Is the Action of Calcium in the Coagulation of Blood Stoichiometric or Catalytic?[1]

ARMAND J. QUICK

Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin

By determining the amount of thrombin formed from known quantities of calcium, one is able to conclude whether the action of the latter is catalytic or stoichiometric. A simple method to control accurately the concentration of calcium is to add known amounts to plasma obtained from blood decalcified by passage through Amberlite[2] according to the procedure recently described (*4*). Thrombin may be satisfactorily estimated by allowing a plasma to clot and then (after the thrombin has been inactivated by the normal antithrombin of the plasma) determining the amount of prothrombin remaining.

TABLE 1

QUANTITATIVE RELATIONSHIP BETWEEN PROTHROMBIN ACTIVATION AND THE CALCIUM CONCENTRATION IN HUMAN PLASMA

Mixture	(cc.)	Clotting time of mixture (sec.)	Prothrombin remaining in serum after 1 hr. incubation	
			Time* (sec.)	Per cent of normal (approximate)
Amberlite plasma	0.4			
1 Thromboplastin	0.1	90	11	100
Calcium chloride, 0.0006 M	0.1			
Amberlite plasma	0.4			
2 Thromboplastin	0.1	13½	35	10
Calcium chloride, 0.0025 M	0.1			
Amberlite plasma	0.4			
3 Thromboplastin	0.1	12½	105	2
Calcium chloride, 0.005 M	0.1			

* To 0.1 cc. of serum were added 0.1 cc. of thromboplastin, 0.1 cc. of 0.02 M calcium chloride, and 0.1 cc. of fibrinogen or fresh human plasma treated with tricalcium phosphate. The preparation of reagents and the general procedure have been described previously (*2*).

The results summarized in Table 1 clearly suggest that the relation of calcium concentration to thrombin production is stoichiometric. When the calcium chloride concentration in plasma is 0.00015 M (Mixture 1) or lower, very little prothrombin is changed to thrombin in one hour, even though other conditions, including an excess of thromboplastin, are optimum. The thrombin formed, although very small in amount, coagulates all the fibrinogen because it acts enzymatically. Were the action of calcium catalytic, it would similarly convert all the prothrombin to thrombin. It is only when the calcium concentration of plasma is 0.0012 M (Mixture 3) that nearly all of the prothrombin is consumed or converted.

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[2] Amberlite IR 100 is a phenol-formaldehyde resin which functions as a cation exchanger.

These findings supply another link in the chain of evidence that the reaction or reactions that bring about the production of thrombin from the prothrombin complex, thromboplastin and calcium, are chemical and not enzymatic. That thromboplastin acts stoichiometrically was indicated by the work of Mertz, Seegers, and Smith (*1*) and recently confirmed by the writer working with hemophilic blood (*5*).

Calcium is closely associated with prothrombin, but the solution of this relationship is contingent upon a fuller understanding of the composition of prothrombin. The concept of the writer (*3*) that it is a complex and not a unitary substance has recently found clinical confirmation (*6*). The writer has studied one family in which three members have a congenitally reduced concentration of one of the components, designated as "B", and recently he has discovered another family in which two brothers have a congenital lowered level of a second principle, which has been named component A. Much study will be required before one can determine how these factors interact, but from available data it appears fairly certain that the reactions follow the law of mass action, and that none of these factors can be considered accelerators or activators in an enzymatic or catalytic sense.

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The Action of the Endotoxin of *Trypanosoma cruzi* (KR) on Malignant Mouse Tumors[1]

WILLIAM M. MALISOFF

Longevity Research Foundation, 254 West 31st Street, New York City

This communication is essentially a verification of the findings of Roskin and Kluyeva (*2*) in producing the substance designated as KR, an extract of the lysed cells of *T. cruzi*, which has a lytic effect on certain malignant tumors.

A culture of *T. cruzi* was obtained which originated from authentic cases of Chagas' disease.[2] Following the Russian prescription, this was allowed to grow on a sterile medium containing rabbit blood at a temperature of 22–24° C. In a period of 14–19 days large colonies of organisms were obtained.

