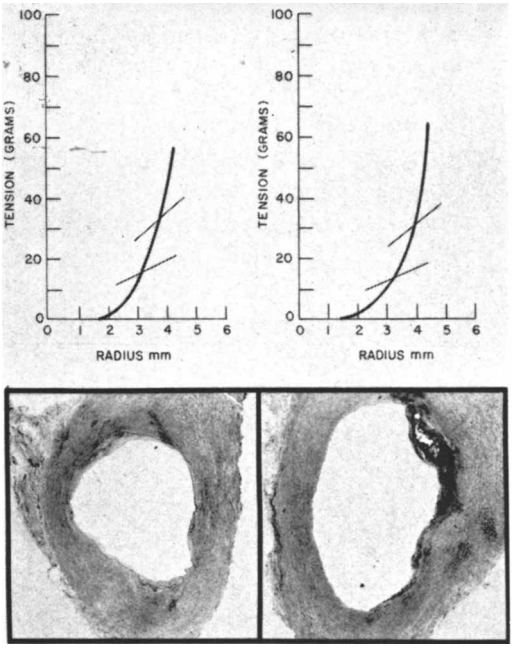

FIG. 3. Tension-length diagrams of the two coronary orifices of a 79-year-old man. The orifice on the right with early atheroma is more flexible than that on the left which shows none. Tension-length curves are marked as in Fig. 1. Sections are stained with Hematoxylin and Sudan IV × 6.5.

coronary orifices is well recognized as one determinant of the pattern of infarction and it seems reasonable to believe that difference in physical property could have similar effect. Because the relative elasticity of the 2 coronary orifices is not directly related to the occurrence of atherosclerosis, change in elasticity could either increase or diminish the affects of atheroma formation upon blood flow. This study does not indicate which possibility is more common.

A previous study has demonstrated that postmortem decalcification produces a consistent increase in size of the femoral artery (4). In this study calcium removal did not produce a comparable change in the coronary orifice and it seems possible that the difference is due to restriction of the coronary orifice by surrounding aortic wall.

Summary. 1. Tension-length diagrams of excised human coronary orifices resemble those obtained from human femoral artery. 2. Wide differences can exist in the two coronary orifices of one individual. These are not directly related to the occurrence of atherosclerosis and may affect coronary blood flow.

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Antihemostatic Effect of Heparin Counteracted by Adenosine Triphosphate.* (32465)

W. O. CRUZ† AND C. P. DIETRICH‡
Laboratory of Hematology, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

The antihemostatic effect of heparin has always been related to the inhibition of blood coagulation and platelet thrombus formation (1). Previous studies in this laboratory have shown that heparin injected in dogs did not affect the normal hemostatic response in about 60% of the animals, inspite of the fact

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† Deceased January 3rd, 1967.

‡ Present Address: Dept. of Physiology & Pharmacology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

that blood taken from great vessels of these dogs remained unclottable for several hours (2) and no significant change in the platelets occurred in the blood after injection of heparin(3). These results cannot be explained on the basis of the usual interpretations, *i.e.,* by the participation of blood coagulation and a passive platelet plug in hemostasis.

Recently, a paper from our laboratory(4) reported suggestive evidence of an early participation of the smooth muscle of the damaged vessels in the control of hemorrhage. In this paper a series of hemostatic inhibitors was investigated and it was found their hemorrhagic activity could be counteracted by adequate concentrations of ATP or ATP-Mg. As many of these inhibitors were inhibitors of cell respiration, they could cause a diminished production of ATP and this was counteracted when adequate ATP concentrations were present in the fluid bathing the preparation. It has been pointed out as an alternative interpretation of these phenomena the possibility that ATP could dislodge some inhibitors (which were mostly myosin ATP-ase inhibitors) by direct competition at the myosin active sites. Since heparin, when tested in the rat tail preparation, belonged to a group of inhibitors that maintained an antihemostatic activity even after removal of the bathing fluid from contact with the scarified skin, it was selected as a suitable tool to test the last mentioned interpretation.

