plasma from normal rats was 116 seconds with 22 of 463 samples (4.8 per cent) showing a prothrombin time of less than 85 seconds. In contrast, the prothrombin time

³ Field, J. B., Scheel, L., Baumann, C. A., and Link, K. P. Unpublished data.

from rats with mammary tumors averaged 80 seconds with samples from 21 of 29 rats (72.4 per cent) giving prothrombin times of less than 85 seconds.

There was a wide variation in the degree of hypoprothrombinemia that the standard dose of anticoagulant induced in individual rats with mammary tumors. But in most of the tumorous rats the hypoprothrombinemic response obtained from testing with a standard dose of anticoagulant after successive resting periods of 2 to 3 weeks was progressively reduced. Thus a typical illustration of the increasing resistance to induced hypoprothrombinemia taken from the protocol of a rat with a medium sized tumor over an 11 week period is 113, 89, 80 seconds. However, there was no fixed correlation between the size or number of developing mammary tumors and the resistance to induced hypoprothrombinemia. In some rats with relatively large tumors a progressive debilitation in general health tended to interfere with such resistance and to increase the degree of hypoprothrombinemia.

Fibrinogen levels of tumorous rats.—Deutsch and Gerarde (3) have recently indicated their belief that the hyperprothrombinemic states, indicated in previous reports from this laboratory (6), reflect primary elevation in fibrinogen levels. The latter condition is usually elicited by the methylxanthine drugs (8) and only rarely by vitamin K (5). However, the fibrinogen levels of rats with mammary tumors as determined by standard procedures (9, 13) averaged 183 mgm. per cent, compared to an average of 274 mgm. per cent for non-tumorous control rats.

Effect of surgical excision of tumors.—After the individual prothrombin activity and degree of induced hypoprothrombinemia had been well established in rats with 1 or 2 mammary tumors, the latter were excised. In a brief manipulation under ether anesthesia accomplished with little bleeding, the well encapsulated masses were readily identified and removed. Repeated determinations of prothrombin activity and the hypoprothrombinemia induced by the anticoagulant were made at intervals of 7 to 10 days. A summary of data from animals surviving 61 days postoperatively and from whom several repeated values were obtained, is given in Table III. Whereas the prothrombin time of 12.5 per cent plasma of this group of rats was 34 seconds in the presence of the tumor, the prothrombin time was 38 seconds following excision of the tumor (compared to an average of 39 seconds in non-tumorous normal rats). The prothrombin time of 12.5 per cent plasma 24 hours after a stand-

ard dose of the anticoagulant was 70 seconds prior to the operation and following excision averaged 106 seconds (compared with 116 seconds in normal rats). The response to the anticoagulant immediately after excising the tumor was often temporarily irregular for 2 to 4 weeks, but ultimately the degree of hypoprothrombinemia induced in 13 of 15 rats was that characteristic of non-tumorous rats. The hypoprothrombinemia elicited in 2 of the rats indicated a persisting resistance for 9 weeks postoperatively.

Liver and pituitary weights.—A survey was made of the weights of pertinent organs from healthy rats

23 mgm. As indicated in Table IV, there was a rough correlation between the weight of the mammary tumor and the size of the liver and pituitary gland. In animals possessing more than one tumor the pertinent organ weights resembled those from animals with a single tumor of the same weight as the weight of the combined tumors. Following excision of the tumors the liver and pituitary weights apparently regressed to values characteristic of the control females (Table III). At death, the livers averaged only 2.5 per cent (fresh weight) of the body weight while the pituitary gland averaged 14.4 mgm.