Only minor changes were made in the original Russian procedures in preparing the extract of the endotoxin. Lysis was accomplished by covering with pyrogen-free distilled water and allowing to stand at refrigerator temperatures overnight. Metaphen at a final concentration of 1:10,000 was used to ensure additional protection against bacterial contamination.

[1] Presented at the Fourth International Cancer Research Congress, September 2–7, 1947, St. Louis, Missouri.

[2] Grateful acknowledgment is made to J. A. Aeschlimann and R. Schnitzer, of Hoffman-La Roche, Inc., Nutley, New Jersey, and to Malcolm Soule, University of Michigan, for cultures designated as 2 and 3. (When the two cultures were combined in one, the designation is 23. Culture 3 was used in practically all our work, unmixed with 2.)

The lysed material was designated and was rated in terms of the number of lysed organisms per milliliter, as determined by counts before lysing. One KR unit is the lysed product of 1,000,000 organisms.[3]

Careful technique is required in the procedure, because *T. cruzi* is very sensitive to temperature, nutrient base, and other variables. Lysis is easily produced, and any inadvertent departure from set conditions might result in a high mortality of the organisms before the proper separations can be made. In addition, a variation in strain might lead to entirely inactive extracts, as reported by T. Hauschka and associates (*1*).

Two kinds of well-established malignant tumors in mice were tried: (a) spontaneous mammary carcinoma and (b) sarcoma 180. Injections of 1 KR unit were made throughout the experiments, unless otherwise noted, as in the toxicity tests. They were made daily, a day being skipped occasionally, as on Sundays. In some cases the animals were used for assay purposes on materials which proved to be inactive, and consequently the treatment with active material was thus delayed or protracted.

Complete observations were made as to the initial condition of the cancers, effects in the gross during treatment, and effects in the gross and in the histology when animals were autopsied, with special attention to the tumor site, liver, kidney, heart, lungs, etc. Slides were prepared in all cases, unless a particular tissue were autolyzed. Photographic records were taken at frequent intervals.

TABLE 1

EFFECT OF THE ENDOTOXIN OF *T. cruzi* ON MOUSE MALIGNANT TUMORS
(Series A: Spontaneous Mammary Carcinoma)

Mouse	Dates	KR Prep.	No. of injections	Am't tumor left (%)	Histology of organs
Group 1					
1 k	6/16-6/23	61347-23	8	10	Normal
3 k	"	"	8	25	"
6 k	6/16-6/26	"	10	25	"
7 k	"	"	10	20	"
9 k	6/16-6/23	"	8	5	"
11 k	"	"	8	0	"
4 d 7/14	6/16-7/14	"	18	5	"
5 d 7/2	6/16-7/1	"	13	0	"
Group 2					
13 k	6/23-7/22	613	21	0	"
14 k			23	0	"
15 k			12	0	"
18 k accid.			3	50	"
20 k			18	0	"
19 d 7/2			8	80	Autolyzed
Group 3					
21 k	6/30-7/23	627-3	18	0	Normal
23 k			13	0	"
24 k			4	20	"
28 k			21	0	"
32 k			5	65	"
22 d 7/29	2 tumors		14	0	Autolyzed
26 d 7/30			19	10	"
29 d 7/31			16	5	"
30 d 7/3	Very large		3	95	Necrotic liver
33 d 7/29			19	0	Normal
34 d 7/8			5	65	Fatty yellow liver
Group 4					
36 k	7/15-8/14	7104-3	21	0	Normal
40 k			16	0	"
42 k			16	0	"
45 k			19	0	"
38 d 8/14			23	0	"
41 d 7/31			13	5	Autolyzed
46 d 8/3			12	5	"

d = died, k = killed, 0 = no tumor found.

TABLE 2

EFFECT OF THE ENDOTOXIN OF *T. cruzi* ON MOUSE MALIGNANT TUMORS
(Series B: Sarcoma 180)

Mouse	Dates	KR Prep.	No. of injections	Am't tumor left (%)	Histology of organs
52 k 8/14	Start 7/15	7247-3	7	0	Normal
54 k 8/14			7	0	"
57 k 8/14			11	0	"
61 k 8/14			9	0	"
49 d 7/24		710-34A	8	100	Autolyzed
50 d 8/14			10	0	"
51 d 8/3			13	10	"

Mice # 53, 56, 59, 63 still alive 8/28/47 with no visible tumors; injections stopped at 10.