This paper presents evidence that the antihemostatic activity of heparin is connected with its combination on active sites of the myosin molecule and that ATP dislodges heparin from these sites.

Methods. Adult albino rats weighing 60-150 g and anesthetized with thionembutal were used in these experiments.

Physiological methods. Bleeding time determinations. The method used for this has been described previously(4) and consisted essentially of making a scarification with a razor blade in the terminal part of the tail and immersing it in a small test tube containing 1 ml of the solution to be tested, held at 37°C in a water bath. The blood flow from the vessels was observed with a dissecting microscope. Unless otherwise stated, the sequence of experiments was as follows: *a.* The scarified tail was immersed into a saline solution (0.13 M sodium chloride) and the normal bleeding time observed. *b.* A mechanical stimulus was made by rubbing gauze with strong pressure over the scarification, and the tail was immersed again in the test solution and the test bleeding time observed.

This experiment could be repeated at least 10 times at ten minute intervals giving bleeding times in the normal range (½ to 2 minutes). When heparin was used, the tail, after mechanical stimulus, was left in contact with heparin for 2 minutes and then washed with 100 ml of saline (heparin-treated tail). The bleeding time was then observed in 1 ml physiological saline (posterior saline) or a solution of other test substance.

Coagulation time. The coagulation time was determined on the blood taken directly from the wound using a thin capillary tube as previously described(5). The drop of blood formed in the wound was collected in the capillary tube for the clotting test and the rat tail was immersed again in saline for the bleeding time determination.

Microelectrophoresis. Heparin liberated from the rat tail after treatment with the test substances was identified by microelectrophoresis according to the method of Jaques *et al*(6). The slides were prepared with agarose in barbital buffer, pH 8.6 and run in a small microelectrophoresis cell for 7 minutes at 200 volts. The slides were then fixed with Cetavlon, and stained with toluidine blue and decolourized with 95% ethanol for removal of the background.

Biochemical preparations. Heparin. Prior to use 1 ml of heparin (5000 I.U.) (Liquimine Roche) was passed through a column of Sephadex G-25 (Pharmacia, Upsala), 20 cm × 1 cm to remove low molecular weight contaminants. A carbazol determination(7) was used to adjust the heparin concentration in the solution. The Dextran Sulfate used contained the equivalent of 17 I.U. of heparin per mg.

Protein extractions. Plasma was obtained

from dog blood diluted in 4 volumes of saline in plastic tubes, centrifuged for 2 minutes at 2,000 × *g* and used immediately. Serum, obtained after drawing the blood in sodium citrate (10%, 0.1 M), was centrifuged and the plasma obtained clotted with calcium chloride. Fibrinogen was extracted following Seeger's procedure(8). Myosin was prepared according to Szent-Gyorgyi(9) and assayed as follows: 0.1 ml, 0.1 gm % enzyme; 0.02 to 0.2 ml, 10^{-2} M ATP; 0.1 ml, 10^{-1} calcium chloride: 0.1 ml, 2 × 10^{-1} M, pH 7.2, Tris buffer; water to 1 ml; incubation for 5 minutes at 25°. Aliquots were taken for inorganic phosphate determinations according to Fiske and Subarow(10). Blanks were made with the complete system without calcium chloride or ATP. Tropomysin was prepared according to Bailey(11). Actin was prepared by the method of Tsae and Bailey(12) and assayed by adding it to the system described above where calcium chloride was substituted by 1 ml 10^{-1} M magnesium chloride. The actomyosin formed was activated by $MgCl_2$ showing that an active actin was prepared.

Hyaluronic acid, dextran (clinical grade), glucose-6-phosphate, glucose-1-phosphate, ATP§, ADP, AMP, CTP, UTP, GTP, and ITP were purchased from the Nutritional Biochemicals Company and diluted in isotonic saline and the pH corrected to neutrality.