TABLE III: PROTHROMBIN TIME AND INDUCED HYPOPROTHROMBINEMIA IN RATS BEARING MAMMARY TUMORS BEFORE AND AFTER SURGICAL EXCISION OF THE TUMORS*

	Normal prothrombin time (12.5 per cent plasma)		Induced Hypoprothrombinemia in 12.5 per cent plasma (in seconds)		Weight of tumor at removal, gm.	Body, gm.	Liver, gm.	At death Liver as per cent body weight	Pituitary, mgm.
	With tumor	After excision	With tumor	After excision					
Average	34	38	70	106	41	397	10.1	2.54	14.4
Range	32-37	35-41	56-113	67-165	22-68	361-413	8.3-12.5		12.4-17.7

* Of a large group of rats with medium to large mammary tumors, only 6 survived to provide successive values incorporated in the above prothrombin data. Following operative excision of the tumors the rats were subjected to periods of induced hypoprothrombinemia and frequent cardiac punctures. The survival times of the rats used to compile this summary, dating from the operation, was from 14 to 61 days.

TABLE IV: LIVER AND PITUITARY SIZE IN NORMAL RATS AND RATS BEARING MAMMARY TUMORS

	Fresh tumor weight† + S.D., gm.	Body weight + S.D., gm.	Fresh liver weight + S.D., gm.	Liver as % of body weight	Fresh pituitary weight + S.D., mgm.	Number
Normal rats	273 ± 13.0	8.8 ± 0.9	3.22	11.8 ± 0.9	25
Rats bearing spontaneous mammary tumors	345 ± 20.6	9.8 ± 1.3	2.84	13.9 ± 1.6	18
Single	6.0 ± 2.2	353 ± 33.0	12.4 ± 1.3	3.57*	14.4 ± 1.8	13
(Multiple**)	19.3 ± 5.4	364 ± 30.0	14.6 ± 2.9	4.23*	15.6 ± 2.3	30
	53.2 ± 25.5	395 ± 29.4	14.9 ± 2.9	4.36*	15.1 ± 2.6	22
	24.9 ± 15.7	371 ± 30.2	14.8 ± 3.0	4.28*	16.6 ± 1.2	5

S.D. Standard deviation.

† Arbitrarily classified by tumor size.

* Calculated on the net body weight minus tumor. When the gross body and tumor weight is applied as the divisor, these values range from 3.51 per cent to 4.01 per cent.

** In these animals 2 to 3 isolated and separate tumors were found. The weight given is that of the total of all tumors found.

bearing spontaneous mammary tumors. The summary of the weights of the tumor, liver and pituitary gland and the weights of the liver and pituitary gland obtained from healthy non-pregnant non-lactating stock female rats of comparable body weight is given in Table IV. The liver (fresh weight) is approximately 2.9 per cent of the body weight of large normal non-tumorous female rats. However, the ratio in rats of comparable body weight with mammary tumors ranged from 3.6 to 4.4 per cent. Similarly the weight of the pituitary gland in normal non-tumorous rats is approximately 13.9 mgm., whereas in rats with mammary tumors the gland weighed from 14.4 to 16.6 mgm. The largest specimens in each category were (a) a healthy normal female of 346 gm. with a liver of 12.2 gm. (3.5 per cent) and a pituitary gland of 15 mgm. and (b) a rat of 387 gm. with a tumor weighing 10.3 gm. which had a liver weighing 23.3 gm. (6.1 per cent of body weight) and a pituitary gland weighing

Microscopic and chemical examination of livers.—Samples of liver from sacrificed rats bearing mammary tumors were placed in Bouin's fixative and stained in routine fashion with hematoxylin and eosin. A careful microscopic examination of sections from a large series of tumorous rats was made. In every case the liver cells were regular in shape and content and stained in the usual manner. No increase was observed in mitotic figures. The hepatic sinusoids were somewhat prominent and generally an increased vascularity was apparent, but there was no congestion.

Total liver lipids were determined by grinding with sand and extracting with ether and chloroform, combining the extractives and evaporating the solvents. Total moisture of the livers was determined by drying liver slices at 100° F. for 48 hours. The content of total liver lipids and moisture from normal and tumorous animals were similar.

DISCUSSION

A reduction in the number of functioning liver cells has previously been shown to result in a plasma that is mildly hypoprothrombinemic and especially sensitive to the hypoprothrombinemic action of the anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin) (4). A significant increase in liver size was associated with observations of a marked resistance to induced hypoprothrombinemia detectable in normal lactating rats (7). The observations described in the present study are in harmony with the assumption that an increase in the number of normal liver cells produces a state of increased plasma prothrombin activity and a reduced hypoprothrombinemic response to a standard dose of the anticoagulant.