Table 1 is a summary of experiments with a selected group of mice having well-developed, proven, spontaneous mammary carcinoma. Table 2 is a summary of experiments with a selected group of mice having well-developed, proven sarcoma 180.

Figs. 1 and 2 show the appearance of a sarcoma at the start and after complete regression followed by a period of recovery and growth, and the histological evidence of regression under treatment to a completely necrotic condition of the cancer.

The spontaneous mammary carcinoma mice came from a strain in constant use in research, on which numerous controls were tested and showed no spontaneous remissions. The tumors form at about 9 months of age and are well-developed 3–4 weeks later, at which stage they were used in the experimental work. In the controls the mortality is nearly 100 per cent in 2 months. The sarcoma 180 mice were chosen at an advanced stage of tumor development, namely, at 2.5 weeks, when the life expectancy shown by numerous controls was 3 weeks. On groups so screened the spontaneous recovery is well below 10 per cent.

Although an effort was made to make all the preparations of KR of the same strength, the biggest factor of uncertainty was the difficulty of making accurate counts of organisms. There must also be hidden variables in the techniques of seeding cultures, small variations in the conditions of growth and harvesting, in the chemical purification, and in the conditions of lysis which affect the potency. That the preparations were not of equal strength is suggested by the fact that the animal responses were not quite proportional to the total dosage. One preparation of KR (Table 1, group 3) showed a high mortality, attributable possibly to inadequate purification.

The character of the regressions can be gauged from the following typical case histories:

Mouse #21: spontaneous mammary carcinoma. Tumor in the left inguinal region, 1 x 1.5 cm. It was firm and infiltrated

[3] Since the results are due to an active ingredient of the KR, calibration will have to be made in terms of its action on standard tumors. It is suggested that the active ingredient of KR be called *trypanosin*.

into the surrounding tissue. Treatment was started on June 30, 1947, and stopped on July 22, a total of 18 injections being given at a uniform dosage of 1 unit/injection. Shrinkage and marked softening of the tumor was apparent after the 4th daily

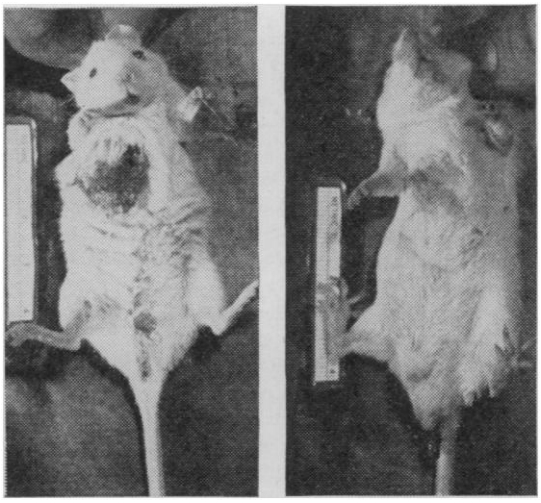

FIG. 1. Regression of sarcoma 180 and recovery thereafter (mouse #54). *Left:* before treatment. *Right:* one month after 7 injections.

injection; after the 7th injection, only a small nodule could be palpated; after the 12th, no tumor at all could be palpated. The animal was killed and autopsied on August 14. No tumor was found. A 2-mm. nodule consisting of fibrous tissue was