Results. Bleeding times of more than 30 minutes were obtained when heparin is allowed to be in contact with the scarified area in the rat tail for 2 minutes. This antihemostatic effect remained even after extensive wash of the tail with saline. This effect was called the "heparin residual effect." Table I shows that the "heparin residual effect" is roughly proportional to heparin concentration (0.05 to 5 mg/ml) and the time of contact of the heparin solution with the scarified area. (At least 1 minute of contact with 2 mg/ml of heparin solution to give bleeding times of more than 30 minutes.) The blood flowing from the wound in the "heparin-treated tail" gave normal clotting times showing that heparin is not free to act as an anticoagulant.

§ ATP—adenosinetriphosphate, ADP—adenosinediphosphate, AMP—adenosinemonophosphate, CTP—cytidinetriphosphate, UTP—uridinetriphosphate, GTP—guanosinetriphosphate, ITP—inosinetriphosphate.

TABLE I. Effect of Time of Contact and Heparin Concentration in Producing "The Heparin Residual Effect."

Heparin dose (mg/ml)	Time of contact in solution of heparin (min)	Bleeding time in posterior saline (min)	Coagulation time in saline* (min)
0.05	2	½	2
0.10	2	7–12	3
0.25	2	12–20	—
1.00 to 5.00	1–2	>30	3
10.00	2	>30	4
2.0	¼	1	—
2.0	½	5–10	—
2.0	1	>30	—
2.0	2	>30	—

* Blood collected between the 1st and 3rd min of bleeding in posterior saline.

Fig. 1 shows the microelectrophoresis for heparin of extracts from pieces of the scarified rat tail. For this experiment the scarified area was immersed in 5 mg/ml of heparin for 2 minutes. The tail was then washed extensively with saline and cut in three approximately equal pieces: a. Piece above the scarification; b. Piece below the scarification and c. Piece containing the scarified area. The pieces of tail were lyophilized and ground with a glass rod, mixed with 1 ml of water and protein removed by extraction with phenol(13). The aqueous phase was dried and the residue resuspended in 5 λ of water. An aliquot of 2 λ was applied on the microelectrophoresis slide. The greatest amount of heparin was obtained from the piece containing the scarification.

Table II shows that the "heparin residual effect" depends upon the sulfoamino groups

TABLE II. Effect of Some Mucopolysaccharides in Producing "The Heparin Residual Effect."

Substance	Dose (mg/ml)	Time of contact (min)	Bleeding time in posterior saline (min)
Heparin	1 to 5	2	>30
Desulfated heparin	5	2	½
	5	15	8
Dextran sulfate	5	2	>30
Dextran	5	2	½
Hyaluronic acid	1	2–10	½

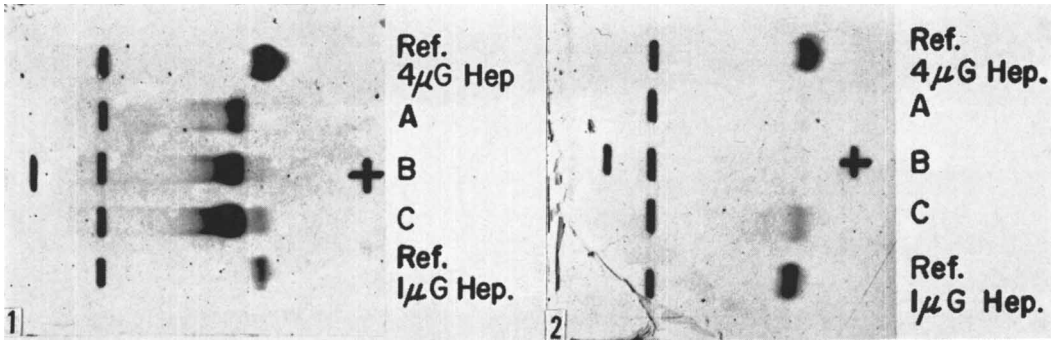

FIG. 1. Microelectrophoregram for heparin of extracts from pieces of the scarified rat tail.
A. Tissue above the cut.
B. Tissue below the cut.
C. Tissue containing the cut. (For details see text.)