It is not improbable that the hepatomegaly arising during the development of a mammary tumor is associated with, or secondary to, what appears to be a true hypertrophy of the pituitary gland. It may be presumed that the pituitary hypertrophy connotes hyperfunction and in the absence of more objective evidence, the increase of the pituitary weight in the tumorous state is offered as a *prima facie* evidence of this rationalization. The nature, if any, of the relationship between the developing mammary tumor and the pituitary gland is an object for further study. However, a relationship between the liver and the pituitary is on a firmer basis. Hypophysectomy has been shown to produce a reduction in liver weight in the pigeon (14) and rat (15) and hepatic cirrhosis in the dog (10), and conversely acromegaly (pituitary hyperfunction) in the dog (12) and man (2) have been associated with marked increases in liver size. At least 2 individual hormonal preparations in the hands of several investigators, prolactin and the growth factor, have induced splanchnomegaly and particularly hepatomegaly (10, 14). When purified prolactin preparations were administered parenterally to rats and rabbits, there developed a tendency to increased plasma prothrombin activity and more generally a reduced hypoprothrombinemic response to a standard dose of the anticoagulant.³ These data were in agreement with those obtained from lactating rats (7) and are consistent with those contained in the present report.

The excess of functional liver tissue apparently imparts to the lactating rat, and rat with a mammary tumor, an enhanced capacity to recover from incipient or induced hypoprothrombinemia. Undoubtedly prothrombin is only one product of liver metabolism increased above normal in liver tissue, and it is possible that further studies will reveal other substances similarly affected. However, it

cannot be assumed that a gross hepatic hyperplasia and consequent increased hepatic function fortifies the animal against all manner of hepatotoxins. In fact, in separate trials we have been unable to demonstrate that the susceptibility of the livers of healthy rats with mammary tumors given chloroform is less than those of non-tumorous rats.⁴ It is surprising that an increased hepatic reserve does not protect the rat against the lethal action of a hepatotoxin such as chloroform.

Independent observations by Mr. Lester Scheel in Prof. Link's laboratory during the summer of 1947 confirm the findings recorded here. In 15 rats with mammary tumors, the average clotting time of 12.5 per cent plasma was 32.9 ± 2.9 seconds compared with a clotting time in non-tumorous female rats of 40 ± 2 seconds. The 6.25 per cent diluted plasma from the tumor rats was 33.2 ± 3 seconds compared with the $47.3 \pm$ seconds of control rats. The increase in "clotting activity" of the plasma of mammary tumor rats was also apparent when 0.3 per cent fibrinogen solution was employed as diluent. Finally, the mean of the plasma fibrinogen of the tumorous rats was 267 mgm. per cent compared with a value of 300 mgm. per cent in normal rats.

SUMMARY

1. The presence in the rat of spontaneous mammary tumors causes a reduction of the normal prothrombin time (12.5 per cent plasma).

2. The hypoprothrombinemia induced by a standard dose (2.5 mgm.) of the anticoagulant, 3,3'-methylenebis (4-hydroxycoumarin), is considerably less in rats with mammary tumors than in non-tumorous controls.

3. The presence of mammary tumors in rats appears to be associated with an increase in the size of the liver and pituitary gland. There is some correlation between the weight of the mammary tumor and weight of liver and pituitary gland. The hyperplastic liver is composed of microscopically normal hepatic cells and the moisture and total lipids of the liver are within normal limits.

4. Following excision of the mammary tumor, the prothrombin activity and the extent of induced hypoprothrombinemia increased to levels resembling those obtained from normal rats. Similarly, the weights of the liver and pituitary gland were similar to those of non-tumorous rats.

5. The hepatomegaly and prothrombin response observed in rats with mammary tumors is similar to data obtained from lactating rats and rats treated with the hormone, prolactin. Rats with mammary

⁴Field, J. B. Unpublished data.

tumors are not less susceptible to the hepatotoxic and lethal action of chloroform.

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Cancer Res 1948;8:172-176.

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