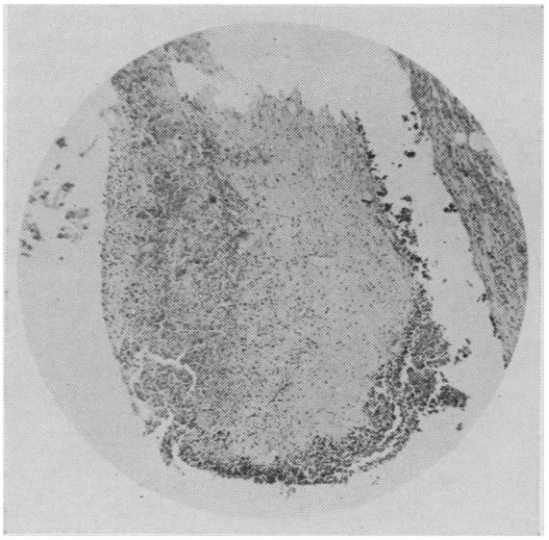

FIG. 2. H and E × 90 photomicrograph showing spontaneous mammary carcinoma completely necrotic after 5 injections with KR (some fibroblastic proliferation present).

present at the original tumor site. All organs were normal under microscopic examination.

Mouse 54: sarcoma 180. The initial tumor was 10 days old, 5.5 cm. in diameter, firm, and partly ulcerated. Injections were started on July 15, 1947, a total of 7 being given in the course of a week. After the 4th injection the tumor became smaller, completely ulcerated, and discharged cheesy material; after the 6th, the tumor was 0.25 cm. in diameter; on the 7th, the tumor was entirely gone, and the original site was completely covered with new epithelium. The mouse was kept alive for 3 weeks after healing, during which time it lost its runted appearance and gained weight. It was then killed and autopsied. No tumor was found. Fibrous tissue was found subcutaneously. All organs were normal in the gross and microscopically.

The KR was also probably of unequal stability because the conditions of preservation at low temperatures differed, as did the time of exposure to the air. The Russian observers claim a period of stability no longer than 10 days. If the same KR was used beyond 10 days, *i.e.* beyond injection 9, there is still some doubt whether the preparation was fully active.

It is apparent that with potent preparations of KR (Table 1, group 2) 10 or more injections on consecutive days can produce 100 per cent remission of tumors with no damage to any organs.

Damage to the liver was noted only in the case of 2 mice, both of which died before any extensive treatment with KR, namely, after the 3rd and 5th injections (mice 30 and 34), and both treated with the same preparation, which was considered impure. Mouse 30 had an exceptionally large tumor at the start. In both cases there was practically no regression, and the consideration comes to mind that possibly both mice had damaged livers to begin with. Otherwise, no organ injury of any kind has been found in 100 animals that have come to autopsy to date.

In Series A (carcinoma) decreases in tumor size were noted in all cases. Complete regression was noted in 25–83 per cent of the cases, depending on the KR preparation used and the number of injections made. In spite of the exploratory nature of the experiments, complete regressions were noted in 55 per cent of the cases, and regressions of better than 50 per cent in over 90 per cent of the cases. Fifteen per cent of the animals were kept alive and have shown no return of the tumors grossly in the course of 1–2 months. This work is being continued.

At the present writing 40 sarcoma 180 mice are undergoing treatment and are healing. Only one group is reported in Table 2, showing complete regression in 9 out of 11 and 1 failure. In 15 controls, untreated by KR, 2 animals showed signs of regression which was sufficiently slow to weed them out from the experiment proper and testing with KR. When regressions occur with KR, the effect is, on the contrary, rapid and clearly connected with the injections. The one complete failure defies explanation at present because of the autolysis of the organs of the animal found some time after death. It must be remembered that we have screened animals for test with active KR when they were as near death as possible.

A set of 14 healthy animals were tested with KR, receiving 4 times the therapeutic dose in a series of 11 injections, and sacrificed. All organs were found normal on autopsy and histological examination.

The experiments are being continued on a larger scale and with a larger variability of the numerous factors that enter into the situation.

Our experiments show that the endotoxin of *T. cruzi* exerts a selective lytic effect on malignant tumors in mice, such as spontaneous mammary carcinoma and sarcoma 180. The

endotoxin is not toxic to normal tissues under the conditions of therapy and in concentrations 4 times as great.