FIG. 2. Microelectrophoregram for heparin of solutions previously immersed with the "heparin-treated tail."
A. 0.1 M (NH_4) HCO_3 solution.
B. 0.1 M (NH_4) HCO_3 solution + 10^{-3} M AMP.
C. 0.1 M (NH_4) HCO_3 + 10^{-3} M ATP. (For details see text.)

of the heparin molecule. Heparin N-desulfated by mild acid hydrolysis loses its antihemostatic effect. On the other hand, dextran sulfate has the same effect as heparin on the rat tail. Substances with similar molecular structures but without sulfate groups, for example dextran and hyaluronic acid, have no effect at all.

TABLE III. Substances Able to Neutralize the "Heparin Residual Effect." (Tests made in "heparin-treated tail.")

Substance	Molarity	Time of contact with the substance (min)	Bleeding time in posterior saline (min)
None	—	—	>30
Sodium phosphate	0.15	2	9
Sodium pyrophosphate	0.15	2	6
Sodium sulfate	0.15	2	6
Magnesium chloride	0.15	2	>30
Calcium chloride	0.15	2	>30
Glucose-6-phosphate	0.10	2–10	>30
Glucose-1-phosphate	0.10	2–10	>30

Controls (heparin-untreated tail) of sodium phosphate, sodium pyrophosphate, sodium sulfate, magnesium chloride, calcium chloride showed antihemostatic action but did not produce "residual effect," while glucose-6-phosphate and glucose-1-phosphate had no effect on the bleeding time.

Some ions, as sulfate, phosphate, and pyrophosphate are able to remove the "heparin residual effect" as can be seen in Table III. These ions have an antihemostatic action in the concentrations used but they do not show the residual effect since their action can be removed by washing with saline. Phosphate esters, calcium and magnesium ions do not change the "heparin residual effect." These results indicate some specificity of phosphate or sulfate ions in removing the "heparin residual effect." When phosphate is attached to other groups the attached group is important in its action upon the "heparin residual effect." (Table IV) Only ATP and ADP were able to remove the "heparin residual effect." Furthermore, in contrast to sulfate and phosphate ions, these nucleotides are hemostatic by themselves, shortening the normal bleeding times. These results show that adenine base is specific in this action. Citidine, uridine, guanidine and inosine have no effect even after 30 minutes contact with the "heparin treated tail."

The removal of heparin by ATP was demonstrated by microelectrophoresis (Fig. 2). For this experiment, physiological saline was replaced by 0.2 M ammonium bicarbonate to give samples free of salts for microelectrophoresis determinations. Control experiments showed that ammonium bicarbonate did not interfere with the bleeding time

TABLE IV. Nucleotide Specificity in the Neutralization of Heparin. (Tests made in "heparin-treated tail.")

Nucleotide	Molarity	Time of contact in the nucleotide solution (min)	Bleeding time (min)	
			In the nucleotide solution	In posterior saline
None	—	—	—	>30
ATP	10^{-5} to 10^{-2}	2 to 4	1/5 to 1	4–10
ADP	10^{-3}	2 to 4	1/5	5
AMP	10^{-1} to 10^{-3}	2 to 30	>30	>30
CTP	10^{-3}	2 to 30	>30	>30
UTP	10^{-3}	2 to 30	>30	>30
GTP	10^{-3}	2 to 30	>30	>30
ITP	10^{-3}	2 to 30	>30	>30

Controls (heparin-untreated tail) of CTP, UTP, GTP, ITP, AMP showed no action on the normal bleeding time, while ATP and ADP shortened the bleeding time.

and allowed normal hemostasis to take place. The rat tail was immersed for 10 minutes in 1 mg heparin/ml, washed with isotonic saline, and immersed successively for 5 minutes in the following solutions: a. 1 ml ammonium bicarbonate; b. 10^{-3} M AMP in ammonium bicarbonate and c. 10^{-3} M ATP in ammonium bicarbonate. The samples were extracted by the phenol procedure(13) and the water phase evaporated to dryness. The ammonium bicarbonate was then removed by heating the sample three times with small volumes of water followed by lyophilization. The final residue was resuspended in 5 λ water and 2 λ were applied to the agarose slides. Of the solutions tested only ATP removed heparin from the "heparin-treated tail."

These results suggest that heparin is directly linked with some structure (possibly vessels) responsible for the normal hemostatic process. On the other hand, the hemostatic action of ATP and its activity in removing the "heparin residual effect" suggest also that heparin competes with ATP for this structure. Therefore proteins related to the muscular contraction of the vessels were also tested on the "heparin treated tail." Table V shows that among the proteins tested only myosin and this in a very low concentration is able to remove the "heparin residual effect." Proteins involved in blood coagulation such as fibrinogen and thrombin (fresh serum) have no action at all.

Based on these experiments the hypothesis was formulated that heparin competes with ATP *in vivo* for the myosin active center.

TABLE V. Protein Action on the "Heparin Residual Effect." (Tests made in "heparin-treated tail.")

Protein*	Concentration (g %)	Bleeding in "posterior saline"
Plasma	1.50	>30
Serum	2.00	>30
Fibrinogen	0.10 to 0.50	>30
Hemoglobin	1.00	>30
Myosin	2.00	1/2
Myosin	0.02 to 1.00	2 to 5
Myosin	0.01	8
Myosin	0.005	10
Tropomyosin	0.50 to 2.50	>30
Actin G	0.15	>30
Actin F	0.15	>30
Denatured myosin†	0.10	>30

* The "heparin-treated tail" was allowed to be in the protein solution for 2 min.
† Heated for 1 min at 100°C.

In order to verify this hypothesis, an *in vitro* system with myosin, ATP and heparin was prepared. The Lineweaver-Burk plot of myosin enzymatic activity upon ATP is presented in Fig. 3. Myosin is inhibited by heparin and the inhibition is competitive.

Discussion. In spite of the presence of heparin, normal coagulation times were always observed in the experiments described, even when the bleeding times were more than 30 minutes. Substances involved in blood coagulation did not decrease these very prolonged bleeding times.

Inhibitors of platelet plug formation cannot be responsible for the high bleeding times since the concentrations of heparin remaining in the wound were not sufficient to alter the adhesiveness of platelets in the surround-

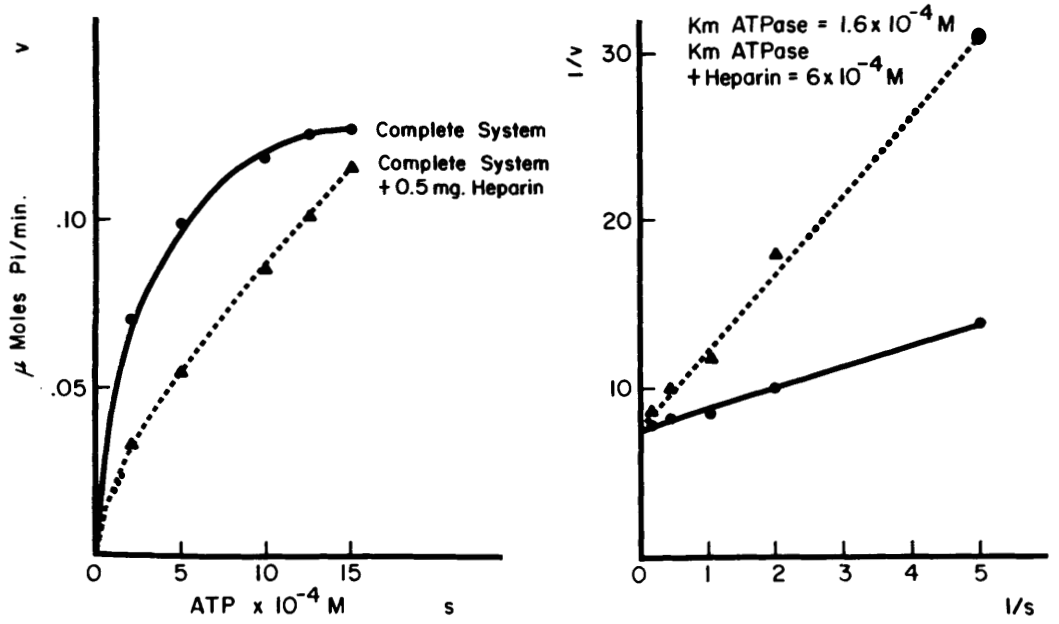

FIG. 3. Dependence of reaction velocity on the concentration of ATP with myosin ATPase:
In the absence of heparin ●——●
In the presence of heparin ▲- - - -▲