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Male Sterility in the Carrot

J. E. WELCH and E. L. GRIMBALL, JR.

U. S. Department of Agriculture, Charleston, South Carolina

Male sterility has been reported in several crop plants including tomato (*3, 6, 9*), flax (*2*), corn (*4, 8*), onion (*5*), sorghum (*1, 10*), barley (*11*), and sugar beet (*7*), and the possibility of utilizing this character in the production of hybrid seed for commercial planting has been pointed out by several investigators. Present horticultural varieties of carrots lack uniformity when environmental conditions deviate from the optimum. By studying the combining ability of paired inbred lines, one in each combination possessing the male-sterile character, it is theoretically possible to obtain extremely uniform carrot varieties which are also superior to those now available in general appearance, productivity, quality, and nutritional value. The feasibility of using inbred lines of corn in producing seed for commercial planting is due to the monoecism of the plant and the ease in making cross-pollinations. In perfect-flowered plants like the carrot it is impractical to employ inbred lines in this way without male sterility.

An apparently male-sterile carrot plant was found in a collection of several dozen being grown for inbreeding in a greenhouse planting in the winter of 1945–46 at the U. S. Regional Vegetable Breeding Laboratory, Charleston, South Carolina. This plant was grown from a root selected in a commercial stock of the variety Tendersweet in the spring of 1945. Caging of certain umbels took place a day or so before the first flowers normally would open, and the caged umbels were observed daily for the appearance of exserted stamens, the stage at which blowflies are introduced into the cages as pollinating agents. The first flies were placed in the cage on February 25, 1946, even though no stamens were evident. A few days later microscopic examination showed that the anthers of this plant were shriveled and brown in color before any petals unfolded. No exserted stamens were found. On March 9, 1946, an umbel of the variety Nantes Strong Top, grown from a root selected from a commercial stock in the spring of 1945, was placed in a test tube of water and introduced into the cage with the apparently male-sterile plant. This procedure was continued with fresh umbels from the Nantes plant. Later the two entire plants were isolated in a single large cage. The seed on the selectivity caged umbels was harvested separately from the other umbels because some seed had set on the male-sterile plant outside the small cage by open pollination before the whole plants were enclosed. The pollen parents of these seeds were unknown. Female fertility of the male-sterile plant appeared normal.

On September 6, 1946, a portion of the hybrid seed was planted under good conditions. The 67 roots which were produced were harvested on January 6 and 28, 1947, and held at 32°–35° F. until they were planted in the field between February 21 and March 18, 1947, at 5 different locations. The histories and internal characteristics of all roots were recorded.

Classification of the flower types of these F_1 plants between June 6 and July 7, 1947, showed 39 male-sterile and 15 normal. The balance of the plants to make the total of 67 planted either had not flowered when the last notes were taken or were lost before classification. No difficulty was encountered in distinguishing between male-sterile and normal plants. The abnormal specimens appeared like the parental male-sterile plant found in the winter 1945–46 greenhouse planting. The mode of inheritance of the male-sterile character is unknown, because so far only a relatively small segregating population has been studied. Further breeding tests will be required before a genetical explanation can be proposed.

In order to determine whether male-sterile plants produce any self-fertile pollen, umbels of four segregates were caged with blowflies. Three of these plants set a few seeds. If enough plants can be grown from these seeds, proof should be obtained as to whether these were really selfed seeds or were cross-pollinated from normal plants by thrips, ants, or some other very small insects that penetrated the fine-mesh cloth cage covering. Umbels were not allowed to touch the cloth, thus eliminating the possibility of insects outside the cages pollinating enclosed flowers pressed against the inside of the cloth. Isolated plantings of single male-sterile plants and other plantings with several male-sterile plants would give further information on the possibility of viable pollen production.

At the time the F_1 population involving male sterility was being classified, several dozen plants in other carrot breeding lines were examined for flowering habit. Four plants were found to possess varying degrees of apparent male sterility. Each plant produced some exserted stamens, but the number was only a small percentage of those which would normally be exhibited. Two of the specimens shed pollen, the viability of which was not determined, but no pollen production by the remaining two plants was observed. All four set an abundance of open-pollinated seed. This partial male sterility was not encountered in classifying the F_1 population which was segregated for the male-sterile character.

The mode of inheritance in the carrot of the male-sterile character, for which segregation data were presented, and the partially male-sterile types with which no controlled crosses were made will not be known until additional breeding tests are completed.

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