ing vessels and in the connective tissue(14, 15). These results support the previous finding that the normal hemostatic process is not related solely to the classical coagulation mechanism and platelet plug formation, at least in the early stages of the process(16). The specificity of ATP, ADP and myosin in removing heparin from the rat tail indicates that the normal hemostatic process is related more to smooth muscular contraction than to coagulation or platelet thrombus formation.

The mechanism by which heparin is fixed so that it is not removed even with several washes with saline is not known. The competitive inhibition by heparin of myosin ATPase *in vitro* suggests that heparin is linked to the active center of the enzyme preventing its normal contraction in the presence of actin.

Summary. In the present paper the antihemostatic effect of heparin on rat tail preparations was studied. It was confirmed that the antihemostatic effect of heparin is not removed after several washes with saline solutions and it was shown that its activity is closely related to the sulfate groups of the heparin molecule. The coagulation time of samples collected from oozing blood in the heparin-treated tail remained in the normal range.

Several salts, nucleotides and proteins were tested for ability to remove this antihemostatic effect. Of the substances tested, phosphate and sulfate ions, ATP, ADP and myosin were able to remove the antihemostatic activity while salts and proteins involved in blood coagulation were without effect. The effect of ATP could be shown to be accompanied by the release of heparin to the bath fluid by a microelectrophoresis technique.

The inhibition by heparin of myosin ATPase activity was demonstrated *in vitro*.

The role of muscular contraction, coagulation, and platelet adhesiveness on hemostasis is discussed.

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Human Anaplastic Thyroid Carcinoma in Tissue Culture.* (32466)

GEORGE W. JONES,† DUSAN SIMKOVIC,‡ JUNE L. BIEDLER, AND CHESTER M. SOUTHAM

Urology Service, Department of Surgery, Memorial Hospital, and Sloan-Kettering Institute for Cancer Research, New York City

Herein is reported the isolation and maintenance in culture of a human anaplastic thyroid carcinoma. Although many stable lines of human cancer cells have been established, we believe that this is the first human thyroid cancer cell line.

Origin and characteristics of the culture. The culture was established from a 1 × 2 cm metastasis of giant and spindle cell thyroid cancer in the kidney of a 73-year-old man (Memorial Hospital No. 26-62-75) on December 30, 1965. The specimen was obtained aseptically approximately 2½ hours after death. The tumor nodule was dissected from the cortex of the kidney and minced into fragments of roughly 3 mm diameter. Ten to 12 of these fragments were placed into milk dilution bottles and fed with 10 cc of Eagle's minimum essential medium(1) containing 20% a–gamma calf serum, with kanamycin sulfate and streptomycin at a concentration of 100 gamma per ml for each. The bottles were laid on their side and incubated at 37°C. No plasma clot or other matrix was used. Medium was changed at approximately one week intervals depending upon pH of the medium and appearance of the cells.

There was a sparse outgrowth of mixed cell types from several of the tissue fragments. It was nearly 4 months before there were complete monolayers in the original bottles. Sub-culturing was first attempted by selectively scraping, with a rubber policeman, those areas where epithelioid cells predominated. Growth of these subcultures was more rapid and consisted predominantly of large and small epithelioid cells with granular cytoplasm (Fig. 1). Subsequent passages were accomplished by trypsinization, utilizing 0.25% trypsin for 5 minutes at 37°C. As

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† Special Fellow in Urology, Memorial Hospital, and Research Fellow, Sloan-Kettering Institute. Present address: Division of Urology, Dept. of Surgery, College of Med., Howard Univ., Washington, D. C.

‡ Eleanor Roosevelt International Cancer Fellow, International Union Against Cancer, Sloan-Kettering Institute, 1965-1966. Present address: Cancer Research Inst., Bratislava, Czechoslovakia.